The influence of protein folding on late stages of the secretion of α-amylases from *Bacillus subtilis*

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Abstract A derivative of the α-amylase from *Bacillus licheniformis* (AmyL) engineered to give an active enzyme with increased net positive charge is secreted by *Bacillus subtilis* with a yield that is significantly lower than that of the native enzyme. This reduction in yield is the result of increased proteolysis during or shortly after translocation through the cytoplasmic membrane. When we compared the overall rate of folding of the engineered derivative (AmyLQS50.5) with that of AmyL it exhibited a greater dependency on Ca^{2+} ions for in vitro folding. When the concentration of Ca^{2+} in the growth medium was increased, so too did the relative yield of AmyLQS50.5. We discuss the importance of secretory protein folding at the membrane/cell wall interface with respect to the yield of native and heterologous proteins from *B. subtilis*.

Key words: Protein secretion; Folding; Cell wall; Calcium ion

1. Introduction

To be competent for translocation across the cytoplasmic membrane, secretory preproteins are prevented from folding into their native conformation in the cytosol of the cell. In *Escherichia coli* this secretion competent state is maintained by soluble cytosolic proteins, termed molecular chaperones, such as SecB [1]. Additionally, there is evidence that the membrane-associated translocation ATPase, SecA, prevents secretory preproteins forming elements of their tertiary structure prior to translocation [2]. During export, secretory preproteins are translocated across the cytoplasmic membrane via the secretory translocase.

Following translocation, secretory proteins fold into their native conformation on the *trans* side of the membrane in a process that is rate limiting for secretion and accompanies the release of the protein from the membrane [3]. In Gram-positive bacteria such as *B. subtilis*, this step is assisted by proteins such as PrsA, a lipoprotein linked to the outer surface of the cytoplasmic membrane which appears to mediate the rapid and efficient folding of secretory proteins into their native conformations [4,5]. In addition, some secretory proteins require cations such as H^+, Ca^{2+} and Fe^{3+} at the membrane-wall interface to promote their folding at this location [3,6].

As a consequence of the turgor pressure generated within the cell the cytoplasmic membrane is likely to be in close contact with the cell wall as secretory proteins emerge from the translocase complex. The *B. subtilis* cell wall is composed of a complex of heteropolymeric peptidoglycan with covalently linked anionic polymers, teichoic acid or teichuronic acid, which confer a high degree of negative charge to the wall [7]. Appropriate concentrations of cations such as Ca^{2+} and Fe^{3+} at the membrane-wall interface are maintained by a combination of the properties of the wall- and membrane-linked lipoteichoic acids [8–10].

The microenvironment into which secretory proteins are translocated is maintained by the cell wall and the proton motive force. Together they contribute to the physico-chemical conditions that are required for rapid and efficient folding of secretory proteins. In addition to influencing the metal cation binding capacity, the charge properties of the cell wall influence the degree to which secretory proteins interact with, and are retarded by, the wall cylinder. Secretory proteins with little or no net charge pass through the wall unretarded [11], while those with an overall positive charge would be expected to be retained within the wall cylinder as a result of electrostatic interactions with negatively charged groups.

To investigate the influence of protein charge on late stages in secretion, we constructed a number of chimeric α-amylases with altered net charge. These proteins were based on AmyL, the α-amylase (EC number 3.2.1.1) of *B. licheniformis* [12,13]. The genes encoding the chimeric α-amylases were constructed using a PCR-based gene splicing technique to splice together specific regions of three related *Bacillus* α-amylases; AmyL, AmyQ (*B. amyloliquefaciens*) and AmyS (*B. stearothermophilus*). In this paper we compare late stages in the secretion of wild-type AmyL (p*I* 7.0) with those of a positively charged chimeric α-amylase, AmyLQS50.5, which has a p*I* of 10.0. Since Ca^{2+} ions are required for the enzymatic activity and structural integrity of α-amylases, we also investigated the role of this cation in the folding of the α-amylases. The data provide an insight into co-/post-translocational stages in the secretion of proteins from *B. subtilis*, particularly with respect to the importance of folding and their interaction with the cell wall.

2. Materials and methods

2.1. Bacterial strains and growth conditions

The α-amylase negative (AmyE) *B. subtilis* strain DN1885 [14] was used as the host for the expression of the wild-type α-amylase from *B. licheniformis* (AmyL, strain DN1885 *xylR*::pKS408) and a chimeric α-amylase with increased net positive charge (AmyLQS50.5, strain DN1885 *xylR*::pKS405B). The α-amylases were expressed from a

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xylose-inducible expression cassette integrated into the *xylR* locus of the *B. subtilis* chromosome [12].

For the determination of α-amylase released into the medium, *B. subtilis* strains were grown in 2×YT medium (1.6% w/v tryptone, 1% w/v yeast extract and 0.5% NaCl). For pulse-labelling, *B. subtilis* strains were grown in Spizizen's minimal salts (SMS; [15]) with 1% w/v ribose as a non-catabolite repressing carbon source. Where required xylose (1% w/v) was included to induce the synthesis of α-amylase. In all cases cultures were grown in an orbital incubator at 37°C with shaking (180 rpm).

2.2. Pulse-labelling of exponentially growing B. subtilis

Cultures were grown in SMS/ribose/xylose to an optical density at 660 nm (OD_{660}) of ∼0.9. The exponentially growing cultures were pulse-labelled with L-[^{35}S]methionine for 1 min and chased with a vast excess of unlabelled methionine [16]. Whole culture (cells+medium) and medium samples were collected at time intervals following the chase and labelled proteins precipitated with trichloroacetic acid. α-Amylases were recovered by immunoprecipitation with a polyclonal anti-AmyL antiserum and subjected to sodium lauryl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and fluorography.

2.3. Determination of α-amylase activity in the culture medium

Cultures of *B. subtilis* were grown in 2×YT/xylose and samples removed at various times for the determination of growth (OD_{660}) and α-amylase activities. $CaCl_2$ was added to the medium to a final concentration of 10 mM or 30 mM. Cells were removed by microcentrifugation and the α-amylase activity in the medium of duplicate samples was determined using the Phadebas α-amylase assay kit (Kabi Pharmacia).

2.4. Unfolding and refolding of α-amylases

AmyL and AmyLQS50.5 were purified from *B. subtilis* culture medium by preparative isoelectric focusing, as described previously [12]. α-Amylases were unfolded with 6 M Guanidine.HCl/1 mM EDTA (pH 7.0) at 47°C for 90 min. Refolding was initiated at 25°C by diluting the denaturant with acetate buffer (0.1 M sodium acetate, pH 7.0) containing 0.5 mM EDTA or the following concentrations of $CaCl_2$: 0.5 mM, 25 mM and 50 mM.

The unfolding-refolding transitions of AmyL and AmyLQS50.5 were followed by measuring variations in intrinsic tryptophan fluorescence using a Hitachi F2000 thermoregulated spectrofluorometer. Wavelengths were fixed at 283 nm for excitation and 340 nm emission.

2.5. Refolding of α-amylases in the presence of isolated cell walls

To determine the influence of the cell wall on the refolding of α-amylases, AmyL and AmyLQS50.5 were unfolded as described above and then refolded in acetate buffer (pH 7.0 and containing 20 mM $CaCl_2$) in the presence (10 mg/ml) and absence of *B. subtilis* cell walls, isolated as described previously [17]. Samples were removed at time intervals following the initiation of refolding and the amount of α-amylase in the fully folded (protease resistant) conformation determined by protease sensitivity [6].

2.6. α-Amylase calcium binding assay

The binding of Ca^{2+} to AmyL and AmyLQS50.5 in their native conformations was assayed on a nitrocellulose membrane by quantitative dot-blot with $^{45}CaCl_2$ [18]. The binding of Ca^{2+} to ovalbumin was used as a negative control. The amount of bound $^{45}CaCl_2$ was quantified by phospho-imaging (Molecular Dynamics Phospho-Imager).

3. Results

3.1. Secretion of wild-type and chimeric α-amylases

The secretion of AmyL and AmyLQS50.5 from exponentially growing *B. subtilis* DN1885 was studied using coupled pulse-chase and immunoprecipitation techniques with subsequent SDS-PAGE. Following autoradiography, the precursor and mature forms of both α-amylases were clearly visible (Fig. 1A, B). The AmyL precursor was processed to the mature form by 5 min post-chase (Fig. 2A). In contrast, the rate of processing of AmyLQS50.5 was much reduced and the

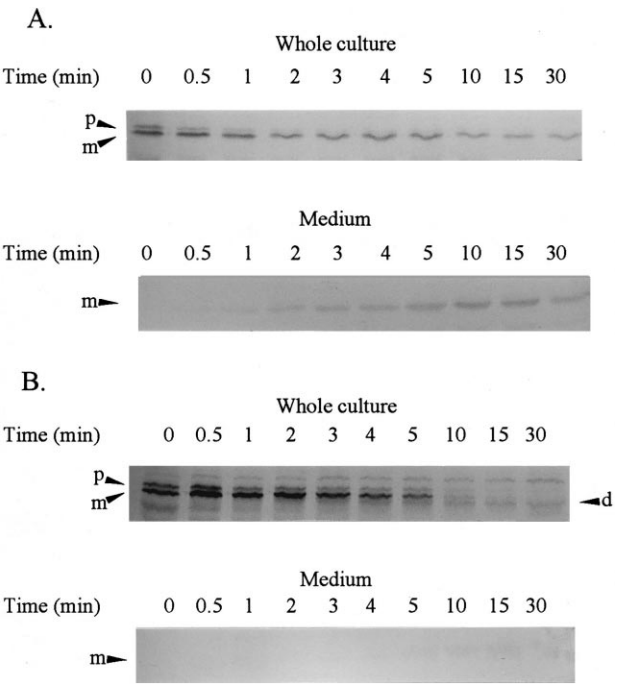

Fig. 1. Secretion of α-amylases from exponentially growing *B. subtilis*. Autoradiographs of representative data from pulse-chase experiments carried out on strains (A) DN1885 *xylR*::pKS408 (AmyL) and (B) DN1885 *xylR*::pKS405B (AmyLQS50.5). Precursor (p), mature (m) and degraded mature (d) forms of AmyL and AmyLQS50.5 immunoprecipitated from whole culture and medium samples are indicated.

AmyLQS50.5 precursor was detectable (∼1% of the total synthesized during the pulse) even at 15 min following the chase (Fig. 2B). Precursor AmyLQS50.5 could not be detected at 30 min post-chase (Fig. 2B).

The amount of mature AmyL in whole culture samples (cells+growth medium) peaked at 1 min post-chase and then declined until it reached a constant level of approximately 20% of the maximum synthesized during the pulse (Fig. 2A). Mature AmyLQS50.5 in whole culture samples peaked at 30 s post-chase and then decreased rapidly until, by 30 min post-chase, no mature protein could be detected and a major degradation product was visible (Fig. 2B). These data confirm

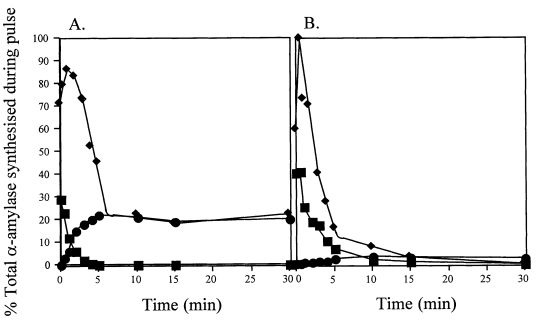

Fig. 2. Quantitation of precursor and mature forms of AmyL (A) and AmyLQS50.5 (B) during secretion. The relative amounts of precursor (■) and mature (♦) α-amylases in whole culture samples and mature α-amylase released into the growth medium (●) were quantified by phospho-imaging and expressed as a percentage of the total α-amylase (precursor+mature) synthesized during the pulse.

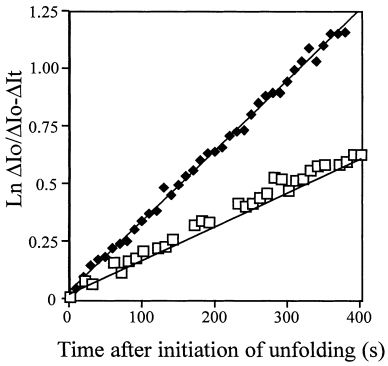

Fig. 3. Kinetics of unfolding of α-amylases. The α-amylases were unfolded in 6 M Guanidine.HCl/1 mM EDTA and the unfolding transition of AmyL and AmyLQS50.5 followed by changes in fluorescent intensity. The rate-constants of unfolding of AmyL (□) and AmyLQS50.5 (♦) were determined from changes in fluorescent intensity and expressed as a semi-logarithmic plot. ΔI_o represents the maximum change in fluorescent intensity during the unfolding reaction and $\Delta I_o - \Delta I_t$ the fluorescent intensity change at a particular time point.

that both AmyL and AmyLQS50.5 were subject to considerable co-/post-translocational proteolytic degradation and are in good agreement with our previous observations [12,13].

Both AmyL and AmyLQS50.5 were released into the medium with equivalent kinetics ($t_{1/2}$ ~4 min, Fig. 2A, B). However, the yield of the chimeric α-amylase reaching the medium in an intact form was reduced to 10% of AmyL as a consequence of the extensive cell-associated proteolytic degradation of this α-amylase (Fig. 2B), and this was confirmed by assaying the α-amylase activity directly (data not shown).

3.2. Unfolding-refolding of α-amylases

In their native conformations and in the presence of the range of proteases secreted by *B. subtilis*, AmyL and AmyLQS50.5 are stable in culture medium for prolonged periods of time [12,19]. This observation, combined with the extensive cell-associated degradation of the α-amylases during secretion (Figs. 1 and 2) and the comparable release kinetics of the α-amylases ($t_{1/2}$ ~4 min), indicates that there is time window between translocation and release into the culture medium of approximately 8 min during which the α-amylases were susceptible to the activity of cell-associated *B. subtilis* proteases, with AmyLQS50.5 affected to a greater extent.

Table 1
Binding of $^{45}CaCl_2$ to proteins at pH 6.0 and pH 7.0

	pH 6.0	pH 7.0
AmyLQS50.5	37.4%	50.1%
Ovalbumin	2.7%	5.6%

The amount of $^{45}CaCl_2$ bound to AmyL, AmyLQS50.5 and ovalbumin at different pH values was quantified using phospho-imaging and expressed as a percentage of that bound by wild-type AmyL.

We argue that a secretory protein with a less efficient folding event on the *trans* side of the membrane is likely to be subjected to more extensive degradation due to the time taken to fold into its native and therefore protease resistant conformation. This hypothesis was tested in vitro by comparing the unfolding-refolding transition kinetics of AmyL and AmyLQS50.5.

The α-amylases were unfolded in the presence of 6 M Guanidine.HCl/1 mM EDTA and the reaction followed by measuring changes in intrinsic tryptophan fluorescence. The rate-constants of unfolding of AmyL and AmyLQS50.5, obtained from semi-logarithmic plot (Fig. 3), show that there was a 3-fold increase in the rate of unfolding of the chimeric α-amylase (0.003 s^{-1}) when compared with AmyL (0.001 s^{-1}). This is indicative of disruptions in the structural integrity of AmyLQS50.5 as compared with AmyL and confirms our previous observations [12].

The α-amylases were refolded at different concentrations of Ca^{2+} and the rate-constants of refolding obtained from semi-logarithmic plots (Fig. 4). In the absence of free Ca^{2+} (i.e. presence of 0.5 mM EDTA) no refolding of AmyLQS50.5 was observed and AmyL exhibited only a small degree of refolding (data not shown). Within the range 0.5 to 50 mM, Ca^{2+} had a negligible effect on the rate-constant of refolding of AmyL which remained constant at 0.018–0.019 s^{-1} (Fig. 4). In contrast, the rate-constant of refolding of AmyLQS50.5 was dependent on Ca^{2+}; at low concentrations (0.5 mM) the rate-constant of refolding (0.006 s^{-1}) was 3-fold lower than that of AmyL (0.018 s^{-1}, Fig. 4). However, when the Ca^{2+} concentration was increased to 25 mM the rate-constant of refolding of AmyLQS50.5 increased to 0.013 s^{-1} (Fig. 4). When the concentration of Ca^{2+} was increased to 50 mM the rate-constants of refolding of AmyL and AmyLQS50.5 were identical at 0.019 s^{-1} (Fig. 4). These data reveal that within the range 0.5–50 mM Ca^{2+}, the rate of refolding of the chimeric α-amylase (AmyLQS50.5) is strongly dependent upon the amount of available Ca^{2+}. Only at a concentration of

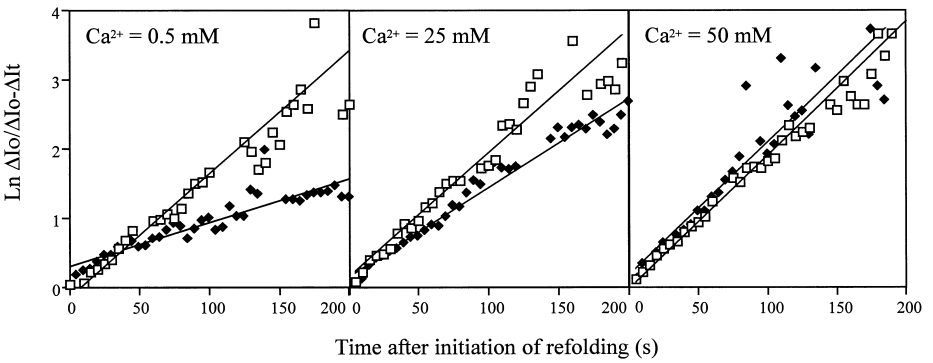

Fig. 4. Kinetics of refolding of α-amylases at different concentrations of calcium. Fluorescent intensity changes were used to construct semi-logarithmic plots which represent the rate-constants of refolding of AmyL (□) and AmyLQS50.5 (♦) at different calcium concentrations. For clarity the raw fluorescent intensity data have not been included.

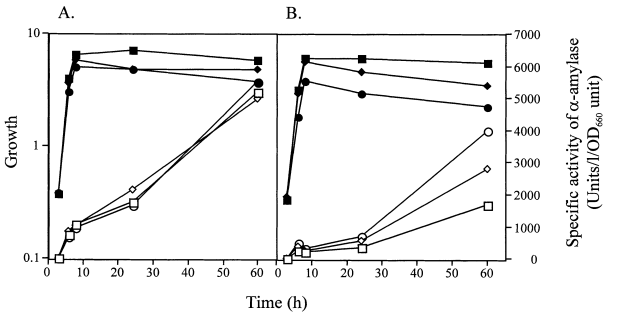

Fig. 5. Yield of α-amylase released into the medium of cultures grown in the presence of different concentrations of calcium. Closed symbols represent growth (OD_{660}) and open symbols specific activity of α-amylase. Cultures of *B. subtilis* DN1885 *xylR*::pKS408 (A) and DN1885 *xylR*::pKS405B (B) were grown in 2×YT/xylose without added $CaCl_2$ (■) and in the presence of 10 mM (◆) or 30 mM $CaCl_2$ (●). One unit of α-amylase activity was defined as the amount of enzyme catalyzing the hydrolysis of 1 μmol glycosidic linkage per min at 37°C.

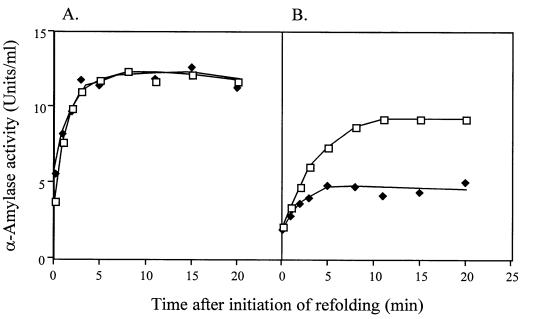

Fig. 6. Kinetics of refolding of α-amylases in the presence of cell wall. AmyL (A) and AmyLQS50.5 (B) were refolded in the presence (◆) or absence (□) of cell wall isolated from *B. subtilis* and the refolding transition monitored by measuring the increase in α-amylase activity with time which accompanies folding into the native conformation.

50 mM did AmyLQS50.5 refold at a rate equivalent to that of the wild-type enzyme AmyL. In addition to exhibiting a more calcium dependent rate-constant of refolding, the yield of folded AmyLQS50.5 was reduced to 50% with respect to AmyL (data not shown).

3.3. Binding of calcium to α-amylases

The degree to which Ca^{2+} binds to AmyL and AmyLQS50.5 was determined by measuring the amount of $^{45}CaCl_2$ bound to purified proteins in their native conformations at different pH values (Table 1). At pH 6.0 or pH 7.0 the amount of Ca^{2+} bound by AmyLQS50.5 was considerably less than that bound by AmyL, with the chimeric enzyme binding only 37.4% or 50.1%, of the Ca^{2+} bound by the wild-type α-amylase, respectively. The binding of Ca^{2+} to a control protein, ovalbumin, remained low ($<6\%$ of AmyL) as expected.

3.4. Yield of α-amylases from B. subtilis grown in the presence of different concentrations of Ca^{2+}

Since we had demonstrated an influence of calcium ions on the in vitro rate of folding of the chimeric α-amylase, we were interested to know whether Ca^{2+} affects the yields of this enzyme in vivo. We therefore determined the yield of α-amylases released into the medium of cultures grown at different concentrations of $CaCl_2$. In the absence of added Ca^{2+} the yield of AmyLQS50.5 released into the culture medium of *B. subtilis* DN1885 *xylR*::pKS405B at 60 h (Fig. 5B) in an active form was only 33% that of *B. subtilis* DN1885 *xylR*::pKS408 (Fig. 5A).

Increasing the Ca^{2+} concentration of the growth medium of DN1885 *xylR*::pKS405B had a marked affect on the yield of AmyLQS50.5 released into the medium in an active form (Fig. 5B) but had little or no affect on the amount of AmyL released from DN1885 *xylR*::pKS408 (Fig. 5A). Growth in the presence of 10 mM Ca^{2+} resulted in a 66% increase in the yield of AmyLQS50.5 which was increased even further (137%) when the concentration was increased to 30 mM. In contrast, growth in the presence of 10 mM Ca^{2+} had a negligible effect on AmyL and 30 mM Ca^{2+} resulted in only a 6% increase in the yield of released α-amylase (Fig. 5A). In addition, the concentration of Ca^{2+} added to the growth medium influenced the final culture density, with higher concentrations resulting in lower culture densities (Fig. 5A, B). This effect of $CaCl_2$ was not investigated.

3.5. The influence of the B. subtilis cell wall on late stages in the secretion of α-amylases

We investigated the refolding of AmyL and AmyLQS50.5 in the presence or absence of cell walls isolated from *B. subtilis*. The method used for monitoring the unfolding-refolding transition was the resistance of α-amylases at different folding states to the activity of an endogenously added protease [6]. This method was selected because the cell wall material itself displayed a significant fluorescent signal at the wavelengths used to measure intrinsic tryptophan fluorescence and masked the refolding signals of the α-amylases (data not shown).

The presence of isolated cell wall in the refolding buffer had little or no affect on the refolding of AmyL (Fig. 6A). However, when AmyLQS50.5 was refolded in the presence of cell wall, the wall prevented approximately 50% of the α-amylase reaching the native (i.e. enzymatically active) conformation (Fig. 6B). These data suggest that the unfolded form of AmyLQS50.5 was in part irreversibly bound to the cell wall or that this α-amylase was partially misfolded in the presence of wall, in agreement with observations from in vivo experiments [11].

4. Discussion

Late (co-/post-translocational) events in the secretion of proteins from *B. subtilis* and related commercially important organisms are central in determining the quality and final yield of secretory proteins released into the culture medium. Consequently, for efficient secretion, the environment into which a secretory protein is released from the membrane must promote rapid and efficient folding. Additionally, for secretory proteins destined for truly extracellular locations, their physico-chemical properties must be such as to minimize interactions with the negatively charged cell wall.

Pulse-chase techniques showed that both AmyL and AmyLQS50.5 were susceptible to proteolytic degradation, although the chimeric α-amylase was affected to a significantly larger extent. Using a combination of in vivo and in vitro techniques we have shown that this increased susceptibility to proteases is, at least in part, a consequence of a

reduced rate of folding of AmyLQS50.5 on the *trans* side of the membrane. We have recently shown that this degradation occurs in a cell-associated location and that a cell wall-associated protease, CWBP52, is involved [19].

The folding of AmyL was unaffected by Ca^{2+} concentration whereas at low concentrations, Ca^{2+} was rate limiting for the folding of AmyLQS50.5. Three amino acid residues constitute the calcium binding site of AmyL, namely asparagine-104, aspartate-200 and histidine-235 [20]. These residues are conserved in AmyLQS50.5 but, as is the case for *B. subtilis* levansucrase [10], AmyL may possess additional, as yet unidentified, low affinity binding sites which were altered during the construction of the chimeric α-amylase. What is clear is that AmyLQS50.5 requires considerably more Ca^{2+} to achieve the same rate of folding as AmyL, possibly because these low affinity binding sites are involved in intermediate stages in the folding pathway. More importantly, it appears that the ion concentration maintained in vivo by the cell wall [10] is not sufficient to overcome this problem completely. The lower yields of the chimeric α-amylase were partially overcome by increasing the concentration of Ca^{2+} in the culture medium and this is likely to be directly related to the increased calcium dependency of the AmyLQS50.5 folding pathway.

The proximity of the cytoplasmic membrane to the cell wall dictates that some degree of interaction must occur between unfolded/partially folded secretory proteins and components of the cell wall. We have shown that the wall has the capacity to affect the yield of refolding of the positively charged AmyLQS50.5 (p*I* 10.0) protein whilst having no overall affect on AmyL (p*I* 7.0), which is neutral in terms of charge. The cell wall also appears to have the capacity to bind large amounts of AmyLQS50.5 when in its fully folded conformation on the basis of electrostatic interactions, whereas little or no binding was observed for AmyL [12]. We have not been able to demonstrate this in vivo.

Our data suggest that the changes engineered into AmyLQS50.5 reduce its affinity for Ca^{2+} and its folding into its native (and protease resistant) conformation. We do not yet know whether the latter is a direct consequence of the former. The subsequent increase in the time taken to fold, however, appears to make AmyLQS50.5 more sensitive to proteolytic degradation. The slower processing of the AmyLQS50.5 precursor into the mature form may contribute to, or be a consequence of, the extended time window for degradation. Further, some degree of wall binding would also be expected for AmyLQS50.5 both during folding and after the native conformation has been obtained, although in the latter case the α-amylase would eventually be released as a consequence of the wall turnover which accompanies growth [21]. In vivo the binding of AmyLQS50.5 to the cell wall in unfolded and partially/completely refolded conformation is likely to increase proteolysis by localizing the protein in the vicinity of cell-associated proteases such as CWBP52. These factors ultimately have the effect of reducing the yield of AmyLQS50.5 released into the medium in an active form.

It is likely that native *Bacillus* secretory proteins have evolved physico-chemical properties that facilitate their rapid and efficient folding on the *trans* side of the membrane while reducing their interactions with components of the cell wall. AmyL itself is not native to *B. subtilis* and this fact may be reflected in the significant cell-associated degradation that this protein undergoes. The high (grams per litre concentrations) secretion capacity of *B. subtilis* and its relatives [22] has meant that these bacteria are important sources of enzymes with commercial applications (e.g. α-amylases, proteases and lipases). However, attempts to secrete non-native proteins to commercially significant levels have largely been unsuccessful. Although AmyLQS50.5 was constructed from three related *Bacillus* α-amylases, the engineered changes result in an α-amylase that appears to behave like a heterologous protein. Late stages in the secretion process have a dramatic influence on secretory protein yield and the events which occur on the *trans* side of the membrane must be given careful consideration when matching potential secretion hosts with native and heterologous proteins of interest.

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