Anionic polymers of *Bacillus subtilis* cell wall modulate the folding rate of secreted proteins

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Abstract

In order to characterize the dynamics of the interaction between the emergent membrane translocated exoprotein and the components of *Bacillus subtilis* cell wall, we examined the kinetics of the in vitro refolding of levansucrase and α-amylase, at pH 7 and 37°C, in the presence of polyphosphates (polyP) of various chain lengths (2 ≤ n ≤ 65). These soluble anionic polymers are considered here to mimic the role of teichoic acids. Even in the absence of calcium, levansucrase rapidly refolded in the presence of polyP of n ≥ 16. In contrast, polyP modulate indirectly the rate of α-amylase refolding via their affinity for calcium. These differential effects might explain that the rate of the cell wall translocation of α-amylase secretion was found to be half that of levansucrase.

Keywords: Protein folding; Alpha-amylase; Levansucrase; Teichoic acid; Cell wall translocation; Bacillus subtilis

1. Introduction

It is now widely accepted that proteins are translocated under a non-native state [1–5]. In *B. subtilis*, proteins emerging on the trans side of the membrane encounter the cell wall, a highly cross-linked semipermeable macromolecule composed of peptidoglycan and teichoic acids. The subsequent translocation of the extracellular proteins through the cell wall requires a rapid and efficient folding into their native conformation in order to prevent the proteolytic action of cell wall associated proteases [6,7] and to minimize interactions with cell wall components. It was proposed that PrsA [8], a lipoprotein linked to the outer surface of the cytoplasmic membrane, assists the folding. Other possible cofactors of folding are metal ions such as calcium or iron which are concentrated at the membrane wall interface [9,10] and mediate the in vitro refolding of several proteins [11–13].

In the present work, we explore the hypothesis that structural components of cell wall could per se facilitate the folding of proteins en route to be secreted. We focused our attention on the possible role of teichoic acids. These phosphorylated polymers, which account for almost 50% of the cell wall dry-weight [14], are linear polyanionic molecules that usually contain between 10 and 50 glycerophosphate residues.
units, displaying affinity for metal ions such as \( \text{Ca}^{2+} \) and possessing a \( K_A \) of \( 4.5 \times 10^4 \text{ M}^{-1} \) [15]. This compound is not commercially available and is difficult to obtain in an homogeneous and pure unmodified state from cell wall extracts [16]. Allowing for that, we chose inorganic short chain length polyphosphates (polyP) as models to mimic the properties of teichoic acids and their effects on the refolding of \( \alpha \)-amylase and levansucrase, two \( B. \ subtilis \) exocellular proteins of which the in vitro reversible unfolding-folding transition at pH 7 and 37°C are well characterized [11,12].

2. Materials and methods

2.1. Materials

PolyP (sodium salts) of various chain lengths were purchased from Sigma.
2.2. Purification and assays of proteins

Levansucrase (stock solution 30 mg ml\(^{-1}\)) was purified from the culture supernatant of induced \textit{B. subtilis} QB112 (\textit{degU32}(Hy), \textit{sacA321}) according to the published procedure [17].

\(\alpha\)-Amylase (stock solution 30 mg ml\(^{-1}\)) was prepared from the culture supernatant of induced \textit{B. subtilis} GM96101 (\textit{degU32}(Hy), \textit{sacA321}, \Delta\textit{sacB}, \Delta\textit{RamyE}) [18], according to the published procedure [19].

2.3. Fluorescence measurements

Changes in intrinsic fluorescence and fluorescence spectra were recorded with a F2000 Hitachi thermostated spectrofluorimeter.

3. Results

3.1. Unfolding-refolding transition of levansucrase and \(\alpha\)-amylase in the presence of polyP and in the absence of calcium

We showed previously [12,20] from in vitro studies that the unfolding-refolding transition of the two proteins was mediated by calcium at pH 7 and 37\(^\circ\)C. In the absence of this metal, the proteins remained in an unfolded state after dilution of denaturing agents. We studied the effect of the presence of 1 mM polyP of various chain lengths under such conditions. Refolding was monitored by measuring changes in the intrinsic fluorescence and the disappearance of proteolytic sensitivity of the refolded state. We observed that levansucrase rapidly and fully refolded in the presence of polyP of \(n \geq 16\). In contrast, these polymers did not promote \(\alpha\)-amylase refolding. The effect of polyP\(_{45}\) (Fig. 1) illustrated this study.

3.2. Unfolding-refolding transition of levansucrase and \(\alpha\)-amylase in the presence of both polyP and calcium

The effects of 1 mM polyP of various chain lengths on the unfolding-refolding transition of each protein, occurring in the presence of 0.5 mM calcium, were monitored by measuring changes in the intrinsic fluorescence intensity at 343 nm as a function of time.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{fig2.png}
\caption{Rate constants of unfolding-refolding transition of levansucrase and \(\alpha\)-amylase in the presence of polyP of various chain lengths and a fixed concentration of calcium. Levansucrase and \(\alpha\)-amylase were unfolded in 5 M Gdn-HCl as described in Fig. 1. The refolding at 37\(^\circ\)C was initiated by diluting the denaturing mixture 200 times in 0.1 M sodium phosphate pH 7, 0.5 mM CaCl\(_2\), 1 mM polyP of chain length indicated. The apparent first order rate constants of refolding were determined from the regression adjustment, by the least square method, of changes in the intensity of fluorescence at 343 nm as a function of time [11,12]. Levansucrase (○); \(\alpha\)-amylase (●).}
\end{figure}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{fig3.png}
\caption{Kinetics of unfolding-refolding transition of levansucrase and \(\alpha\)-amylase with respect to polyP\(_{65}\) concentration and a fixed concentration of calcium. Levansucrase and \(\alpha\)-amylase were unfolded in 5 M Gdn-HCl as described before. The refolding was initiated by diluting the denaturing mixture 200 times in 0.1 M sodium phosphate, pH 7, 0.5 mM CaCl\(_2\) containing 0–100 \(\mu\)M polyP\(_{65}\). The rate constants of refolding were determined from changes in the fluorescence intensity at 343 nm as a function of time. Levansucrase (○); \(\alpha\)-amylase (●).}
\end{figure}
Ca\textsuperscript{2+}, at pH 7 and 37\degree C, are shown in Fig. 2. The rate constants of levansucrase refolding increased with the chain length of polyP, whereas an opposite result was obtained for \( \alpha \)-amylase. PolyP of \( n > 5 \) totally inhibited the refolding of this latter protein. The variation in the folding rate constant of each protein versus the concentration of polyP \( 65 \) in the folding mixture was studied (Fig. 3). We noted that both proteins refolded at an equivalent rate in the absence of polyP \( 65 \). A gradual increase of polyP \( 65 \) concentration decreased the folding rate constant of \( \alpha \)-amylase, whereas it had the opposite effect on the folding rate of levansucrase.

3.3. How does polyP inhibit \( \alpha \)-amylase refolding?

Two hypotheses can account for the inhibition of \( \alpha \)-amylase refolding: either polyP stabilize the unfolded form of the protein through electrostatic interactions or polyP, via their high affinity to calcium (\( K_A = 6.3 \times 10^6 \) M\(^{-1} \) for polyP \( 60 \) [21]), decrease the amount of free calcium needed to promote protein refolding. To test the second hypothesis, we studied whether inhibition of refolding by polyP could be abolished by the addition of a large excess of calcium. This hypothesis seems to be correct, as shown in Fig. 4.

4. Discussion

PolyP of \( n > 16 \) act as cofactors of levansucrase folding even in the absence of calcium, \( \alpha \)-amylase, in contrast, needs free calcium to promote its folding. If polyP mimic in vitro, at least partially, the role played by teichoic acids in the cell wall environment, one would expect that levansucrase emerging from the translocase complex fold more rapidly than \( \alpha \)-amylase. This could explain the difference in the release kinetics of the two proteins, \( t_{1/2} \) of 1 min and 2 min, for levansucrase and \( \alpha \)-amylase, respectively. Furthermore, we show that a high affinity of calcium for polyP inhibits \( \alpha \)-amylase folding mediated by this metal ion. This might be an indication that modulation of teichoic acid anionic charge via alanylation [22] controls calcium affinity in such a way that free calcium remains available for an efficient folding of exoproteins that have properties in common with \( \alpha \)-amylase. On the other hand, we cannot rule out the possibility that the polyP themselves contribute to the efficiency of levansucrase secretion. Although no counterpart of \( E. \) coli polyP kinase was found in \( B. \) subtilis [23], it is reasonable to suggest that different enzymic pathways might promote the synthesis of short chain length polyP in this bacterium [24].

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References


