FEBS Letters 349 (1994) 281–285

FEBS 14298

Ability of MBP or RBP signal peptides to influence folding and in vitro translocation of wild-type and hybrid precursors***

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Received 16 May 1994; revised version received 16 June 1994

Abstract

Maltose-binding protein (MBP), whose export in *E. coli* is dependent upon the chaperone SecB, and ribose-binding protein (RBP), whose export is SecB-independent, have been used to generate hybrid secretory proteins. Here, in vitro techniques were used to analyze MBP, RBP, RBP-MBP (RBP signal and MBP mature), and MBP-RBP (MBP signal and RBP mature). In protease-protection experiments, RBP folded considerably faster than MBP, RBP-MBP, or MBP-RBP. Only the folding properties of proteins containing the MBP mature moiety were influenced by SecB. In post-translational translocation assays, MBP exhibited the highest translocation efficiency. The hybrids RBP-MBP and MBP-RBP showed intermediate levels, and RBP translocation was not detected in these assays. These experiments demonstrate the influence of the signal peptide in determining folding properties and translocation efficiency of precursor secretory proteins.

Key words: Protein translocation; Protein folding; Signal peptide; MBP; RBP; SecB

1. Introduction

Maltose-binding protein (MBP) and ribose-binding protein (RBP) are periplasmic proteins used as models in protein export studies in *Escherichia coli*. In vivo and in vitro studies have provided information about the fate of secreted proteins (for reviews, see [1,2]). Secretory proteins are synthesized in the cytoplasm as precursors with an amino-terminal signal peptide that facilitates entry into the export pathway and translocation across the membrane. Following translocation, the signal peptide is removed from the precursor protein by signal peptidase, and the mature moiety is released into the periplasm. In addition to interacting with components of the export machinery, the signal peptide influences the ability of precursors to maintain a loosely folded conformation that is compatible with export [3]. Fluorescence spectroscopy studies comparing the folding rates of purified precursor and mature MBP and RBP have shown that the presence of the signal peptide retards the rate of folding of precursors, but does not affect their rate of unfolding [4–7]. Secretory proteins also rely on chaperones to retard their folding or inhibit aggregation, and to facilitate their entry into the export pathway. As a chaperone for a subset of periplasmic and outer membrane proteins, SecB helps to maintain export competence of precursor proteins and targets proteins to SecA, the next component of the export machinery [8–10]. Efficient export of MBP requires the chaperone SecB, whereas export of RBP is unaffected in cells lacking SecB [11,12].

Previously, in vitro techniques have been employed to study translocation of precursors into inverted membrane vesicles. However, in vitro translocation of precursors is not nearly as efficient as translocation in vivo, as has been demonstrated in studies with MBP [9,13,14], OmpA [15], PhoE [16], and OmpF-Lpp hybrids [17]. In vitro translocation experiments established the role of SecB in maintaining MBP in a loosely-folded, export competent conformation [9,13,14] and in inhibiting the aggregation of PhoE and OmpA to enhance the translocation of these precursors [18,19].

In this paper, properties of MBP, RBP, and the hybrid proteins MBP-RBP and RBP-MBP were analyzed. The hybrid proteins consist of precursors with the MBP and RBP signal peptides exchanged precisely at the signal peptidase processing sites. Previous in vivo studies with MBP, RBP, and hybrid precursors noted differences in the strength of the export defect conferred by altered MBP or RBP signal peptides and in the ability of these defects to be suppressed by cells harboring *prlA* alleles [12,20]. The goal of this work was to compare the ability of the RBP and MBP signal peptides to modulate folding or to promote in vitro translocation of wild-type or hy-

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***This article is dedicated to the memory of Phil Bassford Jr.

SSDI 0014-5793(94)00684-N

brid precursors. In addition, the ability of SecB to further influence the folding of precursors was examined.

2. Materials and methods

2.1. Bacterial strains

E. coli K-12 strain MC4100 [21] was used to generate components for the in vitro translation and translocation experiments. An *E. coli* strain, BAR1091 [22], was used for plasmid constructions. A SecB-null mutant, CK1953 [23], was used for production of S-100 fractions lacking SecB. A SecB-overproducing strain, MC4100 harboring pDC2 [23], was used to generate S-100 fractions containing high levels of SecB.

2.2. Plasmids and plasmid constructions

The plasmids used in the in vitro experiments were derived from pJF2 [24] which contains the *malE* gene under transcriptional regulation of the *lacUV5* promoter-operator, and the M13 phage intergenic region. The construction of plasmids pSMS41 (encoding RBP-MBP), pDNC186 (RBP), and pDNC197 (MBP-RBP), has been described previously in detail [12]. All constructs were confirmed by sequencing as previously described [25].

2.3. In vitro translation and translocation

Components used in the in vitro experiments were prepared as described previously [8,13]. The S-100 cellular fractions were generated from SecB-null cells (CK1953) and SecB-overproducing cells (MC4100 harboring pDC2). Western blot analysis using anti-SecB antibody confirmed the SecB content of these cellular extracts. Membrane-free ribosomes and inverted cytoplasmic membrane vesicles were prepared from wild-type cells (MC4100). All plasmid DNA species were prepared using lysis in alkaline-SDS [26], banding in CsCl and concentration to 1 μg/ml. Incorporation of [^{35}S]methionine (Amersham) was used to quantitate protein synthesis. Values obtained from at least three sets of assays were averaged for all experiments.

2.4. In vitro assay of posttranslational protein folding

Folding assays were performed as described previously [9] with minor modifications. MBP, RBP, and hybrid proteins were synthesized in the absence of membranes for 8 min at 37°C using an S-100 fraction prepared from SecB-null (SecB$^-$) or SecB-overproducing (SecB^{++}) strains supplemented with ribosomes. Translation was terminated by the addition of 1 mg/ml cold methionine and 50 μg/ml chloramphenicol. At each time point after the arrest of translation, a sample was removed and diluted into ice-cold 40 mM triethanolamine-acetate, pH 7.8, containing 0.5% methionine. The following steps were carried out at 4°C. Half of each sample was treated with proteinase K (60 μg/ml; Boehringer Mannheim) for 30 min, followed by termination of the reaction with 5 mM PMSF. The other half of the samples were treated identically but in the absence of protease.

Samples were solubilized by boiling in 1% SDS. MBP, RBP, and hybrid proteins were immunoprecipitated with the appropriate antisera, and analyzed by SDS-PAGE and autoradiography. Radioactive counts were quantitated using an Ambis radioanalytic imaging system (Ambis Systems, San Diego, CA). The percentage of protein sensitive to proteolysis was determined by quantitating the amount of precursor and mature protein following protease treatment using the method of Randall and Hardy [27].

2.5. Assay of posttranslational translocation efficiency

In vitro translocation assays were conducted as described previously [13]. MBP, RBP, and hybrid proteins were synthesized in the absence of membranes for 8 min as above using the S-100 fraction (from SecB-overproducing cells), and ribosomes. The SecB^{++} S100 fraction was used in these experiments for optimal translocation. Translation was quenched by the addition of 1 mg/ml methionine and 50 μg/ml chloramphenicol. Samples were removed from the translation mixture at each time point, and incubation was continued for an additional 15 min at 37°C either in the presence or absence of added membrane vesicles. Samples were placed in an ice-water bath for 5 min, solubilized by boiling in 1% SDS, immunoprecipitated, and analyzed by SDS-PAGE and autoradiography. The percentage of protein processed was calculated the same as for the folding assays.

3. Results

3.1. In vitro folding assays comparing MBP, RBP, and hybrids in the presence or absence of SecB

In addition to promoting entry into the export pathway, signal peptides act to modulate folding (and hence, export efficiency) of the mature moiety. Since the chaperone SecB is an additional factor that maintains the export competence of MBP, folding kinetics of hybrids were examined in the presence or absence of SecB. Protein folding is indicated by the loss of sensitivity to proteinase K degradation. It is important to note that protease treatment not only degrades loosely folded proteins but removes the signal peptide from folded precursor protein as well. Results for MBP and RBP-MBP are presented in Fig. 1A. The SecB-dependent MBP exhib-

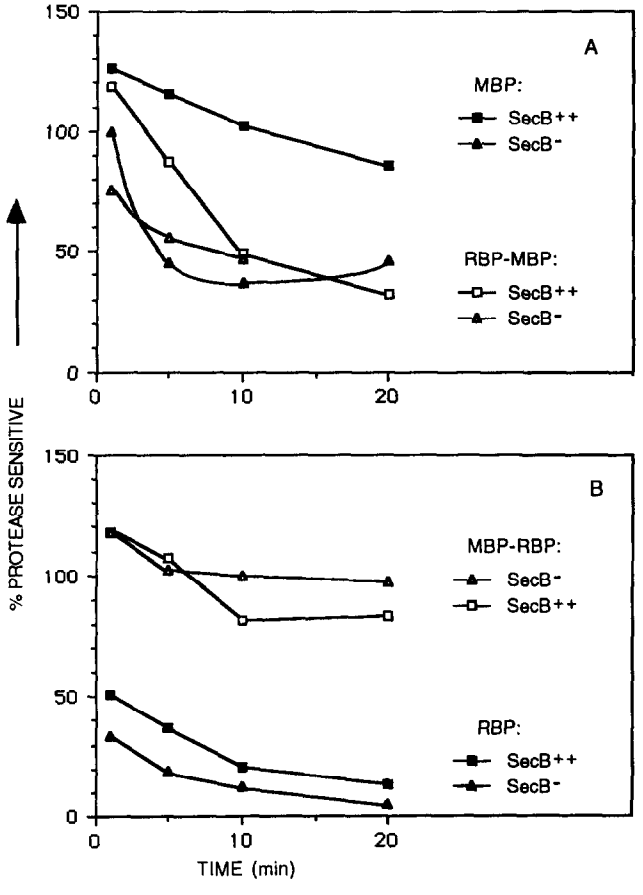

Fig. 1. Folding of MBP, RBP, and hybrid proteins synthesized in vitro in the presence or absence of SecB. Proteins were synthesized using ribosome-supplemented S-100 fractions prepared from either SecB-null (SecB$^-$) or SecB-overproducing strains (SecB^{++}), followed by termination of translation. At each timepoint, half of each sample was subjected to proteinase K digestion. The percentage of proteinase K-sensitive protein was calculated using the same method as Randall and Hardy [27] and Weiss et al. [9], and values shown include correction for loss of methionyl residues upon processing. Filled symbols represent MBP in panel A and RBP in panel B. Open symbols represent RBP-MBP in panel A, and MBP-RBP in panel B. Squares represent experiments done with SecB^{++} S-100 fraction; triangles, with SecB$^-$ S-100 fraction.

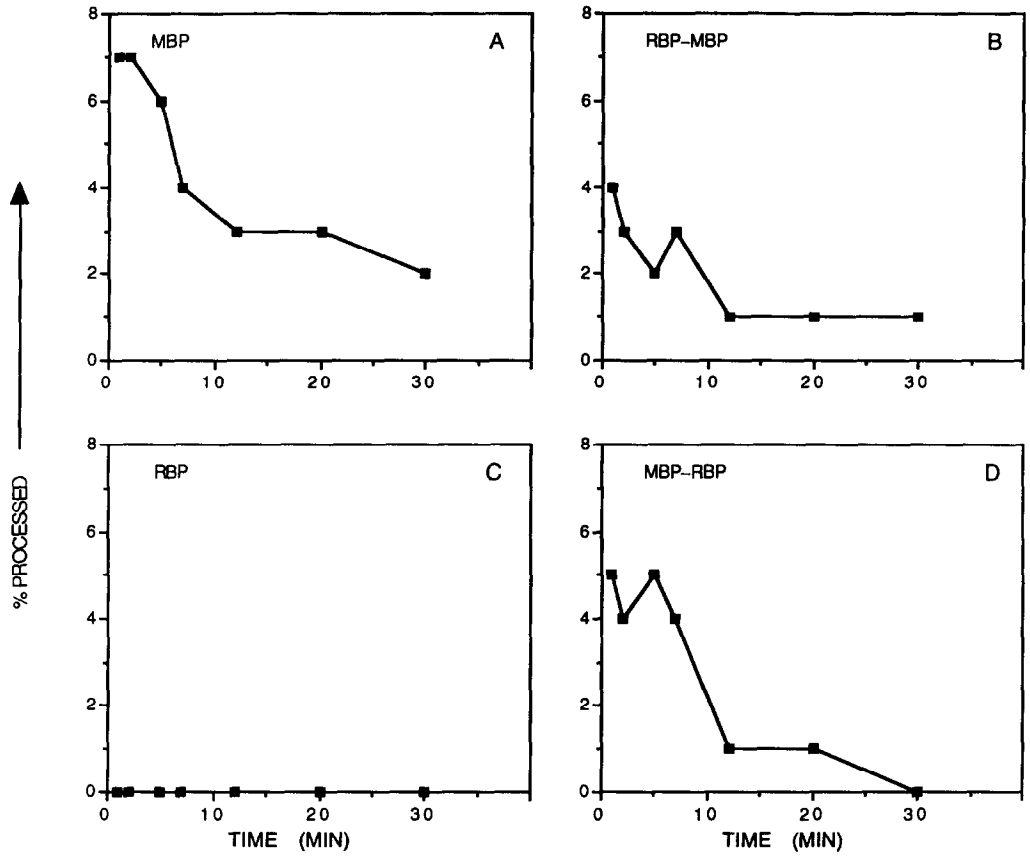

Fig. 2. Post-translational translocation efficiency for MBP, RBP, and hybrids using inverted membrane vesicles. Proteins were synthesized for 8 min using the $SecB^{++}$ S-100 fraction plus ribosomes, followed by termination of translation. At the indicated time points, membrane vesicles were added to the samples, followed by an additional 15 min incubation at 37°C. At each time point, an identical sample was removed and incubated without vesicles. MBP and RBP species were immunoprecipitated and analyzed by SDS-PAGE and autoradiography. The percentage of protein processed was calculated as described in section 2. At each time point, the percentage found in the absence of vesicles was subtracted as background. (A) MBP; (B) RBP-MBP; (C) RBP; and (D) MBP-RBP.

ited slow folding in the presence of excess SecB as supplied in the $SecB^{++}$ S100 fraction (filled squares). This rate of MBP folding using the $SecB^{++}$ fraction is comparable to the rate seen in previous experiments using a SecB-null fraction plus 2 μg of purifed SecB [9]. MBP folded faster in reactions with the $SecB^{-}$ S100 fraction (filled triangles). These results are consistent with previous studies where the influence of SecB on the folding and translocation kinetics of MBP has been examined extensively [1,8,13,14]. In the absence of SecB, the RBP-MBP hybrid folded with kinetics similar to MBP. However, in the presence of SecB, the RBP-MBP hybrid exhibited protease sensitivity but folded faster than MBP. By 10 min, there was little difference in the protease sensitivity of RBP-MBP under $SecB^{++}$ or $SecB^{-}$ conditions.

The accompanying set of experiments for RBP (filled symbols) and MBP-RBP (open symbols) is presented in Fig. 1B. RBP folded very rapidly, with only a slight difference between $SecB^{-}$ (triangles) and $SecB^{++}$ (squares) conditions. MBP-RBP was markedly more protease-sensitive than RBP; in fact it was comparable to the levels seen for MBP. However, unlike MBP, SecB did not influence the kinetics of folding for MBP-RBP. Thus, the MBP signal peptide maintained the protease-sensitive conformation of the MBP-RBP hybrid in a manner that was not dependent on SecB.

3.2. *Translocation efficiency of MBP, RBP, and hybrid proteins*

Previous work has shown that the translocation of MBP occurs both co- and post-translationally [28,29]. The export of RBP has been reported to be exclusively posttranslational [30], and the export of both RBP-MBP and MBP-RBP has been reported to be posttranslational [12]. The in vitro translocation assay was inefficient; less than 10% of the MBP synthesized was translocation-competent (Fig. 2A), consistent with earlier experiments [14]. Replacing the MBP signal peptide with the RBP signal peptide in the RBP-MBP hybrid resulted in a slight reduction in the percentage of precursor protein processed (Fig. 2B). The hybrid MBP-RBP (Fig. 2D) was translocated at levels similar to RBP-MBP. In contrast to MBP or hybrids, RBP translocation was unde-

tectable in this post-translational assay (Fig. 2C). This may indicate that RBP, although capable of post-translational export, only retains export competence for a short time interval after synthesis, and has lost competence by the time membranes are added (9 min after translation is initiated). To test this, the assay was performed with vesicles present during translation. Under these conditions, about 5% of RBP was processed (data not shown).

4. Discussion

The signal peptide plays a multifunctional role during export. Several studies have demonstrated interactions between signal peptides and SecA [31] and lipid bilayers [32]. In addition, the signal peptide modulates folding of the precursor molecule [4–6]. Previous studies with MBP, RBP, and hybrid precursors suggested that the MBP and RBP signal peptides may not be equivalent in their ability to promote export in vivo [12,20]. Our in vitro assays focus on the differences between the ability of RBP and MBP signal peptides to modulate folding or to promote translocation of wild-type and hybrid precursor proteins.

As assayed by accessibility to proteinase K digestion, precursor RBP folded much more rapidly than precursor MBP. As previously observed by Weiss et al. [9], MBP slowly lost its protease-sensitive conformation, and in the presence of SecB this conformation was maintained for a longer time interval. Analysis of the hybrid proteins revealed that the MBP signal peptide was more proficient at retarding folding of precursors than the RBP signal peptide, since MBP-RBP folded much more slowly than RBP. In the absence of SecB, the RBP-MBP hybrid did not fold more rapidly than MBP. Since the proteinase K sensitivity of the RBP-MBP hybrid was not maintained with $SecB^{++}$ extracts as it was for MBP, the ability of SecB to further retard folding of this hybrid was limited. This was somewhat unexpected since the export of the RBP-MBP hybrid is SecB-dependent [12]. Although the MBP signal peptide is not sufficient for SecB interaction [12,33], it may influence the ability of MBP to interact with SecB. A similar conclusion has been reached in studies with another SecB-dependent protein, LamB [34].

Consistent with other studies [9,13,14], our experiments show a correlation between the loss of a proteinase K sensitive conformation and loss of in vitro translocation. Translocation of RBP, which rapidly loses its protease-sensitive conformation, was not detected in our in vitro assays. Prior to this study, RBP translocation has only been analyzed in vivo [7,12,35]. It is surprising to see that RBP was not competent for in vitro translocation in post-translational assays since it is exported in a strictly post-translational mode in vivo. One possibility is that the rapidly folding RBP does not maintain export competence long enough for detection in the in vitro assay. The fact that translocation of RBP was observed with membranes present during translation supports this idea. In addition, some component of the export machinery, such as SecA or a chaperone, may be limiting for RBP in this assay.

Unlike RBP, translocation of the MBP-RBP hybrid was detected in the in vitro assay, suggesting that the MBP signal peptide imparted increased efficiency to the RBP mature moiety. The RBP-MBP hybrid showed slightly reduced translocation efficiency compared to MBP, suggesting that the RBP signal peptide was not as effective at promoting translocation. Translocation of the RBP-MBP hybrid was still more efficient than RBP; however, this could be due to targetting functions provided by SecB. Interaction with SecB appears to be within the mature moiety of MBP [8,12,36,37]. Thus, like modulation of folding, the pattern of in vitro translocation efficiency is a property that can be partially transferred with the signal peptide. While signal peptide modulation of folding is likely to influence the ability of precursors to be translocated, additional parameters such as the ability of signal peptides to interact with components of the export machinery or to associate with membranes would also affect translocation efficiency.

The specific mechanisms by which signal peptides engage the export machinery or modulate precursor folding are unknown. Our results suggest that signal peptides differ over a range of efficiencies in terms of their ability to modulate precursor protein folding and influence in vitro translocation. Some properties of a signal peptide are transferable, implying that signal peptides and mature regions of a specific precursor protein are not necessarily 'matched'. If signal peptides interact with regions in the mature moiety to modulate precursor folding, this interaction must either be non-specific, or with conserved domains, since the RBP and MBP signal peptides behaved in a similar manner with either MBP or RBP mature moieties in the folding assays. Finally, while signal peptides are sufficient to affect some properties of precursor proteins, domains in the mature moiety of precursor proteins also play an important role in determining export competence via their ability to interact with chaperones such as SecB.

Acknowledgements: We wish to thank Dr. Linda L. Randall for critically reading the manuscript.

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