Calcium triggers the refolding of *Bacillus subtilis* chitosanase

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We characterized the reversible folding–unfolding transition of *Bacillus subtilis* exocellular chitosanase from either thermal or urea denaturation of the protein. The transitions were monitored in each case by intrinsic fluorescence changes and resistance to proteolysis. Unfolding and refolding kinetics and differential scanning calorimetry analysis suggested a two-state equilibrium. The equilibrium between the folded and unfolded states was rapidly displaced towards the folded state in the presence of a low concentration of calcium (2–20 mM). The binding titration

curve indicated that chitosanase possesses one weak Ca²⁺-binding site (with an equilibrium affinity constant, K_A , of $0.3 \times 10^3 \text{ M}^{-1}$). These results support the hypothesis that this metal ion, which is accumulated in the cell wall environment of *B. subtilis*, is an effector that influences folding and stability of newly translocated proteins.

Key words: exocellular protein, reversible folding, thermal unfolding, urea denaturation.

INTRODUCTION

It is now widely accepted that the dynamics of folding play an important role during the secretion process of an extracellular protein [1]. Therefore, *in vitro* studies of the unfolding–folding transition under physiological pH and temperature conditions may provide information concerning the pathway of the folding process during secretion and afford opportunities to identify the folding effectors that play a role *in vivo*.

The folding aspect of secretion is especially crucial in *Bacillus subtilis*, since proteins emerging on the *trans* side of the membrane must undergo rapid and efficient folding to prevent the proteolytic action of proteases associated with the cell wall [2]. The question then arises of whether this final conformational transition is spontaneous or controlled by intrinsic factors such as an additive pro-sequence [3], or assisted by effectors that facilitate the folding when the proteins cross the wall, such as the peripheral protein PrsA [4] or metal ions [5], concentrated within the cell wall microenvironment on the external side of the cytoplasmic membrane, or components of the cell-wall matrix such as anionic polymers [6]. We have previously studied two model proteins, levansucrase [7] and α -amylase [8]. These studies enabled us to identify some of the common kinetic features that characterize the folding process of *B. subtilis* secreted proteins. Calcium was found to be a potent effector of the folding process of these proteins, acting as a folding catalyst, since these proteins in their folded form display a very weak affinity for this metal. The two proteins were large (molecular mass > 50 kDa), however, and prone to aggregate in their unfolded form, thus preventing study of the thermodynamic aspects of the folding–unfolding transition.

We anticipated that chitosanase would be a more convenient protein model because of its small molecular mass of 27.4 kDa and its lack of disulphide bonds. Chitosanase (EC 3.2.1.132), however, is secreted at a low level in the *B. subtilis* wild-type strain 168 Marburg [9]. The purification to the milligram scale

required for a complete kinetic and thermodynamic characterization of conformational transition is not possible under these conditions of production. We showed [10] recently that the structural gene *csn* can be expressed under the control of the inducible levansucrase leader region *sacR* in a *degU*₃₂ (Hy) strain.

We demonstrate in this work that exocellular chitosanase is overproduced during the exponential phase of growth. The affinity of the protein for hydroxyapatite makes its purification easy. So far, no information concerning the folding properties of this protein is available, but *B. subtilis* chitosanase has an amino acid composition and functional characteristics that are close to those of its counterpart produced by *Streptomyces* sp. N174 [11]. The thermal unfolding of this latter protein has been studied [11] and the results obtained suggest that the tryptophan side chains most probably play an important role in the stabilization of the protein.

These observations prompted us to use the changes of intrinsic fluorescence properties of *B. subtilis* chitosanase to monitor the conformational transition. Moreover, allowing for the specific environmental context in which this protein is folded *in vivo*, we also paid attention to the kinetics of the protease sensitivity changes accompanying the refolding reaction. The possible role of calcium as a folding effector was studied.

EXPERIMENTAL

Purification of exocellular chitosanase

B. subtilis GM9804 [*degU*₃₂(Hy) *sacA*₃₂₁ Δ *sacR**sacB* *sacR**csn*; Km^R], a derivative of *B. subtilis* Marburg [10], was grown in a 1.5 litre fermentor (Bioflo 3000; New Brunswick Scientific) in minimal medium [12]. The carbon source was 1.5% glucose at the beginning of growth. When the bacterial suspension reached a D_{600} value of 6, fresh minimal medium containing 1% glucose was fed at a constant rate equal to that of the cell suspension outflow (660 ml · h⁻¹). pH and temperature were controlled and maintained at values of 7.0 and 37 °C, respectively. Solubilized

Abbreviations used: DSC, differential scanning calorimetry; K_A , equilibrium affinity constant; MALDI-TOF, matrix-assisted laser-desorption ionization–time-of-flight; $t_{1/2}$, half-life; T_m , melting temperature.

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O₂ was measured with a Clark electrode and an O₂ saturation of 85% was maintained from bubbling through the culture. The speed of agitation was 800 rev./min. Under such conditions, the specific growth rate, m , remained maximum and constant, equal to 0.69 h⁻¹. Growth yield (g of biomass/mol of glucose) was approximately 78 g · mol⁻¹. One absorbance unit at 650 nm of cell suspension corresponded to 70 µg of protein · ml⁻¹.

After centrifugation, the supernatant was treated as follows.

Step 1

Solid hydroxyapatite (Bio-Rad) was resuspended in culture supernatant (3 g · l⁻¹) and the suspension was gently stirred for 20 min. Hydrated hydroxyapatite was then left to settle. The supernatant was discarded and the pellet resuspended in a minimum volume of 0.1 M sodium phosphate, pH 7, and poured into the column. Hydroxyapatite was allowed to pack by gravity and was then washed with 5 vol. of the same buffer. Proteins were eluted with a linear gradient of 0.1–1 M sodium phosphate. Fractions containing chitosanase activity were eluted at 0.6 M phosphate, pH 7, and the fractions containing the enzyme activity were re-concentrated up to 5 ml with a Centricon concentrator (Amicon).

Step 2

For gel-filtration chromatography the protein solution was loaded on to a 40 cm × 2.5 cm Biogel P100 column (fine 45–90 µm; Bio-Rad) pre-equilibrated with 0.1 M sodium phosphate, pH 7. Continuous elution was performed with the same buffer at a constant rate of 8 ml · h⁻¹ at 5 °C. Fractions containing chitosanase activity were analysed by SDS/PAGE (10%, w/v, gel). The purified protein migrated as a single protein band with an apparent molecular mass of 28 kDa. The stock solution of pure protein was 7.5 mg · ml⁻¹ (0.3 mM) in 0.1 M potassium phosphate, pH 7.

Matrix-assisted laser-desorption ionization–time-of-flight (MALDI-TOF) MS analysis

MALDI-TOF spectra of chitosanase were obtained with a Voyager-DE PRO Biospectrometry Workstation mass spectrometer (PE Biosystems). Analyses of the spectra were performed in linear mode, with an accelerating voltage of 25000 V, and a delayed extraction of 800 ns. For subsequent data processing, Data Explorer software (PE Biosystems) was used. Data acquisition was performed over the m/z range 10000–50000. Mass spectra were calibrated externally using the doubly protonated peak of BSA corresponding to m/z 33216.

Enzyme assay

Chitosanase activity was assayed by using solubilized deacetylated chitosan as a substrate. Partially N-acetylated chitosan (degree of acetylation, 20%) prepared from crab shells was purchased from Sigma. Stock solution was prepared according to Uchida and Ohtakara [13]. The reaction mixture contained 0.2% substrate in 0.1 M sodium acetate buffer, pH 5.8. After addition of the enzyme solution, the mixtures were incubated at 37 °C. Reactions were stopped by boiling for 10 min. The amount of reducing sugar was determined using dinitrosalysilate acid according to Miller [14]. One unit of chitosanase activity was taken as the amount of enzyme that produced 1 µmol of reducing sugar (glucosamine base)/min.

Fluorescence measurements

Changes in intrinsic fluorescence and fluorescence spectra of chitosanase were recorded with a F2000 Hitachi thermoregulated spectrofluorimeter.

CD measurements

CD measurements were made with a Jasco J720 thermoregulated spectropolarimeter using cells of 1 mm path length at 37 °C.

Differential scanning calorimetry (DSC) analysis

Calorimetric measurements were carried out in a MicroCal VP-DSC differential scanning microcalorimeter.

RESULTS

Production and purification of exocellular chitosanase in strain GM9804

Strain GM9804 [10], growing in a continuous fermentor, produced exocellular chitosanase at a constant differential rate of synthesis (4% of total protein). Analysis of culture supernatant by SDS/PAGE showed that chitosanase was the major protein released into the growth medium (results not shown).

Exocellular chitosanase was purified as described in the Experimental section. The purified enzyme was homogeneous on SDS/PAGE. Its molecular mass evaluated by MALDI-TOF MS was 27424 Da, as expected from the nucleotide sequence of the structural gene *csn* [15] and the cleavage of the signal peptide. N-terminal sequencing confirmed that the precursor form of the protein is processed at the expected site. Kinetic parameters of the enzyme were evaluated by the release of reducing sugar from soluble chitosan. K_m and V_{max} of 1.2 ± 0.1 mg · ml⁻¹ and 340 ± 15 units · mg⁻¹ respectively, were similar to parameters of chitosanases found in various other bacteria [16].

Chitosanase unfolding by urea

Under native conditions (0.1 M potassium phosphate, pH 7, 37 °C), chitosanase displayed a high intrinsic fluorescence emission when excited at 280 nm (Figure 1A). The emission spectrum was maximal at 325 nm. From the CD spectrum (Figure 1B), according to the deconvolution method of Yang et al. [17], the secondary structure contents of the protein were evaluated as 53.8% α -helix, 21.7% turn and 24.5% unordered. Incubation of the protein for a long time in the presence of various proteases demonstrated the insensitivity of chitosanase to proteolysis.

In the presence of 6 M urea, under the same conditions of pH and temperature, a large quenching of intrinsic fluorescence and a large red shift of the maximum were observed (Figure 1A). In such conditions, the CD spectrum indicated that the unfolded form of the protein is devoid of any secondary structures. Moreover, chitosanase rapidly lost its resistance to proteolysis in the presence of 6 M urea, as shown below.

Kinetics of chitosanase unfolding and refolding reactions

The kinetics of unfolding and refolding were determined by monitoring both the intrinsic fluorescence (Figure 2) and the changes in protease sensitivity that accompanied the transition (Figure 3). We observed that unfolded chitosanase recovered the fluorescence and protease-resistance properties of its native form when the concentration of urea was lower than 2 M. Unfolding and refolding kinetics were both monophasic. The evaluation of half-life ($t_{1/2}$) gave a $t_{1/2}$ value of 78.8 ± 0.9 s for the unfolding

Figure 1 Characterization of chitosanase in native conditions and in the presence of 6 M urea

(A) Intrinsic fluorescence spectra of chitosanase under native (trace a) and denaturing (trace b) conditions at pH 7 and 37 °C. The final concentration of chitosanase was 0.4 μ M in each case. Trace a, chitosanase in 0.1 M potassium phosphate, pH 7/0.5 mM EDTA; trace b, chitosanase in 6 M urea in the same buffer. Traces of fluorescence emission spectra were recorded after pre-incubation of the samples for 15 min at 37 °C. Protein spectra were corrected by the spectra of a reference solution without protein but otherwise identical. The excitation wavelength was 280 nm. (B) Far-UV CD spectra of chitosanase under native (trace a) and denaturing (trace b) conditions. CD spectra, acquired by means of a Jasco J720 spectropolarimeter with cuvettes of 1 mm pathlength thermoregulated at 37 °C, are shown as mean residue molar ellipticity ($\text{deg} \cdot \text{cm}^2 \cdot \text{dmol}^{-1}$) as a function of wavelength after subtraction of a blank spectrum (buffer alone). The final concentration of chitosanase was 10 μ M in each case. Conditions for each trace were as for (A).

process in the presence of 6 M urea and a $t_{1/2}$ of 73.7 ± 0.8 s for the refolding process in the presence of 1.75 M urea. Under the latter conditions (Figure 3), the unfolding–refolding transition of chitosanase monitored by resistance to proteolysis was achieved within the same time range ($t_{1/2} \approx 1$ min).

Kinetics of the unfolding and refolding of chitosanase as a function of the concentration of urea lead to the construction of a chevron plot (Figure 4). The rate constants evaluated from extrapolation in the absence of urea were $k_u^0 = (6.4 \pm 0.7) \times 10^{-5} \text{ s}^{-1}$ for unfolding and $k_f^0 = 6.7 \pm 1.7 \text{ s}^{-1}$ for refolding.

Additional evidence for the absence of a partially folded intermediate during unfolding or refolding processes

The finding that the unfolding and refolding processes are monophasic events, plus the observation that the refolding

reaction monitored by two different methods showed a similar time course, suggested that the unfolding–folding transition occurs as a two-state process. Among other criteria considered to be good indicators of the absence of transiently populated intermediates [18] is the absence of any significant fluorescence changes during refolding (Figure 5) when this event was monitored at the wavelength of the isosbestic point of the native and unfolded emission spectra ($\lambda = 355$ nm, evaluated from Figure 1). Such results are as one would expect when no transient intermediate is accumulated during unfolding–refolding transition.

Conformational stability of chitosanase

Since the results obtained indicated that transition occurs as a two-state process we evaluated the free energy of denaturation of

Figure 2 Kinetics of chitosanase unfolding (A) and refolding (B) monitored by fluorescence intensity change at pH 7 and 37 °C

(A) Unfolding was promoted by mixing 1.5 μ l of chitosanase stock solution (7.5 mg $\cdot$ ml $^{-1}$) with 0.9 ml of 6 M urea in 0.1 M potassium phosphate, pH 7/1 mM EDTA (preincubated at 37 °C). Traces of fluorescence intensity changes were recorded at 325 nm (the excitation wavelength was 280 nm). (B) Refolding, 1.5 μ l of chitosanase stock solution (7.5 mg $\cdot$ ml $^{-1}$) was mixed with 4.5 μ l of 8 M urea in the same buffer. After 15 min of incubation at 37 °C, denaturing mixture was diluted into 0.9 ml of buffer containing 1.75 M urea preincubated at 37 °C. Trace of fluorescence intensity changes were recorded at 325 nm (the excitation wavelength was 280 nm). The final concentration of chitosanase was 0.45 μ M in each case, and the dead time of mixing was 8–10 s.

the protein. Native or previously denatured chitosanase was exposed for a long period, 18 h, to various concentrations of urea in 0.1 M potassium phosphate buffer, pH 7, at 37 °C. The denaturation profiles (Figure 6A) can be analysed assuming a

Figure 3 Kinetics of the unfolding–refolding transition of chitosanase measured by resistance to proteolysis at 37 °C

Unfolding was promoted by mixing 7 μ l of stock solution of chitosanase (7.5 mg $\cdot$ ml $^{-1}$) with 21 μ l of 8 M urea, pH 7 (final concentration, 6 M). Samples (2 μ l) were withdrawn at the times indicated and quickly mixed with 100 μ l of 2 M urea/0.1 M sodium phosphate, pH 7, containing 20 μ g $\cdot$ ml $^{-1}$ subtilisin (Sub). After 5 min of incubation 4 μ l of 0.1 M PMSF was added. Refolding (arrow 2) was initiated after 10 min of unfolding by mixing 16 μ l of unfolding mixture with 784 μ l of 1.75 M urea/0.1 M sodium phosphate, pH 7. Samples (100 μ l) were withdrawn at the times indicated and quickly mixed with 2 μ l of subtilisin (1 mg $\cdot$ ml $^{-1}$) and incubated for 5 min. PMSF was then added and 50 μ l samples were subjected to SDS/PAGE and 5 μ l were assayed for chitosanase activity. Enzyme activity was measured as described in the Experimental section. (A) SDS/PAGE analysis of the samples. (B) $\square$, Curve obtained from scanning the coloured bands of the SDS gels; $\bullet$, curve obtained from enzyme activity.

two-state model. The denaturation equilibrium constant, K_U , is given by:

$$K_U = e^{-(\Delta G_U/RT)} = \frac{f_U}{f_N} = \frac{IF - IF_N}{IF_U - IF} \quad (1)$$

where f_U and f_N are the relative protein fractions in their unfolded and native forms, respectively, and IF_N , IF_U and IF represent the intensity of fluorescence at 325 nm of the native form, the totally unfolded form and a mixture of both forms, respectively.

Figure 4 Urea dependence of unfolding and refolding apparent rate constant

Plot of $\ln k_{\text{obs}}$ of unfolding (■) and refolding (□) against urea concentration. The rate constant k_{obs} was evaluated from the kinetics of chitosanase unfolding and refolding monitored by fluorescence intensity changes at pH 7 in 0.1 M sodium phosphate and 37 °C at various concentrations of urea. For refolding, chitosanase was first unfolded in 6 M urea for 15 min; the final urea concentration was obtained by dilution in the same buffer. All assays were performed with 0.8 μM chitosanase.

Figure 5 Kinetics of chitosanase refolding monitored by the change of fluorescence intensity at simultaneously 325 nm, trace (a) and 355 nm, trace (b)

The wavelength of the isosbestic point of the spectra emission of native and unfolded forms of the protein was 355 nm. Experiments were performed as described in the legend to Figure 2.

The free energy for the unfolding of chitosanase in the transition region varied linearly with the denaturant (Figure 6B). According to the following equation [19]:

$$\Delta G_0 = \Delta G_0^{\text{H}_2\text{O}} - m_u[\text{urea}] \quad (2)$$

the Gibbs free energy difference between the unfolded and native states, $\Delta G_0^{\text{H}_2\text{O}}$, was estimated by extrapolation to be $33.4 \pm 2.3 \text{ kJ} \cdot \text{mol}^{-1}$. This estimate is in agreement with the values calculated by the kinetic approach (Figure 4). Indeed, $K_U = k_u^0/k_r^0 = (9.5 \pm 1.3) \times 10^{-6}$ gives $\Delta G_U^0 = 29.8 \pm 3.1 \text{ kJ} \cdot \text{mol}^{-1}$. The parameter m_u , equal to $-12.5 \pm 0.8 \text{ kJ} \cdot \text{mol}^{-1} \cdot \text{M}^{-1}$, reflects the dependence of the free energy on the urea concentration.

Figure 6 Unfolding and refolding transition curve of chitosanase at pH 7 and 37 °C

(A) Chitosanase in the native (●) or denatured (▲) form was exposed at 37 °C to various concentrations of urea in 0.1 M potassium phosphate, pH 7/0.5 mM EDTA for 18 h. The final concentration of chitosanase was 0.35 μM . The fraction of the protein remaining folded was evaluated from the fluorescence intensity of the sample at 325 nm (the excitation wavelength was 280 nm). An aliquot of each sample was treated with subtilisin and submitted to SDS/PAGE analysis as described in Figure 3, allowing evaluation of the protein fraction remaining resistant to proteolysis (○). (B) Representation of free energy changes of unfolding in the transition region measured by fluorescence intensity.

Thermal unfolding of chitosanase

The thermal unfolding process of chitosanase was first investigated by DSC to evaluate thermodynamic parameters. The heat absorption curve profile (Figure 7, top panel) showed only one sharp peak of heat capacity with a maximum at the melting temperature (T_m) of 60.06 ± 0.02 °C. The total calorimetric denaturation enthalpy of the transition was evaluated to be $251 \text{ kcal} \cdot \text{mol}^{-1}$ from the area under the curve. The DSC

Figure 7 Thermal unfolding of chitosanase measured by DSC (top panel) and spectroscopy (bottom panel)

Top panel: plot of heat capacity (C_p) against temperature for chitosanase ($10.6 \mu\text{M}$) at a scan rate of $60^\circ\text{C} \cdot \text{h}^{-1}$ in 0.1 M potassium phosphate, pH 7. Bottom panel: fluorescence changes at 325 nm . Chitosanase ($0.5 \mu\text{M}$) was diluted in 0.1 M potassium phosphate at pH 7, 37°C . Temperature was raised from 37°C to 64°C at a constant rate of $2^\circ\text{C}/90 \text{ s}$, and was then rapidly shifted down to 37°C within $\approx 30 \text{ s}$ (arrow). Traces of fluorescence intensity changes were recorded at 325 nm (the excitation wavelength was 280 nm). The curve (●) obtained from enzyme activity recovered after incubation of samples in the presence of subtilisin. Enzyme activity was measured as described in the Experimental section.

experiments, however, were conducted using $10.6 \mu\text{M}$ protein, a concentration at which the protein solution was visibly cloudy after heating to 75°C . Then the aggregation of the unfolded protein in this condition of high concentration prevented the reversibility of thermal unfolding and may explain the observation that the post-transition baseline was lower than the pre-transition baseline.

Reversible thermal unfolding was obtained in the same conditions of pH and ionic strength when the unfolding–refolding

Figure 8 Effect of calcium on the unfolding–refolding transition of chitosanase in the presence of 2.2 M urea at 37°C

Unfolding was promoted by mixing chitosanase ($0.3 \mu\text{M}$) in 6 M urea. After 5 min , unfolded chitosanase was diluted in 0.1 M sodium acetate, pH 7, containing 2.2 M urea. After stabilization at 37°C , calcium was added at final concentrations in the refolding mixture of 2 mM (b), 5 mM (c), 10 mM (d) and 20 mM (e). (a) Control without calcium. Ionic strength was kept constant in each condition by the addition of NaCl. The unfolding–refolding transition was measured by monitoring the fluorescence intensity changes as a function of time.

transition was monitored by changes in the intrinsic fluorescence of the protein (Figure 7, bottom panel). The experiments were carried out using a chitosanase concentration 20 times lower than those used for DSC analysis. The midpoint of the transition curve led to an estimate of 59°C for the T_m , which is in good agreement with the DSC approach.

Calcium triggers the unfolding–refolding transition of chitosanase

We focused our attention on calcium as a cofactor that could assist in the folding of chitosanase in the cell wall micro-environment of *B. subtilis*. We tested the effect of the addition of calcium to chitosanase in an unfolded stabilized state. Such stabilization was obtained, as shown above, either in the presence of urea or at a temperature above the T_m .

In a medium containing phosphate, however, the formation of an insoluble Ca^{2+} –phosphate complex limits the use of this metal ion within the concentration range of 0 – 1 mM . Therefore acetate buffer was more convenient to explore the effect of a wider range of calcium concentrations on the conformational equilibrium of chitosanase. Study of the unfolding–refolding transition of the protein in acetate buffer using the same method as described above led to the conclusion that in this buffer chitosanase was in the unfolded state in the presence of a urea concentration higher than 2 M and that the T_m was 50°C , in contrast to that in the phosphate buffer. Allowing for these data we analysed the calcium effect on the conformational transition of the protein.

We observed first (Figure 8) that in the presence of 2.2 M urea addition of calcium shifted the conformational equilibrium of chitosanase from the unfolded form towards the native form. The rate of refolding is dependent on the concentration of calcium.

Secondly, calcium promoted refolding of the thermal unfolded chitosanase (Figure 9). In acetate buffer, the protein was rapidly

Figure 9 Calcium triggers the transition of chitosanase from the thermal unfolded state to the native state

Thermal unfolding was promoted by mixing 5 μ l of stock solution of chitosanase (7.5 mg $\cdot$ ml $^{-1}$) with 1 ml of 0.1 M sodium acetate, pH 7, preincubated at 52 $^{\circ}$ C. Unfolding and refolding were measured by monitoring fluorescence intensity changes and by resistance to subtilisin degradation. (A) Traces represent fluorescence intensity as a function of time (excitation wavelength, 280 nm; emission wavelength, 325 nm). Changes in the protease sensitivity (●) were evaluated as described for Figure 7. After 8 min of unfolding (indicated by an arrow), 10 μ l of 2 M calcium in 0.1 M potassium acetate, pH 7, was added. (B) Fluorescence spectra of chitosanase. Trace a, after 8 min of incubation at 52 $^{\circ}$ C, just before addition of calcium. Trace b, a short time (20 s) after addition of calcium.

unfolded ($t_{1/2} \approx 6$ min) when incubated at 52 $^{\circ}$ C. Addition of 20 mM calcium to this solution of the unfolded form triggers an instantaneous shift towards a form displaying the fluorescence and protease-resistance properties of the native form.

Calcium-binding titration curve of chitosanase

At 58 $^{\circ}$ C, unfolding was complete even in the presence of calcium; however, the rate of unfolding was calcium-dependent. The $t_{1/2}$ of chitosanase unfolding was determined at a series of calcium

Figure 10 Calcium-binding titration curve of chitosanase from the rate of the folding-unfolding transition at 58 $^{\circ}$ C

Chitosanase (0.4 μ M) was dissolved in acetate buffer containing various amounts of calcium. The ionic strength was maintained constant by the addition of NaCl. The rate of the folding-unfolding transitions was measured by the intrinsic fluorescence intensity changes as a function of time at 58 $^{\circ}$ C in 0.1 M sodium acetate, pH 7 (excitation wavelength, 280 nm; emission wavelength, 325 nm). The $t_{1/2}$ of unfolding as a function of the $p[Ca^{2+}]$ was established. The theoretical titration for a single binding site is $p[Ca^{2+}] = \log K_A + \log ([E]/[E - Ca^{2+}])$, where K_A is the equilibrium affinity constant of the protein for calcium.

concentrations (Figure 10). The titration curve closely fits the theoretical curve established for a single binding site [20]. A weak Ca^{2+} -binding site was identified; the value of the apparent equilibrium affinity constant (K_A) of the chitosanase for calcium was 0.3×10^3 M $^{-1}$.

DISCUSSION

The aim of this work was to characterize the kinetic and thermodynamic aspects of the unfolding-refolding transition of *B. subtilis* chitosanase and to identify the folding modulators of this protein which could play a role inside the cell wall space during the last stage of the secretion process. Transition of the native state of the protein to an unfolded state characterized by its specific fluorescence emission spectrum and protease sensitivity was obtained in the presence of a high concentration of urea or from thermal denaturation. Refolding occurred rapidly under physiological conditions of pH, ionic strength and temperature. The most striking feature observed was calcium's role as a folding effector. Results indicated that the refolding of chitosanase was metal-ion-dependent under conditions of temperature, 52 $^{\circ}$ C, or denaturant concentration, 2.2 M urea, which

Figure 11 Amino acid sequence alignment of the calcium-binding site of chitosanase

The numbers indicate positions of the residues in the primary sequence. The calcium-binding site (in bold) is located in the region 186–198. The residues in α -helices predicted by Garnier [22] are underlined. Ca^{2+} ions are probably co-ordinated by oxygen atoms in side chains of the amino acids that are boxed.

are quite different from the usual conditions of *B. subtilis* growth in the laboratory. Two hypotheses can be proposed to explain this: either this property is involved in the process of adaptation of *B. subtilis* to specific growth conditions, since this micro-organism is known to grow within a temperature range of 20–55 °C, or this folding modulator is essential, in the conditions of the cell wall microenvironment, in promoting prompt folding to prevent the proteolytic action of cell-wall-associated proteases. Unfortunately, it has not been possible up until now to test the latter hypothesis, since too little is known about the physico-chemical properties of the cell wall compartment to mimic, *in vitro*, this specific microenvironment. Nevertheless, it has been shown that various *B. subtilis* exocellular proteins exhibit a weak affinity for calcium and that the presence of this cation facilitates their folding [7,8,20]. Within the frame of such a hypothesis, the calcium-binding site of these proteins can be considered to be a functional domain playing a role in secretion efficiency. We addressed the identification of a calcium-binding site on chitosanase according to the prediction method presented on the website <http://npsa-pbil.ibcp.fr>. Only one segment (Asp¹⁸⁶–Asn¹⁹⁸) flanked by two α -helices was identified as a possible EF-hand motif (Figure 11).

Finally, the question arises as to why chitosanase displays such a low affinity for calcium ($K_A = 0.3 \times 10^3 \text{ M}^{-1}$). Two hypotheses may be put forward. First, the ability of the *B. subtilis* cell wall to concentrate calcium ions preserves a high concentration of calcium (> 2 mM) on the external side of the cytoplasmic membrane [5], allowing the protein–metal interaction to occur despite a low affinity constant. Secondly, it was demonstrated [21] that K_A of a synthetic helix–loop–helix calcium-binding unit is modulated by the dielectric constant of the medium. A hydrophobic medium strongly increased the affinity constant. Therefore, the affinity of chitosanase for calcium within the cell wall microenvironment is probably different from those determined in this work.

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