Kinetics of the unfolding–folding transition of \textit{Bacillus subtilis} levansucrase precursor

Pier A. Scotti*, Régis Chambert, Marie-Françoise Petit-Glatron

Institut Jacques Monod, CNRS, Université Paris VII Laboratoire Génétique et Membranes, Tour 43, 2, place Jussieu, 75251 Paris Cedex 05, France

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Abstract The reversible folding–unfolding transition of mature and precursor forms of \textit{Bacillus subtilis} levansucrase were compared under physiological conditions of pH and temperature. The time constant of the folding reaction was not modified by the presence of the signal sequence and the precursor in the native form was slightly more resistant to the denaturing action of urea. However, the folding pathway could be different for each protein since a domain of the mature levansucrase underwent an independent transition which is not observed during the renaturation process of prelevansucrase.

Key words: Folding; Signal sequence; Prelevansucrase; \textit{Bacillus subtilis}

1. Introduction

Signal sequences play a key role in both the efficient and selective targeting of nascent protein chains and the translocation process across the membrane [1]. It has also been suggested that signal sequences can act as intramolecular chaperons. By lowering the folding rate of the protein en route to be secreted, they could preserve an unfolded competent state for secretion. The precursor forms of some exported proteins from \textit{E. coli}, maltose-binding protein, ribose-binding protein [2] and \textit{β}-lactamase [3] refolded more slowly in vitro than the corresponding mature forms. In contrast, it was shown that several precursor forms of mitochondrial proteins refolded with similar yield and kinetics as their presequence-free counterparts [4].

We proposed here to address this issue with levansucrase, a protein secreted by \textit{Bacillus subtilis}. This approach has been made possible as the precursor form of levansucrase can be obtained in reasonable quantities, under pure native state, from the expression of its structural gene in yeast [5]. Moreover, previous studies led us to define the in vitro conditions for monitoring the reversible folding–unfolding transition of this protein [6].

2. Materials and methods

2.1. Purification of precursor and mature forms of levansucrase

The precursor form was purified from the membrane fraction of the yeast strain \textit{WSacBA} according to the published procedure [5]. From 10 g of wet weight cells, 1 mg of precursor was obtained under pure state. The mature levansucrase was prepared from the supernatant of the induced culture of \textit{B. subtilis} QB112 strain [7].

2.2. Fluorescence measurement

Variations of intrinsic fluorescence of the proteins were measured with a F2000 Hitachi thermoregulated spectrofluorimeter. Wavelengths were fixed to 280 nm for excitation and 336 nm for emission.

2.3. Levansucrase assay

Levansucrase activity was estimated by measuring its saccharolytic activity. The amount of glucose released was estimated according to the Somogyi procedure [8].

2.4. Gel electrophoresis and immunoblotting

Proteins were analyzed on 10% (w/v) SDS polyacrylamide gels. Electrophoresis and immunoblotting were performed as previously described [9].

2.5. Enzymes and products

Subtilisin from \textit{B. subtilis} was supplied by Sigma. Urea was purchased from Merk.

3. Results

3.1. Reversible unfolding–folding transition of precursor and mature forms of levansucrase measured by intrinsic fluorescence

The purified form of levansucrase and prelevansucrase (Fig. 1a) were first unfolded in 7 M urea. The renaturation was promoted by dilution of the denaturant (0.5 M final concentration) and monitored by measuring the changes of intrinsic fluorescence intensity of these proteins with respect to the time (Fig. 1b). The recovery of the fluorescence property of native proteins was total and followed the same kinetics for both forms, \( t_{1/2} = 75 \) s and \( t_{1/2} = 71 \) s for mature and precursor forms, respectively. The semi-logarithmic plots of fluorescence intensity changes were linear (Fig. 1c). The results suggested that the unfolding–folding transitions could be approximated by a simple two state model which kinetics is described by the following equation: \( I(t) = (I_1-I_0) e^{-t/t} \) where \( I, I_0, I_1 \) are the fluorescence intensity values at any time, zero time and infinite time respectively. The time constant \( t \) (relaxation time) could be calculated from the slope of the straight lines obtained in Fig. 1c.

3.2. Comparison of relaxation times for the folding–unfolding transition of mature and precursor levansucrases

The relaxation times of the folding–unfolding transition were obtained by monitoring the changes in intrinsic fluorescence of the proteins induced by the addition or the dilution of urea (Fig. 2). The presence of the leader sequence did not significantly alter the time constant for refolding. The folding time constants extrapolated at 0 M urea pH 7, 0.1 M potassium phosphate, 25°C, were 52 ± 5 s and 47 ± 5 s for levansucrase and prelevansucrase, respectively. Moreover the urea concentrations of the midpoint of denaturation were not very different, 1.8 M for prelevansucrase and 1.5 M for the mature form. These later data were in good agreement with the value of 1.6 M, previ-
Fig. 1. Kinetics of levansucrase and prelevansucrase refolding measured by fluorescence intensity changes. The purity of the two protein solutions was checked by SDS-PAGE 10% (a). Samples (5 μl) of each protein stock solution, 300 μg ml⁻¹ were loaded on acrylamide gels and stained with Coomassie blue. Lane 1: precursor form of levansucrase purified from W0 sacB4 yeast strain; lane 2: purified levansucrase from B. subtilis QB112 culture supernatant. Molecular mass of protein markers (in kDa) are indicated on the left. Unfolding was promoted by mixing 10 μl of protein stock solutions with 100 μl of 5 M urea in 0.1 M potassium phosphate pH 7 at 25°C. After 10 min refolding was promoted by adding 0.9 ml of the same buffer. Fluorescence intensity was monitored at 25°C. Traces (1: prelevansucrase; 2: mature levansucrase) of fluorescence intensity changes (b) and semi-logarithmic representation (c) are given.

3.3. Unfolding–refolding transition of levansucrase and prelevansucrase measured by proteinase sensitivity

The proteinase resistance acquisition has been successfully used to study the refolding of mature levansucrase. In its native state, but not under the unfolded state, the protein was resistant to subtilisin degradation [6]. The appearance of a subtilisin-resistant form was analyzed by enzymatic activity and by immunoblotting. Both proteins recovered concomitantly their proteinase resistance (Fig. 3). An half time of 35 s was evaluated for each refolding kinetics (Fig. 3a). However, a 20,000 mol wt. peptide resistant to proteolysis and possessing one or several epitopes of the native protein was observed during the initial phase of the folding reaction, only with the mature levansucrase (Fig. 3b). It may be reasonably supposed that this peptide results from the fast refolding of an independent domain of the protein leading to a folding intermediate partially resistant to proteolysis. This finding suggests that either the folding pathway is different for each protein or the NH2-terminal extension leads to a protease sensitive intermediate. Furthermore the leader sequence was removed in the presence of subtilisin. This result was confirmed by incubating native prelevansucrase with subtilisin (Fig. 4). The 53 kDa form was rapidly processed to a stable 50 kDa form. Such a processing was also reported for precursor forms of maltose-binding protein and of ribose-binding protein incubated with proteinase K [2].

4. Discussion

As most of the secretory proteins, B. subtilis levansucrase is synthesized under a precursor form. It has been proposed and demonstrated in vitro that the presence of a signal sequence delays the unfolding-folding transition of some proteins [2,3]. However such property cannot be extended to all secretory

Fig. 2. Relaxation times of folding transitions at various concentrations of urea. The relaxation times τ, in seconds, for folding (filled symbols) and unfolding (open symbols) transitions were obtained by monitoring the changes in protein fluorescence as in Fig. 1. Experiments were carried out at 25°C in 0.1 M potassium phosphate pH 7. Unfolding was promoted by mixing 10 μl of each protein stock solution in 1 ml of urea solution at various concentrations; mature levansucrase (○); prelevansucrase (△). Refolding was obtained by mixing 10 μl of protein with 8 M urea. After 10 min, urea was diluted with phosphate buffer to obtain the final concentrations indicated. The volume was 1 ml in each case; mature levansucrase (●); prelevansucrase (○).
We reported here that the presence of levansucrase leader sequence decreased the unfolding rate promoted by urea. Thus, the protein is more stable in the presence of this denaturing agent with its signal sequence although this later remains accessible to proteolytic degradation suggesting that it is not buried in the final tertiary structure. On the other hand, under physiological conditions of pH and temperature the precursor and the mature forms of levansucrase refolded with very similar rates. However the pathway of the folding reaction might be different since an intermediate partially resistant to protease degradation was detected only with the mature form. The presequence could prevent the formation of such an intermediate or could confer an enhanced protease sensitivity. Whatever the hypothesis chosen, it is difficult to ascribe a function in the secretion process to this precursor property. Comparing the folding process of precursor and mature forms in vitro gives attractive results per se, but does not afford direct informations about the secretion mechanism. We have to keep in mind that cytosolic factors could modulate the kinetics of prelevansucrase folding via specific interactions with the signal sequence. As molecular chaperons homologous to E. coli GroEL and DnaK have been characterized in B. subtilis [10-12] it would be of great interest to investigate in vitro the effects of these proteins on the folding of the prelevansucrase.

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References