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Bacillus subtilis α-amylase: interactions of a partially folded conformer with small unilamellar vesicles

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Abstract

We studied the interactions between conformers of exocellular α-amylase and small unilamellar vesicles (SUV) composed of the major membrane lipids of *Bacillus subtilis* under physiological conditions of pH, temperature and ionic strength. Using fluorescence spectroscopy, surface plasmon resonance (SPR) and phase separation, we show that the native α-amylase has no affinity for the SUV, whereas a partially folded form, displaying structural properties in common with the competent state for secretion, binds to the vesicles ($K_A \approx 10^5\ M^{-1}$). This association prevented its subsequent folding. The complex was destabilized in the presence of PrsA, a major peripheric lipoprotein of *B. subtilis* which displays a strong affinity for SUV ($K_A \approx 1.5 \times 10^8\ M^{-1}$). Vesicles coated with PrsA lost their ability to bind the partially folded conformer.

The approach in vitro, in which our aim was to mimic the last stage of α-amylase translocation, indicates that PrsA possibly helps, in vivo, the secreted protein to acquire its native conformation by modulating the interaction between the latter and the lipid polar heads on the trans side of the cytoplasmic membrane.

Keywords: *Bacillus subtilis*; Protein folding; Small unilamellar vesicle; Surface plasmon resonance; PrsA lipoprotein

1. Introduction

The dynamics of folding plays a decisive role during the translocation process undergone by imported or exported proteins [1–3]. There is considerable evidence that translocation involves a partially folded intermediate (competent state) [4] which emerges on the extracellular side of the translocation complex. However, it has been demonstrated, in vitro, that protein conformers, which possess, like competent state, significant secondary structures but lack native tertiary structure, exhibit membrane association properties [5–7]. Such interactions could trap the partially folded protein during its secretion in a cell-associated state, thus preventing both its subsequent folding and its release into the external medium. One can postulate that to avoid such an eventuality, either the kinetics of folding transition is faster than the membrane binding step or a membrane component shields the partially folded protein from interactions with lipid polar heads. To examine this hypothesis in the case of the secretion mechanism of *Bacillus subtilis*, we propose here an in vitro approach using small unilamellar vesicles (SUV) composed of the main lipids that make up the *B. subtilis* cytoplasmic membrane [8] and α-amylase as a model of secreted protein. We have shown that a partially folded form of this protein sharing common structural features with the competent state for secretion can be stabilized under physiological conditions of pH, temperature and ionic strength [9]. The strong interaction that we observed between the partially folded conformer and SUV led us to search for a potential modulator present on the trans side of the *B. subtilis* membrane. The lipoprotein PrsA was a good candidate. This major extracytoplasmic protein is bound to the outer face of the cytoplasmic membrane of *B. subtilis* [10]. It has been suggested that it can function as an extracytoplasmic chaperone during the last stage of protein secretion [11]. But no evidence documenting the in vitro activity of PrsA has yet been published. Alternatively, it has been proposed [12] that PrsA may protect secreted proteins from degradation either by acting as a proteinase inhibitor or by shielding the newly

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secreted proteins from proteinases. Interestingly, this abundant protein, whose concentration remains unchanged throughout growth phases ($2-3 \times 10^4$ molecules per cell) [13,14], can cover 15% to 20% of the outer surface of the cytoplasmic membrane.

Using fluorescence spectroscopy, surface plasmon resonance (SPR) and phase separation, we investigated the interactions between partially folded α-amylase and SUV and their modulation by PrsA.

2. Materials and methods

2.1. Purification of exocellular α-amylase

α-Amylase was prepared from the culture supernatant of *B. subtilis* GM96101 (*degU32* (Hy), *sacA321*, Δ*sacRsacB*, *sacRamyE*), a derivative of *B. subtilis* 168 [15]. A stock solution of pure protein (10 mg/ml) was prepared in 0.1 M potassium phosphate, pH 7.

2.2. Assay of α-amylase

α-Amylase activity was assayed at 37 °C, using *p*-nitrophenylmaltotrioside as substrate (bio-Merieux) at pH 6.3 in 0.1 M potassium phosphate. One enzyme unit corresponds to 25 μg of pure α-amylase.

2.3. SUV preparation

A mixture of dry phospholipids (Sigma) was prepared with 35% phosphatidyl-ethanolamine, diheptadecanoyl (C17:0), 15% phosphatidyl-glycerol, dipalmitoyl (C16:0) and 50% cardiolipin. This mixture was dissolved in chloroform and the lipids were deposited as a thin film by removing the chloroform under a stream of nitrogen and desiccated under high vacuum for 10 min to remove residual chloroform. The lipids were dispersed in 0.2 M sodium acetate, pH 7, by vortex mixing. The resultant lipid dispersion (5 ml) was then sonicated in an ultrasonicator using a tungsten microtip (diameter of 3 mm) for 45 min (until clear). Tungsten dust was removed by centrifuging the homogenate for 10 min at $1250 \times g$. The resulting vesicles (SUV) were at a final lipid concentration of 2 mM.

2.4. SPR analysis

Biosensor experiments were carried out with a BIAcore 2000 analytical system (Biacore, Uppsala, Sweden) using an HPA sensor chip (Biacore) composed of long chain alkanethiol molecules covalently linked to the gold surface to form a hydrophobic monolayer. The running buffer was 0.2 M sodium acetate pH 7, containing 1 mM EDTA. All solutions were freshly prepared, degassed and filtered through a 0.22 μm filter. All binding experiments were carried out at 25 °C.

2.5. Formation of monolayer membrane

The alkanethiol surface of the sensor chip HPA was cleaned by injecting a solution of 40 mM octyl glucoside (100 μl) at 10 μl/min. The suspension of SUV (0.5 mM) was then injected over the chip surface at 2 μl/min. Any

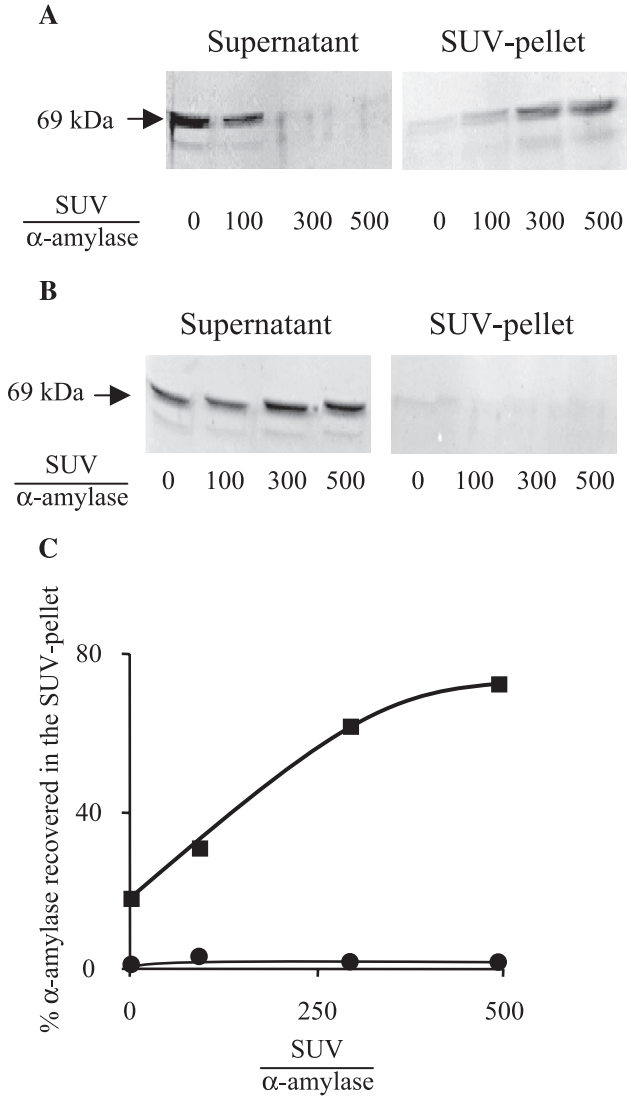

Fig. 1. Partition of native and partially folded conformers of α-amylase between aqueous and lipid phases. (A) For each assay, α-amylase was first totally unfolded by mixing 1.5 μl α-amylase stock solution (1 mg/ml) with 6 μl 6 M guanidium chloride. After 10-min incubation at 37 °C, the folding intermediate was stabilized by diluting the denaturing mixture with 0.15 ml 0.2 M sodium acetate pH 7, 1 mM EDTA. After 1 min incubation, various amounts of SUV stock suspension (2 mM lipid) were added. The lipid phase of each sample was collected by centrifugation at $435{,}700 \times g$ after 5 min incubation at 37 °C. The supernatants and resuspended pellets were analysed by SDS-PAGE. (B) 1.5 μl of α-amylase stock solution (1 mg/ml) was mixed with 0.15 ml 0.2 M sodium acetate pH 7, 1 mM EDTA, containing various amounts of SUV. After 1 min incubation, the lipid phase of each sample was collected as above. (C) Quantification of the percent of native (●) and partially folded conformer (■) of α-amylase in SUV pellet was obtained from densitometric tracings of the Coomassie blue-stained gels.

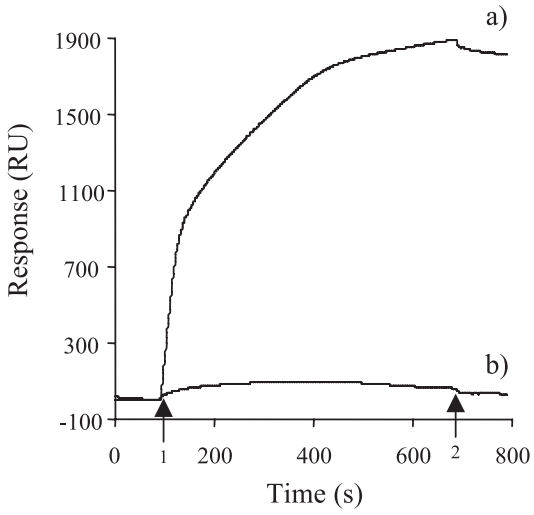

Fig. 2. SPR sensorgrams of the binding of native (a) and partially folded conformers (b) of α-amylase to an immobilized lipid monolayer. A stable lipid monolayer was prepared on the sensor chip as described in Materials and methods. The kinetics of the association of α-amylase with the monolayer was monitored by injecting the folding intermediate (a) or the native form (b) (0.3 μM) at 20 μl/min. The α-amylase folding intermediate was prepared as described in Materials and methods. Arrows 1 and 2 indicated the start and end of α-amylase injection.

multilamellar structures were removed from the lipid surface by injecting sodium hydroxide (30 μl, 10 mM) at 100 μ1/min. This resulted in a stable baseline to give a response signal of 2400 RU [16]. The lipid surface was used to study the protein binding to the lipid membrane.

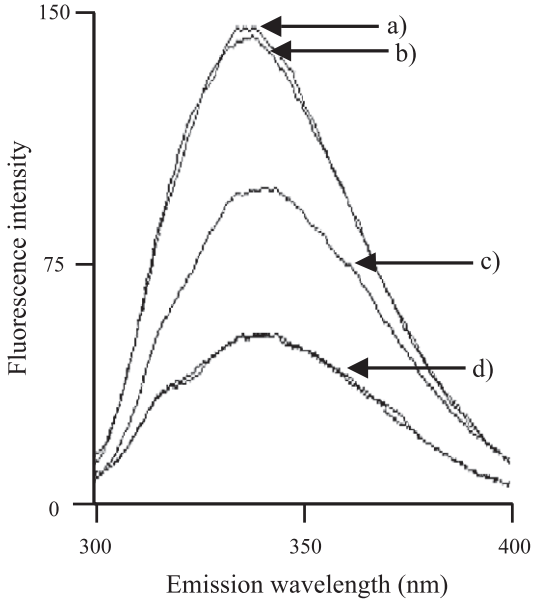

Fig. 3. Intrinsic fluorescence spectra of native and partially folded α-amylase in the absence and presence of SUV. Intrinsic fluorescence spectra of α-amylase were recorded at 37 °C in 0.2 M sodium acetate pH 7, containing 1 mM EDTA. The final concentration of α-amylase was 0.15 μM in each case. The excitation wavelength was 280 nm. (a) Native α-amylase, (b) native α-amylase in the presence of SUV (lipid/protein molar ratio of 300), (c) folding intermediate and (d) folding intermediate in the presence of SUV (lipid/protein molar ratio of 300). For (d), two traces of fluorescence emission spectra were recorded at a time interval of 10 min. Protein spectra were corrected by the spectrum of a reference solution without protein.

2.6. Preparation in vitro of the α-amylase folding intermediate

The folding intermediate of α-amylase was obtained by a two-step procedure: First, the wholly unfolded form was

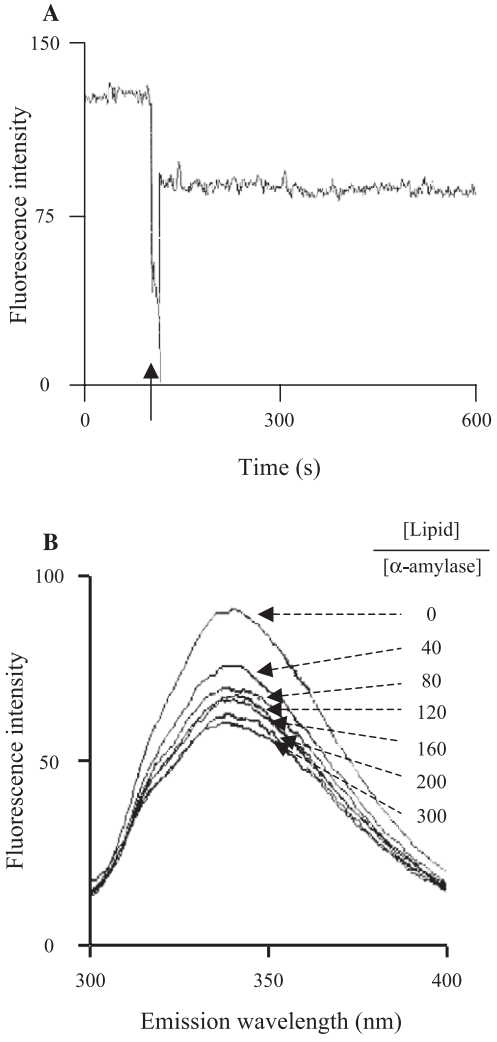

Fig. 4. Intrinsic fluorescence changes of partially folded α-amylase in the presence of SUV. An aliquot (l μl) of α-amylase stock solution (10 mg ml^{-1}) was mixed with 4 μl 6 M guanidium chloride. After 10 min incubation at 37 °C, the denaturing mixture was diluted with 1 ml 0.1 M sodium phosphate pH 7 containing 1 mM EDTA. (A) Kinetics of association between SUV and α-amylase folding intermediate measured by fluorescence intensity changes. The fluorescence intensity of the solution of α-amylase folding intermediate (0.1 μM, pH 7, 37 °C) was recorded for 100 s. The formation of the complex was promoted by adding (arrow) 20 μl SUV stock suspension (lipid/α-amylase ratio of 300). The excitation wavelength was 280 nm and the emission wavelength 338 nm. (B) Fluorescence spectra of α-amylase folding intermediate in the presence of various amounts of SUV. A 0.15 μM solution of the folding intermediate was prepared as described in Fig. 3, and the fluorescence spectra (excitation wavelength was 280 nm) were recorded 5 min after each successive addition of 3 μl of SUV stock suspension (2 mM lipid). The lipid/protein molar ratios tested were 0 to 300.

obtained by mixing the native protein with 5 M guanidium chloride for 10 min at pH 7 and 37 °C. The folding intermediate was then obtained by diluting the denaturing mixture 100 fold in acetate buffer pH 7, containing 1 mM EDTA at 37 °C [9]. The second step of the refolding process does not occur in the absence of free calcium, so that the folding intermediate accumulated.

2.7. Fluorescence measurements

Changes in the intrinsic fluorescence and fluorescence spectra of α-amylase were recorded with a F2000 Hitachi thermoregulated spectrofluorimeter.

3. Results

3.1. Partially folded B. subtilis α-amylase displays affinity for SUV

We have previously defined the conditions under which a folding intermediate of α-amylase is stabilized [9]. This partially folded protein accumulated when α-amylase was allowed to refold in the absence of calcium.

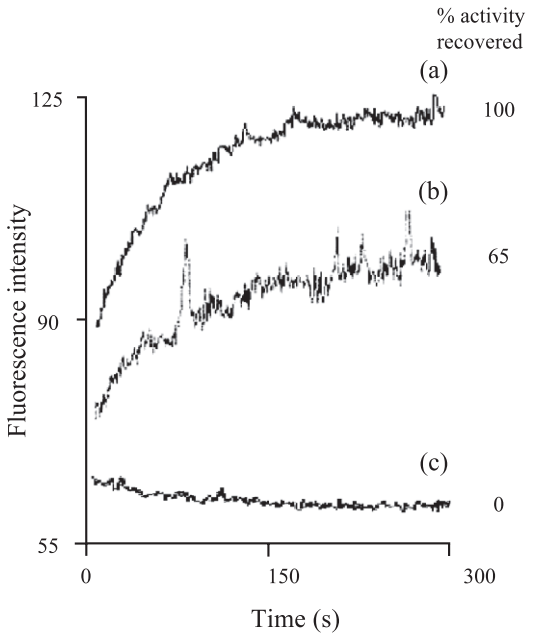

Fig. 5. Refolding transition of α-amylase measured by intrinsic fluorescence changes in the presence of SUV at 37 °C and pH 7. Traces show the fluorescence intensity as a function of time (excitation wavelength 280 nm, emission wavelength 338 nm). One microliter α-amylase stock solution (10 mg ml^{-1}) was mixed with 4 μl 6 M guanidium chloride. After 10 min incubation at 37 °C, the denaturing mixture was diluted with 1 ml 0.1 M sodium phosphate pH 7, containing 0.5 mM calcium and various concentrations of SUV. (a) No SUV added, (b) lipid/α-amylase ratio of 100 and (c) lipid/α-amylase ratio of 300. α-Amylase activity was assayed using 5 μl aliquot of each sample taken 3 h after the beginning of refolding.

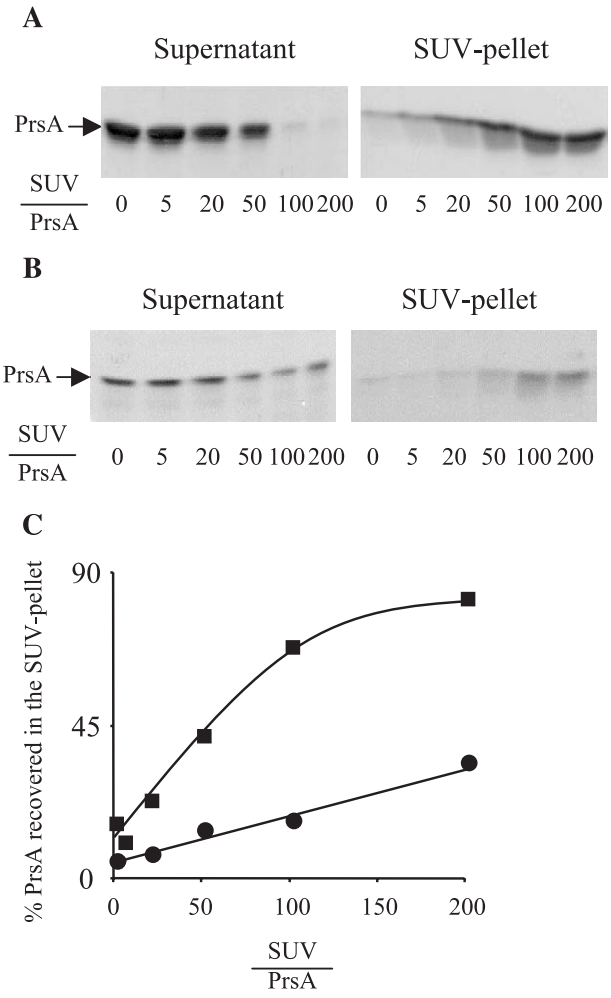

Fig. 6. Partition of lipomodified PrsA and non-lipomodified PrsA between aqueous and lipid phases. Five microliter stock solution (0.4 mg/ml) of lipomodified PrsA (A) and 3 μl stock solution (0.7 mg/ml) of non-lipomodified PrsA (B) were incubated with 60 μl 0.2 M sodium acetate pH 7, 0.5 mM calcium plus SUV. After 15 min incubation at 37 °C, the samples were then centrifuged at 435,700 × *g*. The supernatants and pellets were analysed by SDS-PAGE. (C) Quantification of the percent of PrsA (■) and non-lipomodified PrsA (●) in SUV-pellet.

We first investigated the interaction of this α-amylase conformer with SUV by measuring the partition of the protein between the aqueous and lipid phases at 37 °C and pH 7. The amount of folding intermediate carried down with the SUV-pellet increased as the ratio of lipid to protein increased (Fig. 1A). In contrast, the native form of α-amylase had no affinity for SUV (Fig. 1B).

The different interactions of the two conformers with the lipid bilayer were examined by SPR (Fig. 2). The SPR signal underwent a significant change when a solution of the folding intermediate was injected over a lipid monolayer immobilized on the sensor chip. In contrast, injection of native α-amylase produced only a very small signal. The SPR sensorgram, however, showed that the association phase of the folding intermediate binding was complex with multiphase kinetics that prevented us measuring an association constant

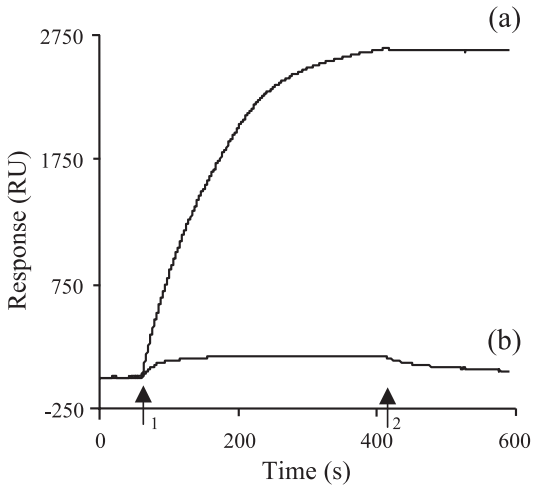

Fig. 7. SPR sensorgram of the binding of lipomodified PrsA and non-lipomodified PrsA to an immobilized lipid monolayer. Stable lipid monolayer was formed on the sensor chip as described in Materials and methods; the kinetics of protein association with the vesicles was monitored by injecting (a) lipomodified PrsA (0.5 μM) or (b) non-lipomodified PrsA (1 μM) at 2 μl/min. Arrows 1 and 2 indicate the start and end of protein injection, respectively.

even when attempts were made to fit these data to complex binding interaction. We have to keep in mind that such a quantitative analysis of binding is based on the hypothesis that proteins did not aggregate on the biosensor surface. This was probably not true for the folding intermediate since the protein is prone to aggregate at high concentrations [17]. This condition may prevail on the biosensor surface since it was estimated that a signal of 1000 response units (RU) corresponds to a protein surface concentration of approximately 10 mg ml^{-1} [18].

Specific interactions between the folding intermediate of α-amylase and SUV can be demonstrated by the changes in the intrinsic fluorescence properties of the protein (Fig. 3). These experiments were carried out with low protein concentrations to prevent aggregation [17]. The fluorescence intensity of native α-amylase was not altered by adding SUV, suggesting that the quenching of the partially folded α-amylase resulted from its interactions with SUV. The association with SUV did not seem to entail aggregation of the protein since no shift in the maximum of its fluorescence emission spectrum was observed (Fig. 3). This indicates that the protein was adsorbed onto the SUV rather than inserted into the lipid bilayer. Association was rapid, as demonstrated by the quick change in intrinsic fluorescence of the α-amylase folding intermediate subsequent to its mixing with SUV (Fig. 4A). Association occurs during the dead time of the manual mixing (5–8 s). The transition of the protein binding was visualized by the gradual quenching of the intrinsic fluorescence intensity that accompanied the increase in the ratio of SUV to protein (Fig. 4B). An affinity constant was calculated by fitting the fraction of binding to lipid concentration ($K_A \approx 10^5$) [19].

3.2. The binding of folding intermediate to SUV prevented its subsequent refolding

Refolding of the stabilized folding intermediate is readily triggered by adding calcium at pH 7 and 37 °C as shown previously [9]. Therefore, we tested the effect of increasing SUV concentrations on the kinetics of refolding (Fig. 5) and found that SUV prevented the refolding monitored by recovering the fluorescence characteristic of the native α-amylase state. This effect depended on the SUV concentration. Refolding was completely inhibited when all the folding intermediate was complexed with SUV. Thus, association was faster than refolding and it prevented the subsequent refolding of the protein. The recovery of α-

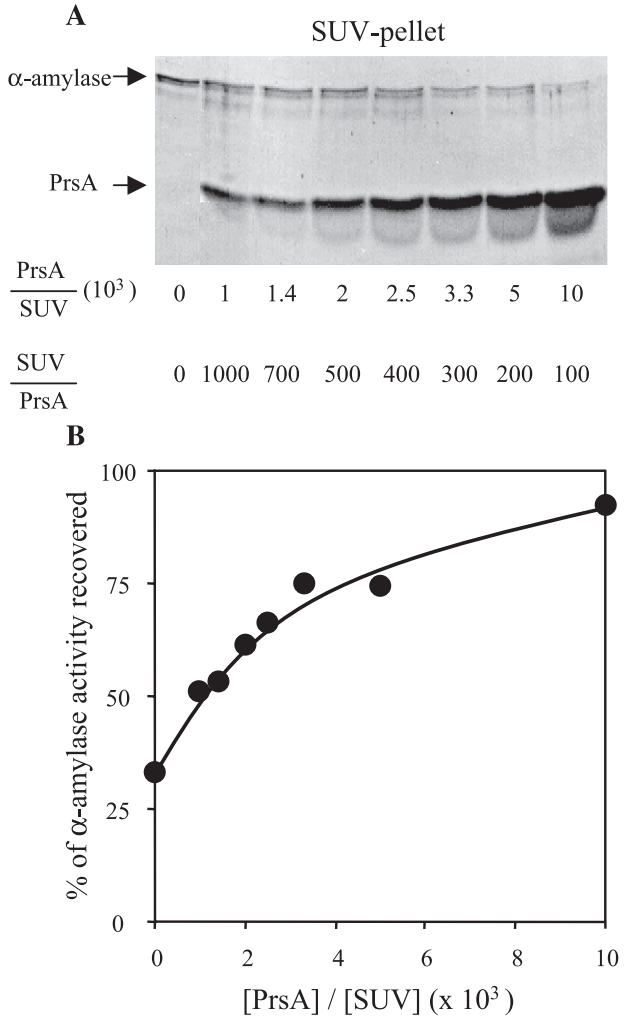

Fig. 8. Effect of increasing PrsA-SUV ratio on the ability of SUV to bind the partially folded conformer of α-amylase. (A) SUV stock solution (3.5 μl, 2 mM lipid) was diluted into 70 μl 0.2 M sodium acetate pH 7, 0.5 mM calcium containing increasing concentrations of lipomodified PrsA. After 15 min incubation at 37 °C, unfolded α-amylase (1 μg) was then added to each sample. The mixtures were incubated for 10 min and centrifuged at 435 700 × *g*. The pellets were solubilized into 70 μl 10% SDS and analysed by SDS-PAGE. (B) The α-amylase activity in the supernatants was measured as described in Materials and methods.

amylase activity agreed well with the changes in the intrinsic fluorescence properties (Fig. 5).

3.3. Native PrsA has a strong affinity for SUV

We tested the affinity of native PrsA for SUV using the same techniques with the protein in its lipomodified or non-lipomodified form [10]. Phase separation experiments (Fig. 6) showed that the lipomodified PrsA had a high affinity for SUV. We determined the kinetics of PrsA binding to lipid monolayer immobilized on the SPR biosensor from SPR sensorgram (Fig. 7). This affinity was mainly due to the presence of the lipid anchor as suggested by the results we obtained with the protein devoid of its lipid anchor. The affinity constant obtained by fitting the binding curve to a simple model with two binding partners was $1.5 \times 10^8\ M^{-1}$ [20]. We calculated that the entire biosensor surface was coated with PrsA from the magnitude of the SPR signal change associated with the binding of the protein [16].

The association of PrsA with SUV did not alter the intrinsic fluorescence properties of the protein (result not shown), indicating that the protein was adsorbed onto the lipid monolayer via its lipid anchor and that its tertiary structure was not modified by the association.

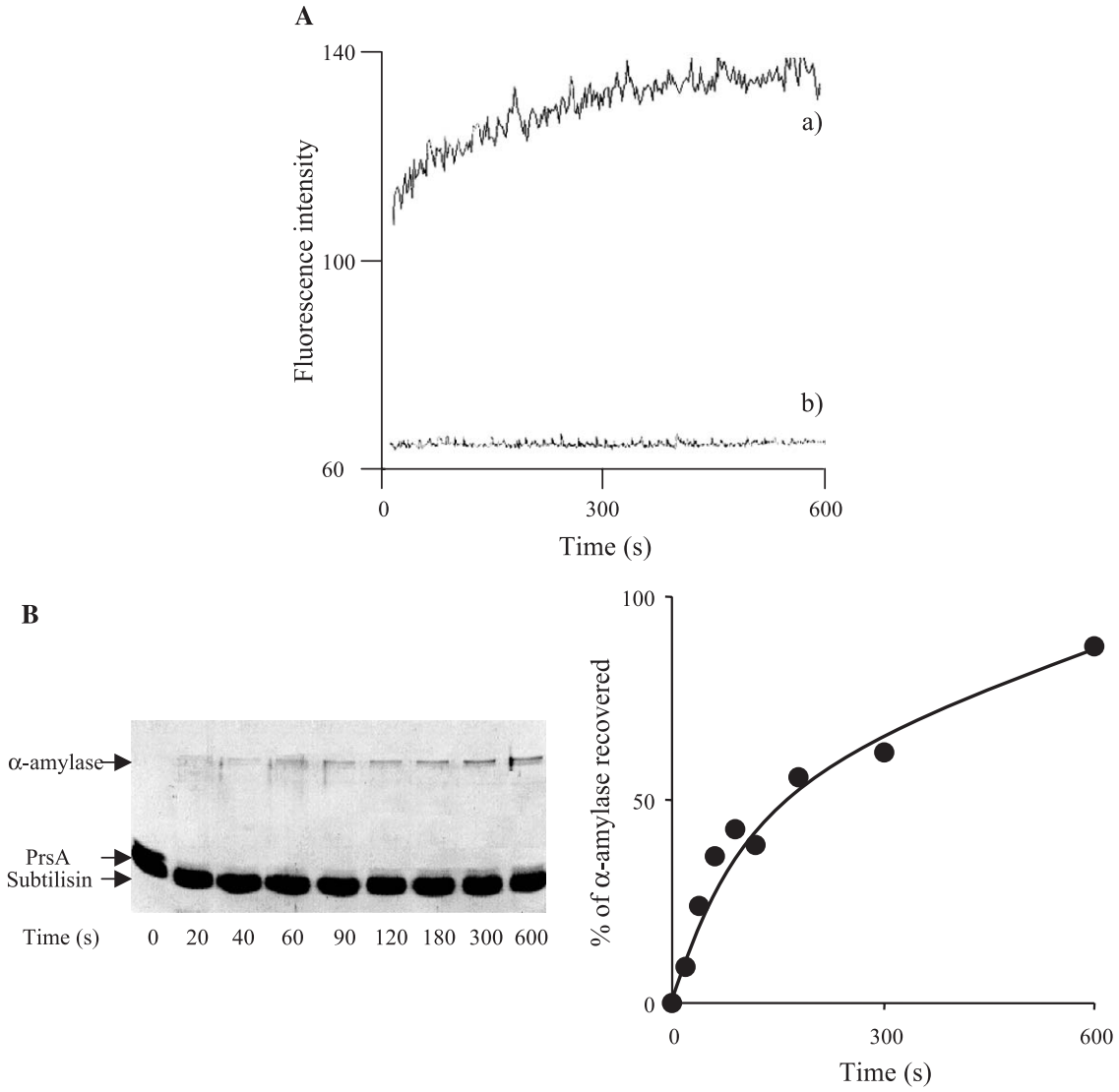

Fig. 9. Kinetics of α-amylase refolding in the presence of the SUV–PrsA complex. (A) Kinetics of α-amylase refolding measured by fluorescence intensity changes. SUV suspension (1 ml, 40 μM lipid) in 0.2 M sodium acetate pH 7, 1 mM calcium containing 0.25 μM of lipomodified PrsA was incubated at 37 °C. After 10 min incubation, 0.15 μM α-amylase folding intermediate was added and fluorescence intensity changes were recorded at 338 nm (a). The excitation wavelength was 280 nm. Control without α-amylase folding intermediate (b). (B) Kinetics of α-amylase refolding measured by resistance to proteolytic degradation. SUV suspension (1 ml, 40 μM lipid) in 0.2 M sodium acetate pH 7, 1 mM calcium containing 0.25 μM of lipomodified PrsA was incubated at 37 °C. After 10 min incubation, 0.15 μM of α-amylase folding intermediate was added. Aliquots of 70 μl of this mixture were withdrawn at intervals and mixed with 1.5 μl subtilisin solution (1 mg/ml). After 5 min, 3 μl of 0.1 M PMSF was added. The samples were then analyzed by SDS-PAGE and proteins quantified by densitometry of the Coomassie blue-stained gels using the NIH Image program.

3.4. Presence of PrsA on SUV surface prevents the interaction between SUV and α-amylase folding intermediate

When SUV were gradually coated with PrsA before being mixed with α-amylase folding intermediate, we observed (Fig. 8A) that the amount of the unfolded protein trapped in the lipid phase decreased gradually. Conversely, the amount of refolded α-amylase recovered in the SUV supernatant increased (Fig. 8B). When SUV were completely coated with PrsA, we observed that the kinetic parameters of the folding transition of the α-amylase folding intermediate monitored by fluorescence intensity (Fig. 9A) or protease sensitivity (Fig. 9B) were in the same order of magnitude as those measured in the absence of SUV.

3.5. PrsA destabilizes the interaction between SUV and α-amylase folding intermediate allowing subsequent refolding

We next examined the stability of the SUV–α-amylase folding intermediate complex in the presence of PrsA. In such conditions, the refolding transition of α-amylase monitored by fluorescence was slow (Fig. 10). The level of enzyme activity recovery was approximately 70%, 30 min after adding PrsA. Two questions can thus be posed: Is PrsA a catalyst with the α-amylase folding intermediate the substrate or does PrsA destabilize the SUV–α-amylase interaction because of its high affinity for SUV? The second hypothesis is supported by the increase of α-amylase refolding with the PrsA concentration (Fig. 11), which strongly suggests that the two proteins compete for SUV binding sites.

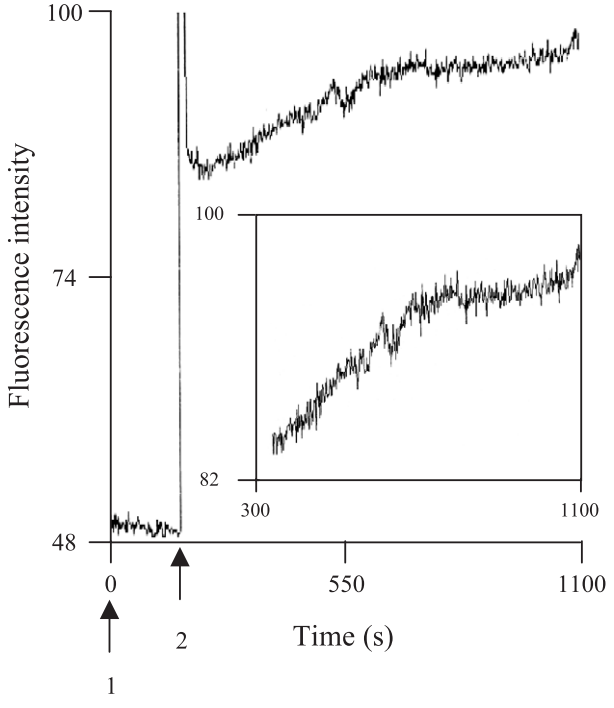

Fig. 10. Kinetics of α-amylase refolding triggered by the addition of PrsA to preformed SUV–α-amylase complex. SUV suspension (1 ml, 40 μM lipid) in 0.2 M sodium acetate pH 7 was incubated at 37 °C. After 5 min, partially folded α-amylase (0.15 μM) was added (arrow 1). Fluorescence intensity was recorded for 3 min, then lipomodified PrsA (0.25 μM) was added (arrow 2). The excitation wavelength was 280 nm, and the emission wavelength was 338 nm. Enlargement of the trace between 300 and 1100 s is shown in the inset.

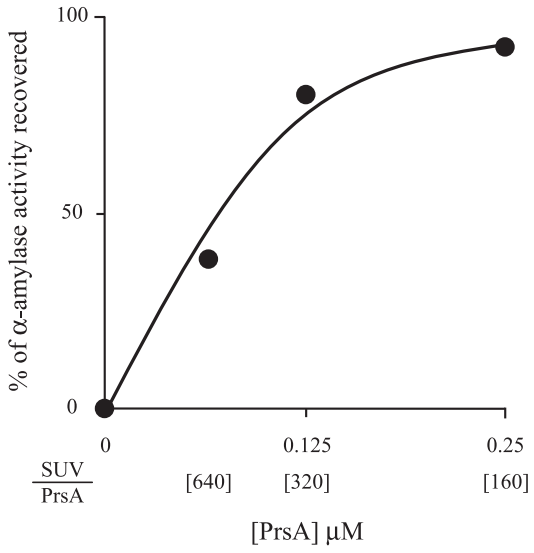

Fig. 11. Effect of PrsA concentration on the yield of α-amylase refolding from preformed SUV–α-amylase complex. SUV suspension (1 ml, 40 μM lipid) in 0.2 M sodium acetate pH 7 was incubated at 37 °C. After 5 min, partially folded α-amylase (0.15 μM) was added. The reaction mixture was divided into fractions of 200 μl to which various amounts of lipomodified PrsA were added. After 1 h incubation at 37 °C, α-amylase activity was assayed from an aliquot of each fraction. There was no significant modification of the results after 3 h incubation. The lipid/PrsA molar ratios are indicated in brackets.

4. Discussion

Our results indicate that the affinity of α-amylase for SUV composed of the major membrane lipids of *B. subtilis* depends on its conformation. The native form did not bind vesicles, whereas a partially folded conformer, stabilized under physiological conditions of pH, temperature and ionic strength and used as a model for the competent state for secretion, was able to interact with the membrane surface of the model membrane to form a complex preventing the subsequent refolding of the protein. Since such an interaction takes place at a pH value higher than the isoelectric point of the protein, it is tempting to postulate that hydrophobic interactions are able to overcome the unfavourable electrostatic repulsion. The exposure of hydrophobic residues at the surface of the partially folded protein could make partial membrane insertion easier.

We can rule out that this association is the result of protein aggregation on the membrane surface for the following reasons. First, the experiments were carried out at low

concentrations of protein to prevent any aggregation [17]. Second, we demonstrate here that the association of the protein with SUV entailed only a modest (30%) quenching of the amplitude of the intrinsic fluorescence spectrum of the protein. Moreover, the wavelength of maximal fluorescence was not red-shifted as one would expect for a denaturation event. Finally, the association process has been shown to be much more rapid than the aggregation reaction.

The results obtained in this work show that the *B. subtilis* peripheric lipoprotein PrsA modulates the interactions between partially folded conformer of α-amylase and the membrane surface. The lipomodified form of PrsA displays a higher affinity for SUV binding sites than the folding intermediate of α-amylase. PrsA must either prevent the formation of the SUV–α-amylase complex or destabilize it. These findings shed light on the possible effect in vivo of this protein on the process of protein secretion.

In a recently published work, which was aimed at characterizing in vivo the role of PrsA, Wahlstrom, et al. using a very different approach, have also provided evidence that PrsA could prevent unproductive interactions of exported protein with the negatively charged matrix of the cytoplasmic membrane–cell wall interface and thus facilitate folding [21].

This abundant lipoprotein may be, during protein secretion, an essential component of the micro-environment that is required for spontaneous folding of secreted proteins during the secretion process rather than a protein folding catalyst as previously suggested [11].

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