Reversible thermal unfolding of *Bacillus subtilis* levansucrase is modulated by Fe^{3+} and Ca^{2+}

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The equilibrium transition curves for thermal unfolding of levansucrase were established at several pH values. At pH 7 and within the temperature range of bacterial growth, the unfolded form is predominant. However, under such conditions, refolding is promoted by the only addition of Ca^{2+} or Fe^{3+}. We propose that the tertiary structure flexibility of levansucrase plays a key role in its secretion process.

Thermal unfolding; Protein secretion; Levansucrase; *Bacillus subtilis*

1. INTRODUCTION

It is generally accepted that a protein is competent for transfer through the membrane only if it possesses a loosely folded conformation [1,2]. Thus, molecular mechanisms which modulate in vivo the folding of exoproteins are presently the subject of intensive investigations [3].

From studies of the levansucrase translocation process, we focused our attention on the possible role of multivalent metallic ions as folding modulators [4].

The question arises: can levansucrase, in the absence of such metals, exist in the unfolded form under the usual pH and temperature conditions occurring in the cytosol of *Bacillus subtilis* cells? If this is the case, what are the metals that are able to promote its spontaneous refolding?

2. MATERIALS AND METHODS

2.1. *Purification of exocellular levansucrase*

Levansucrase was prepared from the culture supernatant of the induced *B. subtilis* QB112 strain according to the published procedure [5].

2.2. *Fluorescence measurements*

Fluorescence was measured with a Jobin and Yvon thermoregulated spectrofluorimeter.

3. RESULTS

3.1. *Thermal folding-unfolding transition of levansucrase*

The change in intrinsic fluorescence of levansucrase has been shown to be strictly correlated with the appearance of proteolytic susceptibility and the loss of the catalytic activity of the enzyme [4]. Thus we used this technique to monitor the tertiary structural changes associated with thermal folding-unfolding transition. As an example, Fig. 1 shows the fluorescence properties of levansucrase incubated at various temperatures in the presence of 0.1 mM EDTA, at pH 7. Each fluorescence emission spectrum was recorded after 30 min incubation of the protein at the chosen temperature. No significant modification was found to occur after this time.

The reversibility of the transition at pH 7 is shown in Fig. 2. At 41°C the unfolded state is reached with a half-time of about 15–20 s; the protein fully recovered the fluorescence properties of its native form when the temperature of the incubation medium was shifted down to 20°C.

Assuming that the two-state equilibrium model is applicable as demonstrated from urea denaturation of levansucrase [4], we can calculate, from Fig. 1, the fraction of the native form, at each temperature of the transition region according to the relation

$$f_N = I - I_U / I_N - I_U$$

where I is the value of the fluorescence intensity at 340 nm at some point in the transition and I_N, I_U representative values for the native and unfolded forms of the protein, respectively. The constructions of the equilibrium transition curves of thermal unfolding of levansucrase at pH 7 in the presence and absence of EDTA are shown in Fig. 3(b) and 3(a).

The same studies were conducted at various pH values. Particular attention was paid, on the one hand, to the pH values where the enzyme possesses its maximum activity, pH 5.5, and, on the other hand, to the pH range of its synthesis secretion by *B. subtilis*, pH

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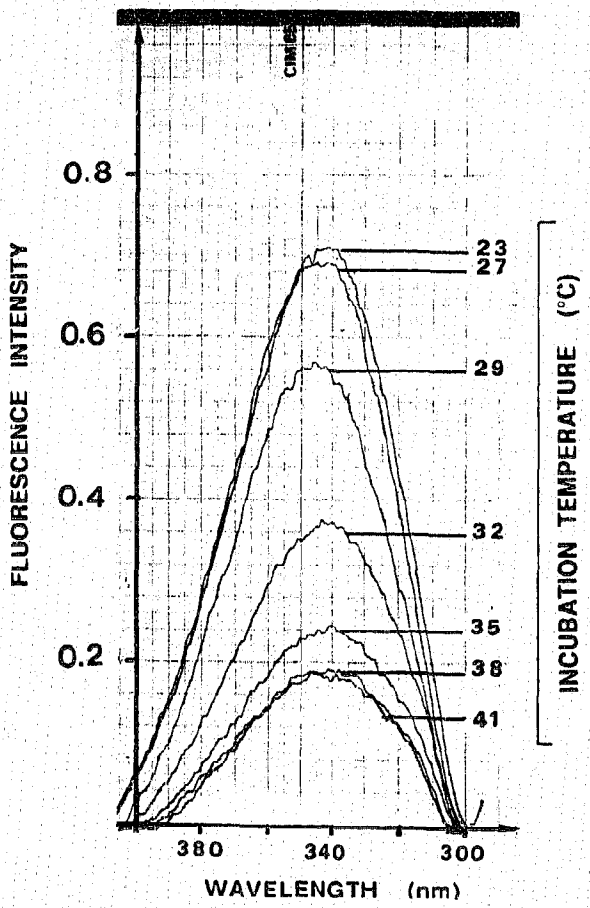

Fig. 1. Fluorescence of levansucrase incubated at various temperatures. Traces of fluorescence emission spectra of each sample, 0.2 μM levansucrase in 0.1 M potassium phosphate, 0.1 mM EDTA, pH 7, were recorded after 30 min incubation at the temperature studied. Excitation wavelength is 285 nm.

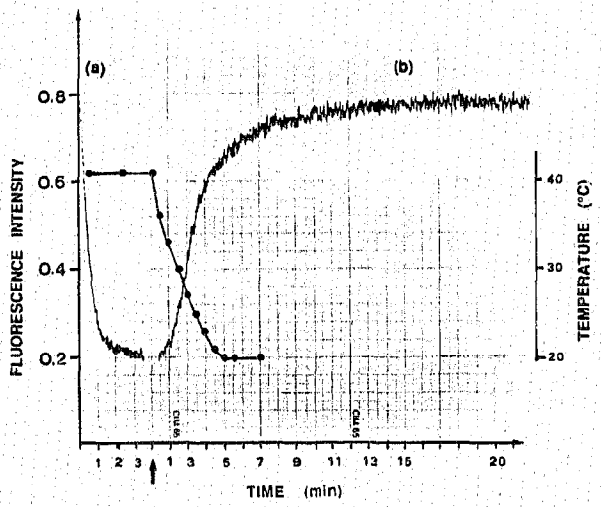

Fig. 2. Kinetics of reversible thermal denaturation of levansucrase measured by fluorescence intensity changes. (a) Unfolding: 6 μl of levansucrase stock solution (0.1 mM) were diluted in 3 ml of 0.1 M potassium phosphate, pH 7, 0.1 mM EDTA, preincubated at 41°C. The fluorescence intensity change of the solution was recorded until the minimum was reached. (b) Refolding: the temperature of the protein solution in the cell of the spectrofluorimeter was decreased to 20°C by the change (arrow) of the fluid in the thermo-regulated circuit. (●) Values of temperature within the cell.

7–7.5. The temperatures of the mid-point (T_m) of the denaturation curves estimated in each case are depicted in Table I. Two conclusions may be drawn from these results: (i) around pH 7, the T_m of the protein is lower than 37°C which is the usual growth temperature of *B. subtilis*. However, under these pH conditions, the T_m is strongly dependent on the presence or absence of a metal chelator; (ii) the metal chelator effect disappeared at pH 5.5, conditions of maximum activity and maximum stability of purified levansucrase. Under such conditions the crystalline form of this protein is not associated with metal [6].

3.2. *Effects of some metals on the folding properties of levansucrase*

The equilibrium transition curve for thermal unfolding of levansucrase at pH 7 in the presence of 50 mM Ca^{2+} is shown in Fig. 3(c). Temperature of the mid-point is 39°C. The comparison of this curve with those obtained in the presence of EDTA (Fig. 3(b)) shows that, in the temperature range of 36–37°C, the protein is present completely in the unfolded state in the absence of free metal and completely present in the folded form in the presence of calcium. It is thus possible to postulate that this metal could act as a folding cofactor under these pH and temperature conditions.

This prediction is correct since, as shown in Fig. 4, the protein previously unfolded at 37°C in the presence of 0.1 mM EDTA was rapidly ($t_{1/2}$ = 120 s) and fully refolded by the only addition of 50 mM calcium. Among the other polyvalent metal ions tested, Mg^{2+}, Ba^{2+}, Mn^{2+} had no effect whereas Sr^{2+} displaced partially the equilibrium. The effect of Fe^{3+}, which displays a strong absorbance at the wavelength of fluorescence emission, was studied by monitoring the change of resistance to degradation by subtilisin [4]. Fig. 5 shows that the addition of Fe^{3+} to the unfolded form promoted refolding at 37°C, pH 7, as observed with calcium. The half-life of the process was in the same order of magnitude in both cases: $t_{1/2}$ = 100–120 s.

4. DISCUSSION

The results presented in this work show that levansucrase possesses a tertiary structure wholly dependent on the presence of Fe^{3+} or Ca^{2+} under the pH and temperature conditions of its synthesis. These observations, added to those published [7], pertaining to the role of Fe^{3+} as a cofactor of the second step of the secretion mechanism of levansucrase, suggest a precise function of such metals during the secretion pathway. We propose the following model for this sequential process. Under the cytosolic conditions, pH 7–7.5 [8], 37°C and

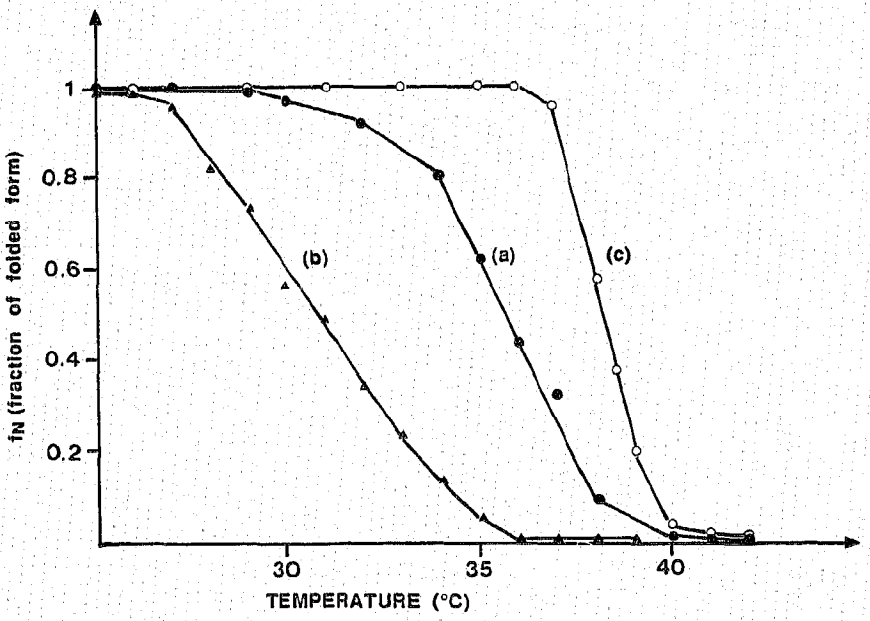

Fig. 3. Equilibrium transition curves for thermal unfolding of levansucrase at pH 7. (a) In 0.1 M potassium phosphate buffer (●); (b) in the same buffer with 0.1 mM EDTA (▲); (c) in 0.1 M potassium acetate buffer, 50 mM $CaCl_2$ (○). The fraction of the native form was calculated at each temperature as described in the text.

low concentrations of free Ca^{2+} or Fe^{3+}, the newly synthesized protein remains in the unfolded state and exposes its hydrophobic groups. The first step is the spontaneous insertion of the protein precursor form into the membrane bilayer [2,9]. Such unfolded membrane form of levansucrase has been isolated and previously characterized [10].

The second step, after the cleavage of the signal peptide, results from an efficient coupling between folding and protein release into the exocellular medium. Such an event, catalyzed by metal ions like Fe^{3+} and Ca^{2+}, occurs on the external side of the membrane since teichoic acid, a component of *B. subtilis* cell surface is known to form a continuous matrix with bound polyvalent metals [11]. This model, on the one hand, rules out the involvement of a specific export machinery for protein translocation in *B. subtilis*, the existence of which was recently questioned [12]. On the other hand, it emphasizes the intrinsic property of the polypeptide

Table I

Values of T_m, temperature of the mid-point of thermal folding-unfolding transition for levansucrase at various pH

pH	5.5	6.5	7	7.5
T_m (°0C)[a] in the absence of EDTA	44.5	40	35.5	31
T_m (°C)[a] in the presence of EDTA (0.1 mM)	43.5	35	30.5	26

[a] The thermal unfolding experiments were carried out in 0.1 M potassium phosphate buffer in the absence and in the presence of 0.1 mM EDTA

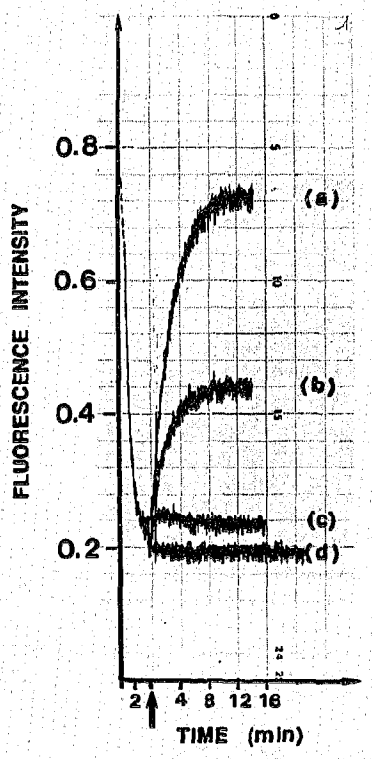

Fig. 4. Unfolding-refolding transition of levansucrase mediated by metals at pH 7 and 37°C. Unfolding was initiated by mixing 6 μl of levansucrase stock solution (0.1 mM) with 2.85 ml of 0.1 M potassium acetate, pH 7, preincubated at 37°C. The refolding assays were promoted by the addition (arrow) of 0.15 ml of 1 M concentration of: (a) $CaCl_2$, (b) $SrCl_2$, (c) $BaCl_2$, (d) $MgCl_2$ and $MnCl_2$. A possible effect of ionic strength variation was tested by the addition of 0.15 ml of 2 M KCl solution (d). Each salt solution was prepared in 0.1 M potassium acetate and adjusted to pH 7.

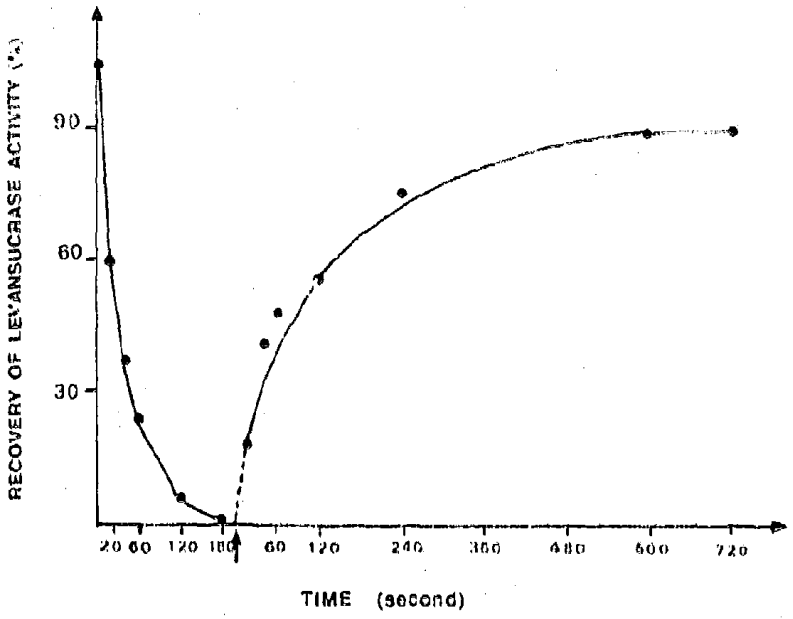

Fig. 5. Unfolding-refolding transition of levansucrase mediated by Fe^{3+}, measured by resistance to degradation by subtilisin. Unfolding was promoted (arrow 1) by mixing 5 μl of enzyme stock solution (0.1 mM) with 2 ml of 0.1 M potassium phosphate, pH 7, 0.1 mM EDTA, preincubated at 37°C. Samples (100 μl) were withdrawn at the times indicated, quickly mixed with 20 μl of a solution of 1 mg·ml^{-1} subtilisin and incubated for 15 min at 22°C. The pH was then adjusted to 5.5 and phenylmethylsulfonyl-fluoride (0.1 mM final concentration) was added. Refolding was initiated (arrow 2) by adding to the renetion mixture 50 μl of ferric-ammonium citrate, pH 7. Fe^{3+} final concentration was 3 mM. Then the samples (100 μl) were withdrawn at the times indicated and treated as above. Samples were assayed for levansuerase activity as described in section 2. Samples were assayed for levansuerase activity [4].

chain flexibility of the exoprotein. Such a key role of the tertiary structure of the protein translocation has been postulated to be very general [2].

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