Characterization of the rate-limiting step of the secretion of *Bacillus subtilis* α -amylase overproduced during the exponential phase of growth

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The *Bacillus subtilis* α -amylase gene, *amyE*, was expressed under the regulated control of *sacR*, the levansucrase leader region. The gene fusion including the complete *amyE* coding sequence with the signal peptide sequence was integrated into the chromosome of a *degU32(Hy)* strain deleted of the *sacB* DNA fragment. In this genetic context, α -amylase is produced in the culture supernatant at a high level (2% of total protein) during the exponential phase of growth upon induction by sucrose. Pulse-chase experiments showed that the rate-limiting step ($t_{1/2} = 120$ s) of the secretion process is the release of a cell-associated precursor form whose signal peptide has been cleaved. The efficiency of this ultimate step of secretion decreased dramatically in the presence of a metal chelator (EDTA) or when the cells were converted to protoplasts. The hypothesis that this step is tightly coupled with the folding process of α -amylase occurring within the cell wall environment was substantiated by *in vitro* folding studies. The unfolding–folding transition, monitored by the resistance to proteolysis, was achieved within the same time range ($t_{1/2} = 60$ s) and required the presence of calcium. This metal requirement could possibly be satisfied *in vivo* by the integrity of the cell wall. The $t_{1/2}$ of the α -amylase release step is double that of levansucrase, although their folding rates are similar. This perhaps indicates that the passage through the cell wall may depend on parietal properties (e.g. metal ion binding and porosity) and on certain intrinsic properties of the protein (molecular mass and folding properties).

Keywords: α -amylase, protein secretion, late step of secretion, folding, *Bacillus subtilis*

INTRODUCTION

The processes involved in the secretion of proteins from *Bacillus subtilis* are still being elucidated. There have been numerous studies of the components of the secretion apparatus and the information borne by the exported proteins (for a review, see Simonen & Palva, 1993). Whether different secretion mechanisms may co-exist in this species is at present not definitely established (Nakamura *et al.*, 1994; Ogura *et al.*, 1996). It is, however, difficult to conduct a comparative study from the data available because of the differences between the model systems studied (genetic backgrounds, growth conditions, model proteins) and the lack of quantitative results. *Bacillus* α -amylase is a good illustration of this difficulty because it has been the subject of numerous

reports. We will focus here on the homologous protein, AmyE, only. Sequencing of the structural gene, *amyE* (Yang *et al.*, 1983), showed an N-terminal extension of the corresponding protein of 41 amino acid residues. The functional signal peptide, which is removed during translocation, includes the first 33 amino acids of this extension (Sasamoto *et al.*, 1989). The structural requirements for efficient processing of the α -amylase signal peptide were investigated by Sakakibara *et al.* (1993). The subsequent proteolytic cleavage of the prosequence depended on the composition of the culture medium and the age of the culture (Takase *et al.*, 1988). Attempts have been made to identify the discrete steps of the secretion pathway. However, no transient cell-associated intermediate has been observed for α -amylase, suggesting that it may be secreted cotranslationally

(Nagata *et al.*, 1974; Mäntsälä & Zalkin, 1979; Had-daoui *et al.*, 1995). These results differ from those obtained for levansucrase. In this latter case, we have shown (Petit-Glatron *et al.*, 1987) that, in a *degU32*(Hy) strain derived from *B. subtilis* 168 Marburg, levansucrase is secreted by a post-translational two-step mechanism. Two precursor forms of the extracellular protein have been identified in the membrane. Their steady-state concentrations are 0.3% (unprocessed form) and 2% (processed form) of total membrane proteins, allowing their biochemical characterization by conventional techniques (Chambert & Petit-Glatron, 1988).

In the present work we re-examined the α -amylase secretion pathway using the same biochemical procedures as those developed to isolate and characterize the transient membrane forms of levansucrase to compare the pathway of their secretion more accurately. Since the production of α -amylase is only 0.25% of that of levansucrase, some doubt could remain as to whether the difference observed between these two proteins would be due to the detectability of the intermediate species, rather than to a difference in the secretion process. The study reported here bears on the mechanism of α -amylase secretion, under conditions in which the expression of its structural gene *amyE* is under the control of the inducible levansucrase leader region, *sacR*, in a *degU32*(Hy) strain. Under these conditions the production of α -amylase was close to that of levansucrase and thus made it possible to compare the secretion mechanisms of these two homologous proteins in the same genetic context.

METHODS

Strains and media. Description of the strains and plasmids used in this work are listed in Table 1. The *B. subtilis* strain

GM96101 contained a *sacR-amyE* fusion which was introduced into the chromosome of strain GM96100. This latter resulted from the complete disruption of the *sacR-sacB* fragment in the QB112 chromosome, which was achieved by double crossing-over recombination between homologous sequences, using pGMS57. pGMS57 was obtained by an *NsiI-BstEII* deletion including the *sacR-sacB* fragment of pLS50 (Steinmetz *et al.*, 1985), followed by ligation of the blunted ends (pGM56) and the subsequent insertion in the *PstI* site of the gene encoding spectinomycin resistance purified from pDG1726 (Guérout-Fleury *et al.*, 1995) (Fig. 1). The double crossing-over events were selected on LB plates supplemented with the appropriate antibiotic (100 µg spectinomycin ml⁻¹). The strain did not produce any extra- or intracellular levansucrase, as tested by activity assays and immunoblotting analysis. Bacteria were grown at 37 °C in minimal medium (Chambert & Petit-Glatron, 1984) supplemented with 1% (w/v) glucose and 0.5 mM CaCl₂.

Plasmids and manipulation of DNA. We used pLS50 (Steinmetz *et al.*, 1985) and pAMY10 (Weickert & Chambliss, 1989) as templates to amplify by PCR (Petit-Glatron & Chambert, 1992) the DNA fragment carrying the *sacB* promoter region (*P_{sacB}*), using oligonucleotides A and B, and the coding sequence for *B. subtilis* α -amylase, *amyE* structural gene, using oligonucleotides C and D. A: 5'-CGCGGATCCTTTTAA-ACCCATCACATATACCTG-3' (forward primer) and B: 5'-CATCGTTGCATGCCTCC-3' (backward primer), with *Bam*HI and *Sph*I sites (shown in bold) in A and B, respectively. C: 5'-GGAGGCATGCAACGATGTTTGCAAAACGATTC-3' (forward primer) and D: 5'-GGGTACCCGCCGGCA-TTTTCTTTTCGGTAAGTCCCGTC-3' (backward primer), with *Sph*I and *Kpn*I-*Nae*I sites (in bold) at the 5' ends of C and D, respectively. The restriction sites were included in the primers to facilitate subsequent in-frame fusion of the regulatory region and the coding sequence, and insertion into the appropriate vector.

The amplified fragments were purified by electroelution after electrophoresis on agarose gel. The two DNA fragment ends

Table 1. Strains and plasmids

Strain/plasmid	Relevant genotype and phenotype	Source or reference
Strains		
QB112	<i>degU32</i> (Hy) <i>sacA321</i>	Lepesant <i>et al.</i> (1976)
GM96100	<i>degU32</i> (Hy) <i>sacA321</i> Δ <i>sacR-sacB</i> (Sp ^R)	This work
GM96101	<i>degU32</i> (Hy) <i>sacA321</i> Δ <i>sacR-sacB</i> <i>sacR-amyE</i> (Km ^R Sp ^R Cm ^R)*	This work
Plasmids		
pLS50	<i>B. subtilis</i> <i>sacR-sacB</i> region inserted in pJH101 (Cm ^R)†	Steinmetz <i>et al.</i> (1985)
pDG1726	pBluescript II SK(+) (Sp ^R)	Guérout-Fleury <i>et al.</i> (1995)
pGMK50	pLS50 (Km ^R)	Petit-Glatron & Chambert (1992)
pGM56	pLS50 Δ <i>sacR-sacB</i>	This work
pGMS57	pGM56 (Sp ^R)	This work
pGMK58	pGMK50 with the <i>Bam</i> HI- <i>Eco</i> RV fragment deleted and the <i>sacR-amyE</i> fusion ligated at these sites	This work
pGM8	pBluescript II SK(-) <i>sacR-amyE</i>	This work

* This construct was created by Campbell-like integration.

† pJH101 is derived from pBR322 by insertion of the 1 kb *cat* gene from pC194 into the *Pvu*II site (Ferrari *et al.*, 1983).

Fig. 1. Construction of plasmid pGMS57. pLS50 was deleted from the *NsiI*–*BstEII* fragment as detailed in Methods. The spectinomycin-resistance gene cassette was inserted into the resulting pGM56 at the *PstI* site. Thin black lines correspond to the original plasmid pBR322. The chloramphenicol-resistance gene was inserted at its *PvuII* site. H1 and H2 boxes represent *B. subtilis* regions flanking the *sacR*–*sacB* sequenced fragment (Steinmetz *et al.*, 1985).

were then blunted by *Pfu* DNA polymerase treatment and inserted into the *SrfI* site of pCR(+) vector according to the recommendations of the supplier (Stratagene). P_{sacB} was isolated after digestion with *Bam*HI and *Sph*I, and ligated into plasmid pCR(+) *amyE* digested with the same endonucleases to give an in-frame fusion between the *sacR* promoter region and *amyE*. The resulting plasmid was used to transform *Escherichia coli* XL-1 Blue. In the plasmids, purified from *E. coli* transformants, exhibiting fragments of the expected size after digestion by various endonucleases, we verified the in-frame fusion between the two DNA fragments by sequencing the amplified fragments, using the Sequenase kit (USB) and double-stranded plasmid DNA. The presence of active α -

amylase was assayed in the cell extracts of *E. coli* transformants. One appropriate plasmid pGM8 was selected and used for construction of strain GM96101.

Construction of strain GM96101. The *Bam*HI–*Nae*I fragment of pGM8 carrying the fusion of P_{sacB} to the *amyE* structural gene, was ligated between the *Bam*HI–*Eco*RV sites in pGMK50 (Petit-Glatron & Chambert, 1992) to give pGMK58, an integrative plasmid in which the *amyE* gene is under the control of P_{sacB} . pGMK58 was integrated by a Campbell-like mechanism into the chromosome of GM96100. The transformants were selected from LB plates containing the appropriate antibiotic. We confirmed the presence of the P_{sacB} –*amyE* fusion in the transformants by PCR using oligonucleotides A and D. One of the transformants containing the P_{sacB} –*amyE* fusion and exhibiting sucrose-inducible expression of α -amylase was chosen for further analysis and named GM96101.

Enzyme assays. The activity of levansucrase was assayed in an acetone/water mixture (50/50, v/v) in the presence of 50 mM sucrose at pH 6, as described previously (Chambert & Petit-Glatron, 1989). In these conditions one enzyme unit corresponds to 6 μ g pure protein. α -Amylase activity was assayed at 37 °C, using *p*-nitrophenyl-maltotriose as a substrate as recommended by the supplier (bio-Merieux, France). One enzyme unit corresponds to 25 μ g pure α -amylase. Phosphoglucose isomerase activity was monitored by A_{340} , using a Unicam UV2 spectrophotometer, as described by Noltmann (1966).

Gel electrophoresis and immunoblotting. Proteins were analysed by 10% (w/v) SDS-PAGE and the cell-associated forms of α -amylase were analysed by immunoblotting as described previously (Petit-Glatron *et al.*, 1987).

Pulse-chase experiments. In the usual conditions of growth, *B. subtilis* cells were induced with sucrose (60 mM final concentration) and pulse-labelled at $OD_{600} = 2$ by adding 0.25 mCi (9 mBq) [35 S]methionine (800 Ci mmol $^{-1}$) to 1 ml culture suspension maintained at 37 °C. After a pulse period of 45 s, non-radioactive methionine (4 mM final concentration) was added. Samples of 0.2 ml were withdrawn at intervals and all reactions were immediately stopped by diluting the samples threefold with ice-cold stopping buffer (0.1 M sodium phosphate, pH 7, containing 2.4 M KCl, 200 μ g

Fig. 2. Production of exocellular α -amylase by strain GM96101 and levansucrase by strain QB112 in the presence of various sucrose concentrations. Cells, grown in minimal medium supplemented with 1% glucose at 37 °C, pH 7, were induced at $OD_{600} = 0.3$ by sucrose at various concentrations. The differential rate of synthesis of α -amylase or levansucrase for each concentration of sucrose was evaluated from the amount of the enzyme released in the culture supernatant in respect to OD_{600} of the cell suspensions during the exponential phase of growth. ($\square$) α -Amylase produced by strain GM96101; ($\bullet$) levansucrase produced by strain QB112.

Fig. 3. Secretion of α -amylase in strain GM96101 and levansucrase in strain QB112 induced by sucrose. Cells non-induced or induced with sucrose (60 mM final concentration) were grown in minimal medium supplemented with 1% glucose and 0.5 mM calcium. At $OD_{600} = 1.5$, a 0.3 ml aliquot of each culture was pulse-labelled with 0.15 mCi [35 S]methionine and incubated for 10 min at 37 °C. Samples were centrifuged and supernatants were analysed by SDS-PAGE. Molecular mass standards are indicated on the right. Lanes: 1 and 2, GM96101 induced and non-induced; 3 and 4, QB112 induced and non-induced.

Fig. 4. Secretion of α -amylase during the cell growth of strain GM96101. Bacteria from strain GM96101 induced with 60 mM sucrose were grown in minimal medium supplemented with 1% glucose and 0.5 mM calcium at 37 °C. Samples were withdrawn at intervals, measured for their absorbance at 600 nm (●) and centrifuged. α -Amylase was assayed in the supernatants of each sample (□).

chloramphenicol ml⁻¹ and 0.2 mM PMSF). Cell suspensions were centrifuged and the supernatants were dialysed against TNE buffer (50 mM Tris/HCl, pH 8, containing 150 mM NaCl and 5 mM EDTA) overnight at 4 °C and diluted fivefold in TNET (TNE buffer containing 1%, v/v, Triton X-100). The bacterial pellets were washed with ice-cold stopping-buffer without KCl, resuspended in 0.3 ml TNES (TNE buffer containing 2%, w/v, SDS) and diluted fivefold in TNET. Cells were disrupted by sonication. The suspensions were incubated for 5 min at 95 °C. Antibodies against *B. subtilis* α -amylase (20 μ l) and 10%, w/v, Protein-A-Sepharose (80 μ l) (Sigma) in TNET were then added to 0.3 ml dialysed supernatants and to the disrupted cells. After overnight incubation at 4 °C, the immunoprecipitates were recovered by centrifugation. The pellets were washed three times with 1 ml TNET and finally resuspended in electrophoresis sample buffer. The samples were boiled for 3 min and analysed by SDS-PAGE.

In conditions that allow the conversion of cells to protoplasts (Merchante *et al.*, 1995), an exponential-phase culture ($OD_{600} = 2.5$) of *B. subtilis* induced with sucrose (60 mM final concentration) was resuspended in TMS buffer (50 mM Tris/HCl pH 7, 16 mM MgCl₂, 1 M sucrose, 1%, w/v, glucose) containing 200 μ g lysozyme ml⁻¹ (final $OD_{600} = 5$). After 1 h incubation at 37 °C with gentle shaking, the pulse-chase experiment was conducted as described above, except that the samples withdrawn at intervals were diluted threefold in ice-cold TMS buffer containing 200 μ g chloramphenicol ml⁻¹ and 0.2 mM PMSF. The supernatants were collected. Protoplasts were resuspended in a hypotonic solution (50 mM Tris/HCl, pH 7, 5 mM MgCl₂) and sonicated (three times for 30 s). Supernatant and protoplast fractions were treated as described above to obtain α -amylase immunoprecipitates, which were subsequently analysed by SDS-PAGE.

Fig. 5. Kinetics of the release of a processed precursor form of α -amylase. Bacteria of strain GM96101 were induced with 60 mM sucrose in the exponential phase of growth. At $OD_{600} = 1.5$, a 1 ml aliquot of the culture suspension was pulse-labelled for 45 s with 0.25 mCi [35 S]methionine and chased with a large excess of non-radioactive methionine (4 mM final concentration). Samples (0.2 ml) were removed at intervals, treated and analysed as described in Methods. (a) SDS-PAGE of labelled extracellular and cell-associated α -amylase. (b) Kinetics of the appearance of labelled extracellular α -amylase (□) and of disappearance of labelled cell-associated α -amylase (●). α -Amylase was quantified by cutting out and counting the radioactive bands from the SDS gel.

Fig. 6. Flow cytometry analysis of protoplast and intact cell populations. Strain GM96101 was induced by 60 mM sucrose under the usual culture conditions. At an OD_{600} of 2.5, cells were centrifuged and resuspended in TMS medium. The suspension was divided in two parts and lysozyme was added to one as described in Methods. After 60 min incubation at 37 °C, samples of intact cells (a) and protoplasts (b) were analysed by flow cytometry. The coordinates FSC and SSC indicate forward and side (90°) scatter. (c) Quantification of (a) and (b). (d) Observation of protoplasts by phase-contrast microscopy; bar, 5 μ m.

Flow cytometry analysis of protoplast formation. Samples for flow cytometry analysis were grown and treated in the conditions described above to obtain protoplasts and intact cells in the same conditions. The flow cytometer used was an Epics Elite ESP flow cytometer (Coulter) equipped with a 488 nm argon ion laser. Standard Epics Elite software was used to quantify the samples. Forward and right-angle scatters were measured on separate detectors with logarithmic gains, the threshold being set to eliminate particles that were much smaller than intact cells or protoplasts.

Unfolding–folding transition. The stock solution of purified α -amylase produced by strain GM96101 was diluted 10-fold in 6 M guanidine hydrochloride, 0.1 M sodium phosphate pH 7, 1 mM EDTA. The unfolding reaction was allowed to proceed for the time specified in the text. For refolding, the guanidine hydrochloride α -amylase solution was diluted 50-fold in 0.1 M sodium phosphate (pH 7) at 37 °C, containing 0.5 mM calcium chloride or 0.5 mM EDTA. Aliquots were removed at intervals, and unfolded α -amylase was digested with subtilisin (50 μ g ml⁻¹) for 10 min at 37 °C. The non-proteolysed α -amylase in each sample was analysed by immunoblotting and assay of α -amylase activity.

Amino acid sequencing of α -amylase. The NH₂-terminal sequence analysis of α -amylase purified from the culture

supernatant was determined by the Edman degradation procedure (Bauw *et al.*, 1989) with a gas-phase sequencer (model 470A; Applied Biosystems) equipped with an on-line phenylthiohydantoin amino acid derivative analyser (model 120A).

RESULTS

α -Amylase overproduction by strain GM96101 is induced by sucrose

The differential rate of α -amylase synthesis was measured during the exponential phase of growth at increasing sucrose concentrations up to 60 mM (2%, w/v) in strain GM96101 grown in minimal medium supplemented with 1% (w/v) glucose and 0.5 mM calcium. Production of α -amylase increased as a function of sucrose concentration (Fig. 2). As for levansucrase, full induction was obtained at 30 mM. The GM96101 strain produces 100-fold more α -amylase than the parent strain and approximately threefold less than the levansucrase produced in strain QB112. In strain GM96101 induced by sucrose, α -amylase was the main protein present in the supernatant as can be observed from pulse

Fig. 7. Kinetics of α -amylase released by protoplasts. The pulse-chase experiment was carried out on protoplasts obtained as described in Methods. (a) Immunoprecipitates of protoplast supernatants and protoplasts were analysed by SDS-PAGE. (b) Kinetics of the appearance of labelled extracellular α -amylase.

experiments, as was the case for levansucrase in strain QB112 (Fig. 3) (Chambert & Petit-Glatron, 1984). In the absence of sucrose, the protein content of the supernatants of strains GM96101 and QB112 are very low.

We observed that α -amylase secretion occurs in the supernatant predominantly during the exponential phase (Fig. 4). Furthermore, the protein remained stable in the culture supernatant. We can conclude from these results that α -amylase overproduction in strain GM96101 is subject to the same regulatory control as levansucrase in strain QB112.

The molecular mass of the secreted α -amylase was 69 kDa (Fig. 3), which is slightly higher than that of α -amylase isolated from supernatant of *B. subtilis* wild-type strain in the late stationary phase (68 kDa) (Haddaoui *et al.*, 1995; Takase *et al.*, 1988). We analysed the N-terminal sequence of the protein produced during the exponential phase of growth.

NH₂-terminal sequence analysis

The NH₂-terminal sequence of the isolated exocellular α -amylase was determined. Most of the released protein had glutamic acid as the NH₂-terminal residue (residue 34 in the deduced nucleotide sequence; Yang *et al.*, 1983), and this residue comes immediately after the signal peptidase cleavage site (Takase *et al.*, 1988). This result is consistent with the absence of a further covalent modification of the protein after its release. Under stationary phase growth conditions, it was previously shown that the protein is subject to a subsequent

Fig. 8. Kinetics of *B. subtilis* α -amylase unfolding–refolding measured by the resistance to proteolysis at 37 °C, pH 7. Unfolding was promoted (arrow 1) by mixing 3 μ l pure α -amylase (stock solution 1 mg ml⁻¹) with 27 μ l 6 M guanidine hydrochloride in 0.1 M sodium phosphate, pH 7, containing 1 mM EDTA. Samples of 3 μ l were withdrawn at the times indicated and quickly mixed with 165 μ l subtilisin solution (50 μ g ml⁻¹) in sodium phosphate, pH 7, containing 0.5 mM CaCl₂. Refolding was initiated (arrow 2) after 3 min of unfolding by mixing 20 μ l unfolding mixture with 1 ml of 0.1 M sodium phosphate, pH 7, containing 0.5 mM CaCl₂ or 0.5 mM EDTA. Samples (150 μ l) were withdrawn at the times indicated and quickly mixed with 15 μ l of a 1 mg subtilisin ml⁻¹ solution, incubated for 10 min. PMSF was then added to the samples. Aliquots of 115 μ l of all the samples were subjected to SDS-PAGE and immunoblotting. Aliquots of 50 μ l were assayed for α -amylase activity. (a) Immunoblot of refolding in the presence of calcium; (b) α -amylase activity: dilution of the denaturant in the presence of calcium (○) or EDTA (●).

proteolysis of its N-terminal part (Takase *et al.*, 1988). Our data confirm that this step is not related to the secretion process.

Secretion of α -amylase analysed by a pulse–chase experiment in strain GM96101

We did not detect a precursor form of α -amylase that included the signal peptide from a pulse–chase experiment (Fig. 5a). This result suggests that the first step of the secretion pathway, which is the cleavage of the signal peptide, either occurs cotranslationally or is a very fast reaction. A cell-associated form with the same molecular mass as the exocellular form slowly disappeared from the cells, however, and labelled α -amylase was concomitantly released in the supernatant with similar kinetics. The mean value of $t_{1/2}$ of release was evaluated to 120 ± 10 s. The yield of the release as

Fig. 9. Effect of EDTA on the production of extracellular α -amylase. (a) SDS-PAGE of labelled extracellular α -amylase from a pulse-chase experiment conducted as described in Methods. EDTA (0.5 mM) was added at the beginning of the chase period. A, in the presence of EDTA; B, control. (b) Kinetics of the appearance of labelled extracellular α -amylase quantified by cutting out and counting the radioactive bands from the SDS gel in cells grown in the presence (●) or absence (○) of 0.5 mM EDTA. (c) Growth (circles) and production of α -amylase in supernatants (squares) of cells grown in the presence (●, ■) or absence (○, □) of 0.5 mM EDTA.

evaluated from radioactivity counts was almost 100%. These data indicated that the processed cell-associated α -amylase is a transient precursor in the secretion process and is released with a high efficiency. This precursor form was strongly bound to the cells, since it remains attached despite a high-ionic-strength washing

of the cells before preparation of cell extracts. The question thus arises as to whether the kinetics of the release of the precursor form was the same when the cells were converted to protoplasts in which the cell-wall matrix has been severely disrupted (Graham & Beveridge, 1994).

α -Amylase secretion by protoplasts

Formation of protoplasts. Protoplasts were obtained following the method of Merchante *et al.* (1995) modified as described in Methods. The formation of protoplasts was monitored by phase-contrast microscopy. Intact cells and protoplasts were clearly discriminated by flow cytometry analysis (Fig. 6). After 1 h incubation at 37 °C, the cells were fully converted to protoplasts. At this time, the production of extracellular α -amylase in the supernatant of protoplast suspension was only 55% of that of intact cells in the same medium (in the absence of lysozyme) at the same optical density. This decrease was not due to the cell lysis occurring during protoplast formation, as measured by the presence of less than 10% of total phosphoglucose isomerase, a cytoplasmic marker, in the protoplast supernatant. To understand why cell-wall disruption significantly reduced α -amylase production, we studied the kinetics of α -amylase release by protoplasts in a pulse-chase experiment.

Kinetics of α -amylase release. The $t_{1/2}$ of α -amylase appearance in the supernatants of protoplasts was 50 s (Fig. 7). Most of the enzyme released by the protoplasts was identical to that secreted by intact cells and a small fraction migrated faster. Nevertheless, about one-third of the labelled protein remained associated with the protoplasts, irrespective of the chase kinetics. Surprisingly, the labelled α -amylase that was not secreted into the external medium migrated like the 68 kDa α -amylase species. This could reflect the effects of protease degradation during the last step of the secretion process or the fractionation process, due to a greater protease sensitivity of this form compared to the species found in the extracellular medium. This effect could also be the result of an inefficient folding of the protein caused by the loss of cell-wall integrity, perhaps because the processed precursor form undergoes conformational modification during the last step of secretion. To confirm this idea, we studied the kinetics of the *in vitro* unfolding–folding transition of α -amylase under the same conditions of pH and temperature as those used for bacterial growth (pH 7, 37 °C).

Kinetics of unfolding–refolding of α -amylase *in vitro*

The unfolding–folding transition was measured by monitoring the appearance and disappearance of the subtilisin-sensitivity of the protein (Fig. 8). In the presence of 6 M guanidine hydrochloride, at pH 7, the unfolding of α -amylase was very rapid. The folding–unfolding transition was almost totally reversible after dilution of the denaturing agent. The $t_{1/2}$ for refolding was 60 s. No refolding was observed in the absence of

calcium. We noticed that the folding reaction and the final release step of secretion have similar time courses.

Efficiency of the ultimate stage of α -amylase release is dramatically decreased in the presence of a metal chelator

To confirm the correlation between the efficiency of α -amylase release and the ability of the protein to fold rapidly in the presence of calcium, we tested the effect of a metal chelator on the kinetics and the yield of the ultimate stage of α -amylase release. The pulse-chase conditions were the same as those used above, except that EDTA (0.5 mM final concentration) was added at the beginning of the chase period at the same time as non-radioactive methionine. We observed that the yield of the labelled protein release was negligible under these conditions when compared with the control (Fig. 9a, b). This effect was not due to a side effect of EDTA, since bacterial growth was not affected during the experiment and the stability of α -amylase secreted before the addition of EDTA remained unchanged (Fig. 9c).

DISCUSSION

We report here on the secretion of *B. subtilis* α -amylase overproduced in a *degU32(Hy)* genetic context, under conditions similar to those of levansucrase. Inducible production of α -amylase was obtained from the expression of the *amyE* gene under the control of the leader region of levansucrase at a level that allows us to compare their secretion kinetics.

Secretion of overproduced α -amylase by the *B. subtilis* *degU32(Hy)* strain appears to be a multi-step process, whose rate-limiting step was the release of the signal-peptidase-processed form of the protein. The secretion pathway of α -amylase has features in common with that of levansucrase (Chambert & Petit-Glatron, 1988; Chambert *et al.*, 1995). Both involve separate steps in the processing of the signal peptide and the release of the protein and, in each case, the rate-limiting step is the final step of the process. The kinetic parameters of this step were of the same order of magnitude as those of the folding reaction *in vitro* of levansucrase (Chambert *et al.*, 1995). This sequential mechanism for protein secretion in *B. subtilis*, in which the intrinsic folding properties of secreted proteins play an important role, is possibly based on a common mechanism. The main differences between the α -amylase and levansucrase secretion pathways are firstly the rate of processing of the signal peptide, which is very rapid (or cotranslational) in the case of α -amylase, since we have never demonstrated the existence of a transient non-processed precursor form. Under the same conditions, a levansucrase precursor form with a non-processed signal peptide disappeared with a $t_{1/2}$ of 5 s (Petit-Glatron *et al.*, 1987). Secondly, the higher $t_{1/2}$ reaction of α -amylase release by intact cells was double that of levansucrase (Petit-Glatron *et al.*, 1987). One of the reasons could be the reduced capacity of high-molecular-mass proteins to

pass through the cell wall (Koch, 1995). From the pulse-chase experiments achieved with lysozyme-treated cells (protoplasts), which have severely disrupted cell-wall ultrastructure (Graham & Beveridge, 1994), it could be concluded that cell-wall integrity is required for efficient secretion, since the yield of the protein release in the extracellular medium is lower than that of intact cells. This could favour a role of a cell-wall component in maintaining high concentrations of divalent calcium ions on the external side of the membrane (Petit-Glatron *et al.*, 1993). The latter ion could act as a folding catalyst for α -amylase and levansucrase (Chambert *et al.*, 1995).

These results all indicate that the cell wall is an active partner in the secretion process in *B. subtilis* and not just a limited porosity barrier as it has often been considered to be (Archibald, 1989). In fact, several arguments have been put forward concerning the functional analogy between the Gram-negative outer membrane and the Gram-positive cell wall (Pooley *et al.*, 1996; Beveridge, 1995). Consequently, proteins 'passing through' the thick cell wall in a partially or totally folded state might be subjected to transient physico-chemical association with components of the cell wall. In the case of α -amylase, the higher $t_{1/2}$ of the release step as compared to that of levansucrase could result in a different affinity for one or several components of the cell wall. Whether these components are part of a secretion apparatus *per se* could constitute the basis of a working hypothesis.

ACKNOWLEDGEMENTS

We would like to thank W. Schuman for providing plasmid pAMY10, M. C. Gendron for performing flow cytometry experiments at the Jacques Monod Institute, and F. Denizot for the communication of unpublished sequences. We are grateful to A. Kropfinger for revision of the English text. We thank members of the EBSG (European *Bacillus* Secretion Group) for very helpful discussions. This work was supported in part by EEC grant (BIO4 CT-960097).

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Received 8 April 1997; revised 7 June 1997; accepted 11 June 1997.