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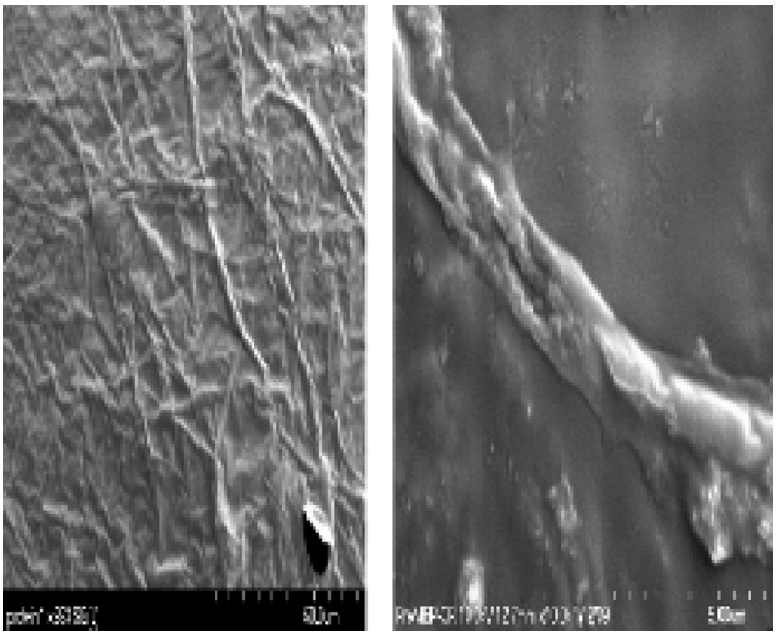
Langmuir, **Article ASAP** • DOI: 10.1021/la900198h • Publication Date (Web): 09 April 2009



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Effect of Crowding Agents, Signal Peptide, and Chaperone SecB on the Folding and Aggregation of *E. coli* Maltose Binding Protein

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Received January 16, 2009. Revised Manuscript Received March 9, 2009

SecB, a soluble cytosolic chaperone component of the Sec export pathway, binds to newly synthesized precursor proteins and prevents their premature aggregation and folding and subsequently targets them to the translocation machinery on the membrane. PreMBP, the precursor form of maltose binding protein, has a 26-residue signal sequence attached to the N-terminus of MBP and is a physiological substrate of SecB. We examine the effect of macromolecular crowding and SecB on the stability and refolding of denatured preMBP and MBP. PreMBP was less stable than MBP ($\Delta T_m = 7 \pm 0.5$ K) in both crowded and uncrowded solutions. Crowding did not cause any substantial changes in the thermal stability of MBP ($\Delta T_m = 1 \pm 0.4$ K) or preMBP ($\Delta T_m = 0 \pm 0.6$ K), as observed in spectroscopically monitored thermal unfolding experiments. However, both MBP and preMBP were prone to aggregation while refolding under crowded conditions. In contrast to MBP aggregates, which were amorphous, preMBP aggregates form amyloid fibrils. Under uncrowded conditions, a molar excess of SecB was able to completely prevent aggregation and promote disaggregation of preformed aggregates of MBP. When a complex of the denatured protein and SecB was preformed, SecB could completely prevent aggregation and promote folding of MBP and preMBP even in crowded solution. Thus, in addition to maintaining substrates in an unfolded, export-competent conformation, SecB also suppresses the aggregation of its substrates in the crowded intracellular environment. SecB is also able to promote passive disaggregation of macroscopic aggregates of MBP in the absence of an energy source such as ATP or additional cofactors. These experiments also demonstrate that signal peptide can greatly influence protein stability and aggregation propensity.

Introduction

Aggregation is a problem frequently encountered during the folding of polypeptide chains both in vitro and in vivo. Protein aggregation is associated with several human diseases,[1,2] and overexpressed proteins are often aggregated making this a major problem in the biotechnology industry. While in vitro studies of protein folding, stability, and function are carried out in dilute aqueous solutions, in vivo folding of nascent polypeptides in the crowded cellular environment generally occurs under unfavorable conditions of very high protein concentrations. Under these conditions, the exposed hydrophobic amino acid residues in nascent polypeptides and folding intermediates may interact inappropriately with other protein chains, leading to misfolding and aggregation.[3] Stresses such as heat shock also lead to the aggregation of proteins.[4,5] Aggregation is considered to be a nonproductive, off-pathway reaction that competes with correct folding reactions.[6] Cells have evolved elaborate protective systems against this and minimize the aggregation problem by producing a family of proteins, called molecular chaperones.[5,7−11] These chaperones bind to exposed hydrophobic surfaces of proteins, thereby sequestering unfolded polypeptide chains and preventing their aggregation.

In the cytoplasm of *E. coli*, the total protein concentration has been estimated to be in the range of 200−300 g/L.[12] Macromolecular crowding results in a reduction of the volume available to one macromolecule as a result of the occupation of space by other macromolecules present in solution. In addition to such excluded volume effects, there can be other complex, nonspecific interactions between macromolecules, especially when multiple macromolecules of different size, shape, and charge are involved. There have been various prior theoretical and experimental studies on the effects of macromolecular crowding on protein stability, folding rates, protein−ligand interactions, and protein aggregation. A summary of the current understanding of macromolecular crowding effects can be found in recent reviews.[13,14] In experimental studies, intracellular macromolecular crowding is typically mimicked by the addition of water-soluble polymers such as dextrans, Ficoll, PEG, and proteins such as hemoglobin and defatted BSA to concentrations ranging from 50 to 300 g/L. It has been observed that well-folded globular proteins typically

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exhibit small increases in stability (~1 kcal/mol in ΔG_u^o or a few degrees in T_m) in the presence of crowding agents.[15−17] These results are consistent with theoretical predictions.[18] In contrast to their modest effect on protein thermodynamic stability, crowding agents can substantially accelerate protein aggregation, fibril formation, and assembly.[19−23] This is probably because crowding can result in an increase in the activity of protein by orders of magnitude.[15,16] Hence, any aggregation-prone intermediates present during folding will show an increased tendency to aggregate. A recent study[24] demonstrated the effect of crowding agents on fibrillation kinetics of four different proteins. Fibrillation of oligomeric proteins was inhibited because of crowding-mediated stabilization of the oligomeric species. However, the fibrillation of monomeric, natively unfolded/disordered proteins was accelerated. In the cell, molecular chaperones can sequester such aggregation-prone intermediates/unfolded states, thus preventing aggregation. Although there has been much work on the molecular mechanisms of chaperone function, the vast majority of in vitro studies have been carried out in dilute aqueous solutions. There are only a few in vitro studies of chaperone function in crowded solutions. It was shown that chaperones GroEL/GroES prevent the aggregation of proteins rhodanese, dihydrofolate reductase, enolase, and GFP[25,26] and PDI reduces the aggregation of reduced lysozyme[23] during refolding in crowded solution. In addition, GroEL was shown to prevent the aggregation of glucose-6-phophate dehydrogenase and glyceraldehyde-3-phosphate dehydrogenase[27,28] during refolding in crowded and uncrowded solution. In all of the above studies, the chaperones appear to act by binding to nonaggregated folding intermediates and preventing their aggregation, not by the dissociation of aggregates that are already formed.

After synthesis in the cytoplasm, many proteins need to be exported to their eventual site of action. The export process typically involves translocation across one or more membrane barriers. A majority of such exported proteins are synthesized as precursors (preproteins) containing an N-terminal signal peptide. Signal peptides are typically 15−40 amino acids long and serve as a molecular address label that is cleaved after or during export by membrane-associated signal peptidases.[29,30] Signal sequences do not share significant sequence homology with each other. However, they have a common characteristic tripartite structure composed of a positively charged N-terminus (n region) followed by a stretch of 7−15 hydrophobic residues (h region) and a more polar region that often contains helix-breaking proline and glycine residues as well as the signal peptide cleavage site (c region).[31,32] The role of the signal peptide as a protein-targeting motif has been well studied. In contrast, its role in directly modulating the stability, folding kinetics, and aggregation properties of the proteins to which it is attached is not well characterized.

Maltose binding protein (MBP) is a large two-domain periplasmic protein of 370 amino acid residues that is involved in maltose uptake and chemotaxis. It is one of the few large proteins that has been well characterized with respect to its folding kinetics and thermodynamic stability.[33−37] It is synthesized in the cytoplasm in its precursor form, preMBP, with an N-terminal signal peptide of 26 residues. It was first shown by Randall and colleagues that signal peptides retard the folding of precursor maltose binding protein (preMBP) and precursor ribose binding protein (preRBP) of *E. coli* by a factor of approximately 5[38] relative to the corresponding mature proteins (MBP and RBP) that lack a signal peptide. A later study[39] reported the thermodynamic characterization of bovine preadrenodoxin and the corresponding mature protein. The preprotein was found to have a reduced thermal transition temperature relative to the mature protein. However, denaturation was irreversible, thus complicating the interpretation of the data. We have previously carried out detailed thermodynamic studies of the stability and folding of MBP and preMBP in dilute aqueous solution and have recently shown that preMBP is less stable than MBP by 2−6 kcal/mol (20−40%),[34−36,40] possibly because the hydrophobic signal peptide interacts favorably with other hydrophobic regions in the unfolded state.

In *E. coli*, the export of most proteins from the cytoplasm to the periplasm occurs via the Sec pathway. The important protein components in this pathway are SecB, a cytosolic chaperone dedicated to protein export, SecA, an ATPase that drives protein movement across the membrane, and SecY, SecE, and SecG, the integral membrane proteins that form a heterotrimeric SecYEG channel. SecA associates peripherally with the membrane and in the presence of ATP activates the SecYEG complex. SecYEG and SecA together form the translocase.[41,42] Most preproteins cannot cross membrane barriers in the folded state. Hence, following synthesis and before folding to the native state, they are bound by cytoplasmic chaperone proteins and maintained in an export-competent unfolded state.[43] SecB has been identified as a chaperone that binds to the precursors of a subset of exported proteins, thereby maintaining them in an export-competent state and delivering them to the translocation apparatus in the membrane.[44,45] In some cases, SecB

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has also been implicated in the prevention of aggregation of its substrate proteins.[44,46]

PreMBP is the physiological substrate of SecB that interacts with SecB in the cytoplasm and is translocated to the plasma membrane for export. There have been numerous in vitro studies of the interaction of SecB with both physiological and model protein substrates.[46−51] These studies have demonstrated that SecB can bind to a variety of unfolded/partially folded proteins with little sequence specificity. However, SecB does not bind to native, folded proteins. Crystal structures of SecB from both *E. coli* and *H. influenza* as well as thermodynamic studies[46,52,53] suggest that substrates bind to a surface-exposed groove on the SecB tetramer. The mechanism(s) and extent of SecB substrate selectivity in vivo are not well understood, given its lack of sequence selectivity in vitro. It had been proposed[49] that the binding of polypeptides in vivo to SecB occurs in kinetic competition with folding. Hence, rapidly folding proteins can escape SecB binding. This kinetic partitioning model was suggested as a possible mechanism for SecB substrate selectivity.[49,54−56] It was based, in part, on the observation that SecB could block the refolding of preMBP but does not block the refolding of faster-folding MBP in vitro. We have recently measured all of the rate constants involved in the interaction of SecB with MBP and preMBP.[33] In contrast to the kinetic partitioning model, the data suggest that the slower folding rate of preMBP relative to that of MBP is not an important factor in SecB selectivity. Both unfolded preMBP and unfolded MBP bind rapidly to SecB with similar values of k_{on} and k_{off} of approximately 10^7 M^{-1} s^{-1} and 0.05 s^{-1} respectively. However, MBP alone is able to fold while bound to SecB with a rate constant of 0.007 s^{-1}, and hence SecB does not block but only slows down the refolding of MBP.[33] The in vivo concentration of free SecB has been estimated to be in the range of 0.4−4 μM,[57,58] and the rate constant of refolding of MBP (k_f) is 0.03 s^{-1}. The ratio of the rate of binding to SecB to the rate of refolding is given by k_{on} [SecB]/k_f. This ratio is in the range of 100−1000, clearly demonstrating that binding to SecB occurs much more rapidly than refolding for both MBP and preMBP. Assuming a minimal free SecB concentration of 0.4 μM, in vivo folding will occur significantly (~10-fold) faster than binding only for proteins with values of $k_f > 40$ s^{-1}. Whereas specific estimates for folding rates of most *E. coli* proteins are not available, a recent study[59] summarized k_f values for 36 two-state and 25 non-two-state folding proteins. Approximately 30% of the two-state and 75% of non-two-state folders had values of $k_f < 40$ s^{-1}. If these results can be extrapolated to the majority of *E. coli* proteins, then it would appear that kinetic partitioning would predict the SecB-mediated blockage of folding for most *E. coli* proteins. In practice, as demonstrated with MBP, many proteins with folding rate constants much slower than 40 s^{-1} can avoid SecB-mediated blockage by folding while bound to SecB. However, the molecular features that permit proteins to fold while bound to SecB remain to be elucidated.

Because signal peptides contain long hydrophobic stretches of sequences, it is likely that these will enhance protein aggregation either during or after folding. Such aggregation may be enhanced in crowded solutions. However, there are no published studies that have examined the combined effects of signal peptides and chaperones on protein aggregation. In the present study, we have therefore examined the effects of crowding agent Ficoll-70 and chaperone SecB on the stability, folding, and aggregation of preMBP (containing signal peptide) and MBP (lacking signal peptide). Interestingly, it was previously observed that whereas both preMBP and MBP refold reversibly at submicromolar concentrations,[34] MBP undergoes complete and spontaneous reversible aggregation when refolded at concentrations in the range of 1.5−28 μM.[40] SecB can reduce but not completely prevent the aggregation of MBP during refolding. However, no corresponding studies on the refolding of preMBP at high concentration were available.

In this work, we have characterized the aggregation properties of preMBP and MBP in crowded and uncrowded solutions in the absence and presence of SecB. In contrast to the reversible aggregation of MBP, preMBP aggregation was found to be irreversible on time scales of up to several days. SecB can block the aggregation of both MBP and preMBP even in crowded solution and can promote the disaggregation of preformed MBP insoluble aggregates. In addition, unlike MBP aggregates, which are amorphous in nature, preMBP aggregates seem to have amyloid-like character as evident from EM as well as thioflavin T and Congo red binding studies.

Materials and Methods

Materials. Ficoll-70, polyethylene glycol (PEG), thioflavin T, Congo red, maltose, Q-Sepharose, trisodium citrate, and glycine were purchased from Sigma Chemical Co. Dextran-70 was from Fluka. Ultrapure guanidine hydrochloride (GdnHCl) and HEPES were obtained from USB. Amylose resin was from New England Biolabs. The remaining chemicals were obtained from local commercial sources and were of analytical grade.

Purification of MBP and preMBP. MBP-deficient strain POP6590 harboring plasmid pMALP2-MBP[60] was used as the source for MBP. Because the wild-type signal peptide on the MBP gets cleaved during its transport into the periplasm from cytoplasm, the same plasmid harboring signal peptide mutation A14E was used for the expression of preMBP as described previously.[34] MBP was isolated by the chloroform shock method[61] and purified by anion exchange chromatography using the fast-flow Q-Sepharose matrix as described previously.[36] PreMBP was purified by affinity chromatography on amylose resin using 10 mM maltose for elution.[34,62] SecB was isolated and purified as described earlier.[63]

In Vitro Aggregation. MBP and preMBP were unfolded in 2.0 M guanidine hydrochloride (GdnHCl) in CGH-10 buffer (10 mM each of citrate, glycine, and HEPES) pH 7.2 containing 150 mM NaCl for at least 3 h prior to the experiments. Bound maltose was removed from preMBP by separation on an NAP-10 column (Amersham Biosciences) in the unfolding buffer mentioned above. Maltose removal was confirmed by refolding the protein at low concentrations and monitoring the fluorescence emission spectrum

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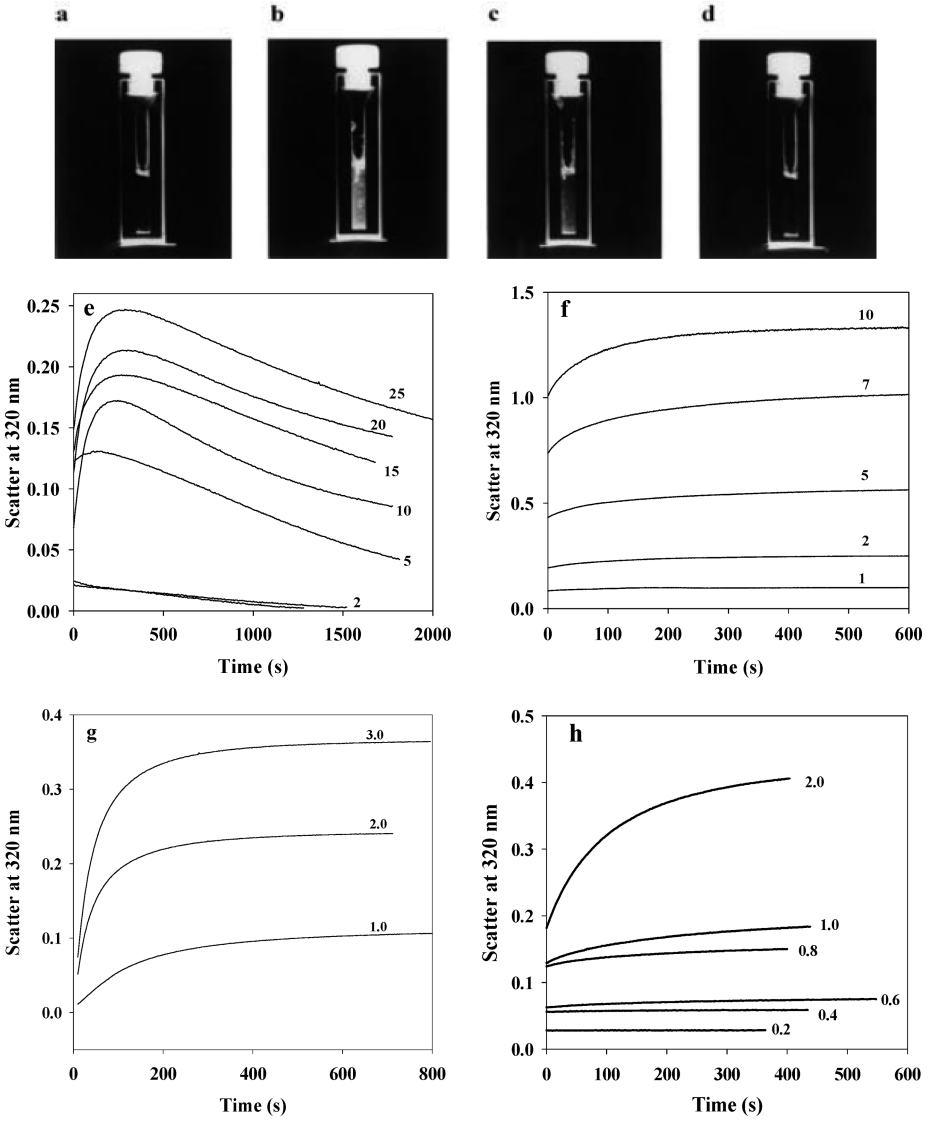

Figure 1. Aggregation during refolding of MBP and preMBP. (a−d) Zero time (before the addition of MBP) and after 30 s, 2 min, and 12 h of equilibration of refolding 25 μM MBP in refolding buffer containing 0.1 M GdnHCl. (e, f) Refolding of various concentrations of MBP in refolding buffer containing 0.2 M GdnHCl without (e) or with (f) 30% Ficoll-70. (g, h) Refolding of various concentrations of preMBP in refolding buffer containing 0.2 M GdnHCl without (g) and with (h) 30% Ficoll-70. Numerical values of protein concentrations in μM are indicated above each curve in e−h. All measurements were made at pH 7.2, 298 K. Data in a−d are reprinted with permission from Protien Science 10, 1635−1644 copyright (2001), The Protein Society.

of the refolded protein.[64] The maltose bound form is red shifted with respect to the maltose free form. Refolding was initiated by diluting the denatured protein into the refolding buffer, CGH-10 at pH 7.2 containing 150 mM NaCl, such that the final GdnHCl concentration was 0.2 M. In preliminary studies, the refolding of MBP under different crowded conditions was investigated by including varying concentrations of Ficoll-70, Dextran-70, and polyethylene glycol (PEG-20) in the refolding buffer to mimic crowding present in vivo.[22] The unfolded protein in 2 M GdnHCl was diluted 10-fold into refolding buffer containing crowding agent in the presence or absence of SecB. After the addition of unfolded protein to the refolding buffer in microcentrifuge tubes, the contents were mixed by constantly taking up and pipetting out the solution using a Pipetman and were then transferred to a cuvette. The mixing could be followed by visual inspection because of the refractive index differences in the solutions being mixed. The approximate mixing time was about 20 s. Aggregation of the refolding protein was followed by monitoring the light-scattering intensity at 320 nm in a Jasco V-530 UV/vis spectrophotometer as a function of time. For more detailed studies, Ficoll-70 was used at a concentration of 30%.

Refolding experiments were carried out as a function of protein concentration and temperature. The thermal unfolding of MBP and refolded preMBP under uncrowded and crowded conditions were studied on a Jasco 715 spectopolarimeter using a Jasco PTC-348WI peltier device. MBP (4.75 μM) or preMBP (3.25 μM) in a rectangular quartz cuvette (1 mm path length) was heated at a rate of 60 K/h from 298 to 353 K, and the change in ellipiticity (mdeg) at 222 nm was followed. Data points were collected at 0.5 K intervals with a 4 s response time and a bandwidth of 2 nm. T_m was calculated by fitting the thermal unfolding data as described earlier.[65]

Effect of SecB on the Aggregation Process. Varying concentrations of SecB were included in the refolding buffer at the time of dilution (t = 0). The ability of SecB to suppress the aggregation of MBP/preMBP during refolding was estimated from the light-scattering intensity at the end of 100 s of refolding.

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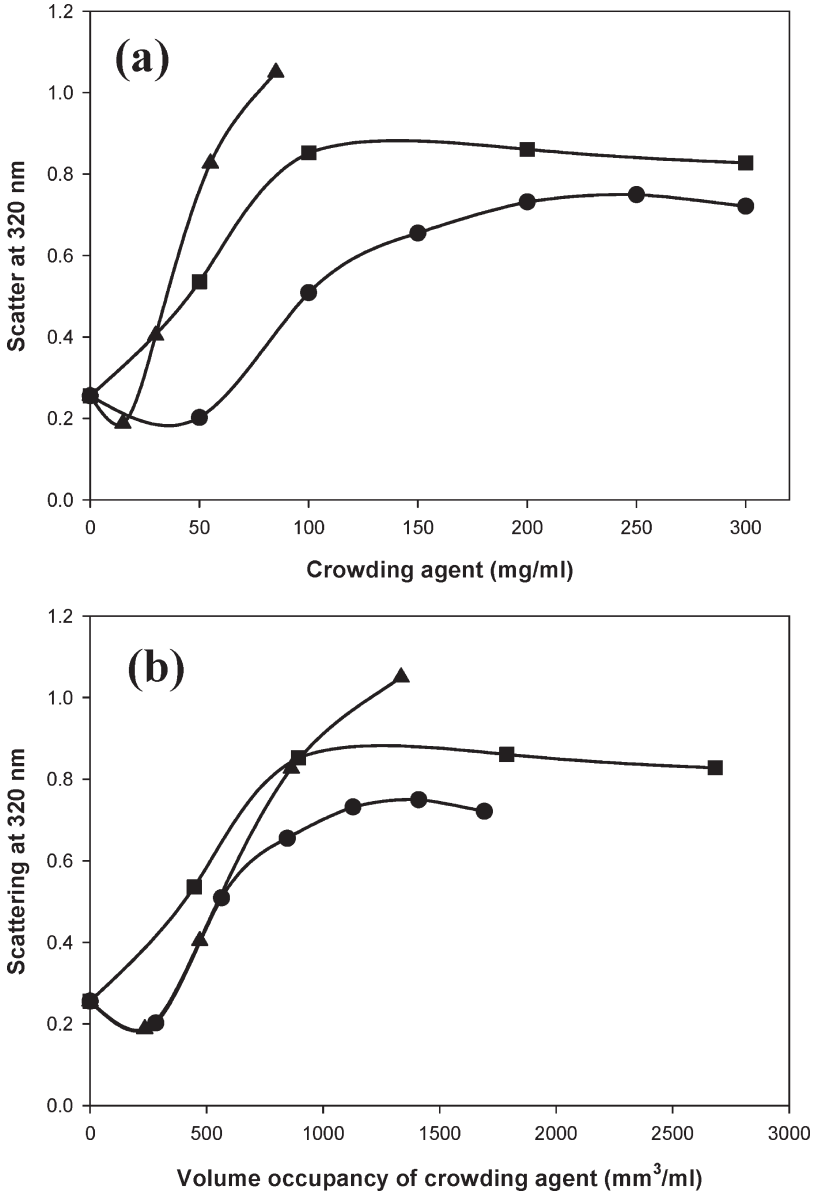

Figure 2. Effect of crowding on the refolding of 5 μM MBP in CGH-10 buffer at pH 7.2 in the absence or presence of varying concentrations of crowding agents Ficoll-70 (●), Dextran-70 (■), and PEG-20 (▲). The extent of scattering at 120 s after the initiation of refolding was monitored by measuring the scattered light intensity at 320 nm. Plot of scattered light intensity vs concentration of crowding agent (a) and volume occupancy (b).

In a separate set of experiments, a complex between varying concentrations of SecB and denatured MBP or preMBP was first formed in the absence of the crowding agent by diluting denatured MBP/preMBP 2-fold into aqueous solution containing a molar excess of SecB. This complex was immediately diluted 5-fold into refolding buffer containing 30% Ficoll-70 to initiate refolding. Aggregation was monitored by light scattering as described above. The aggregate was spun down, and the supernatant was removed. The supernatant was checked for the presence of refolded protein by adding 10 mM maltose to it and measuring the fluorescence emission of the protein upon excitation at 280 nm using a SPEX Fluoromax 3 spectrofluorimeter in a 1.0 cm water-jacketed cell.

Thioflavin T and Congo Red Binding Assays. A stock solution of Congo red was prepared in the refolding buffer. Unfolded MBP or preMBP was refolded by dilution into refolding buffer for about 15 min. Congo red was added to the aggregate in a 5-fold molar excess over the refolding protein concentration and incubated at room temperature for 30 min. UV−vis absorption spectra were then recorded in a Jasco V-530 spectrophotometer using a 1 cm path length quartz cuvette in the wavelength range of 350−700 nm.

To the aggregated MBP and preMBP, thioflavin T was added to a final concentration of 5-fold molar excess over the refolding protein concentration. Fluorescence emission spectra from 460 to 510 nm were then recorded in a SPEX Fluoromax 3 spectrofluorimeter with excitation at 450 nm. The excitation and emission slit widths were set at 3 and 5 nm, respectively.

Scanning Electron Microscopy (SEM). preMBP aggregates obtained during refolding experiments were washed thoroughly with Milli-Q water to remove buffer salts, Ficoll, ThT, and so forth by several rounds of centrifugation and resuspension in water. Finally, the precipitates were gently resuspended in water, and ~5−10 μL aliquots were loaded onto small pieces of carbon tape on an aluminum platform. The samples were dried in a calcium chloride desiccator and then gold-coated in a Hitachi E1010 ion sputterer (12A/200 s.). The coated samples were imaged in a Hitachi S-4700 field-emission SEM in analysis mode using an acceleration voltage of 10 kV, a chamber vacuum of 10^{-9} Pa, and a 12 mm working distance

using the lower secondary electron detector. Several fields were analyzed, and images are displayed as pictures with appropriately indicated magnifications.

Results

Refolding preMBP Is More Prone to Aggregation Than MBP in Aqueous Solution. When MBP is refolded at high protein concentrations ≥5 μM, aggregation (as evident from an increase in light scattering at 320 nm) and visible precipitation are observed. As previously reported,[40] MBP aggregates were found to show the unusual phenomenon of spontaneous resolubilization on a time scale of a few minutes, and nearly all of the protein was recovered in its native form (Figure 1a−d). The amount of scattering increases with increasing protein concentration (Figure 1e). preMBP is the precursor form of MBP and has a signal peptide containing a 15-residue hydrophobic stretch.

We next examined whether the refolding of preMBP at concentrations ≥1 μM is also aggregation-prone. Similar to MBP, preMBP also showed visible aggregation during refolding. The light-scattering intensity at 320 nm indicated that preMBP is more prone to aggregation than MBP, as seen in Figure 1g. The extent of aggregation of preMBP is also proportional to the refolding protein concentration. Unlike MBP aggregation, which is spontaneously reversible, preMBP aggregation was seen to be irreversible on the time scale studied (∼1 day). Appreciable aggregation was observed at concentrations ≥1 and 5 μM for preMBP and MBP, respectively (Figure 1), and the extent of light scattering was roughly comparable after the refolding of 1 μM preMBP and 5 μM MBP. Hence, in most subsequent studies, unless explicitly mentioned, preMBP and MBP were used at concentrations of 1 and 5 μM, respectively.

Refolding of MBP in the Presence of Various Crowding Agents. To study the effects of crowding agents on MBP aggregation, the refolding of 5 μM of MBP was studied as a function of increasing concentrations of Ficoll-70, Dextran-70, and PEG-20 in the refolding buffer. The results are summarized in Figure 2. The volume occupancy of crowding agents was calculated by assuming them to be hard spheres, using the reported radii of 5.5, 6.3, and 5.0 nm for Ficoll-70, Dextran-70, and PEG-20, respectively.[66−68] The extent of aggregation increases linearly with volume fraction occupied in a similar way for all three crowding agents and saturates at a calculated volume occupancy of close to 100%.

Crowding Agents Have Small Effects on Stability but Significantly Enhance Aggregation during the Refolding of Both MBP and preMBP. Because the cellular environment is very crowded with total protein concentrations as high as 300 g/L,[13] intracellular crowded conditions are generally mimicked in vitro by using a 20−30% (w/v) solution of a crowding agent such as Ficoll-70. We therefore carried out the remaining refolding experiments in solutions containing 30% (w/v) Ficoll-70 to mimic macromolecular crowded conditions in the cell. Ficoll-70 (30%) has been previously used by various groups for crowding studies.[26,69−71] We observed that crowding

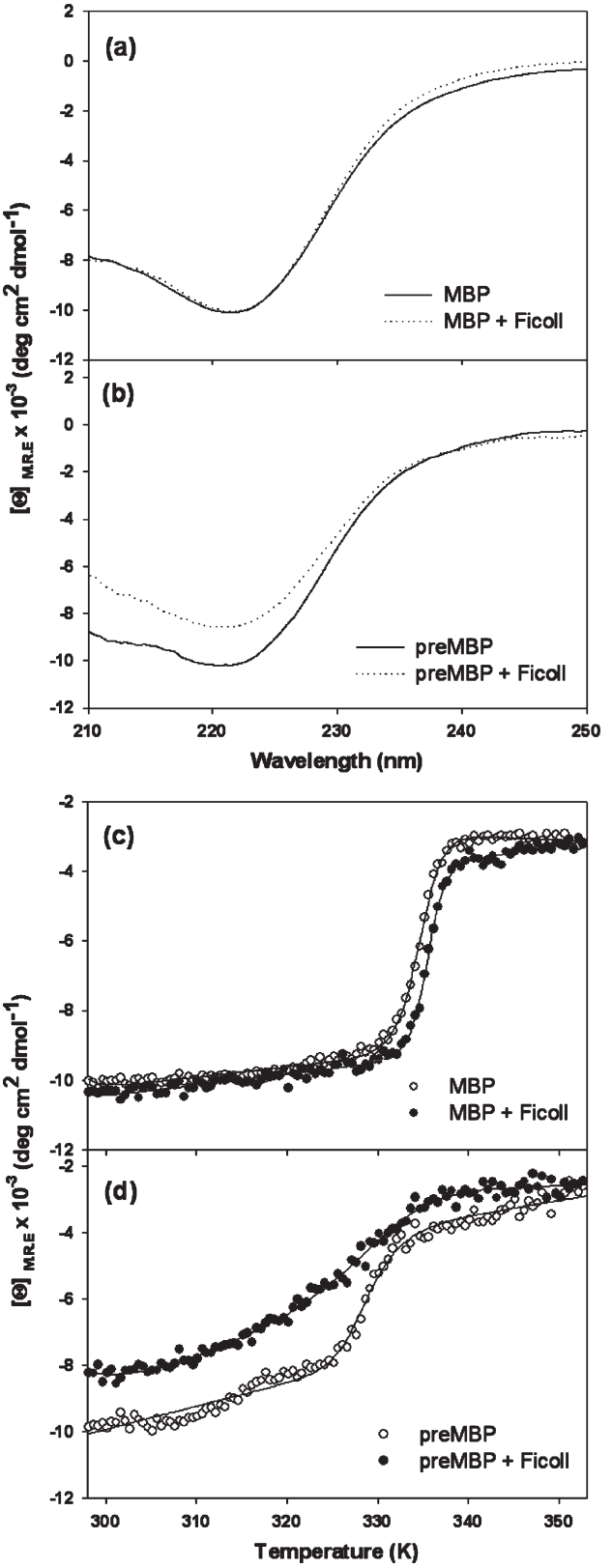

Figure 3. CD spectra and thermal stability of MBP/preMBP. Far-UV CD spectra at 298 K of (a) MBP and (b) preMBP in the presence (···) and absence (−) of 30% Ficoll-70. Thermal denaturation melts of (c) MBP and (d) preMBP in the presence (●) and absence (○) of 30% Ficoll-70 measured at 222 nm. Protein concentrations were 4.75 μM MBP and 3.25 μM preMBP. All measurements were carried out in CGH-10 buffer at pH 7.2 containing 150 mM NaCl and 0.2 M GdnHCl.

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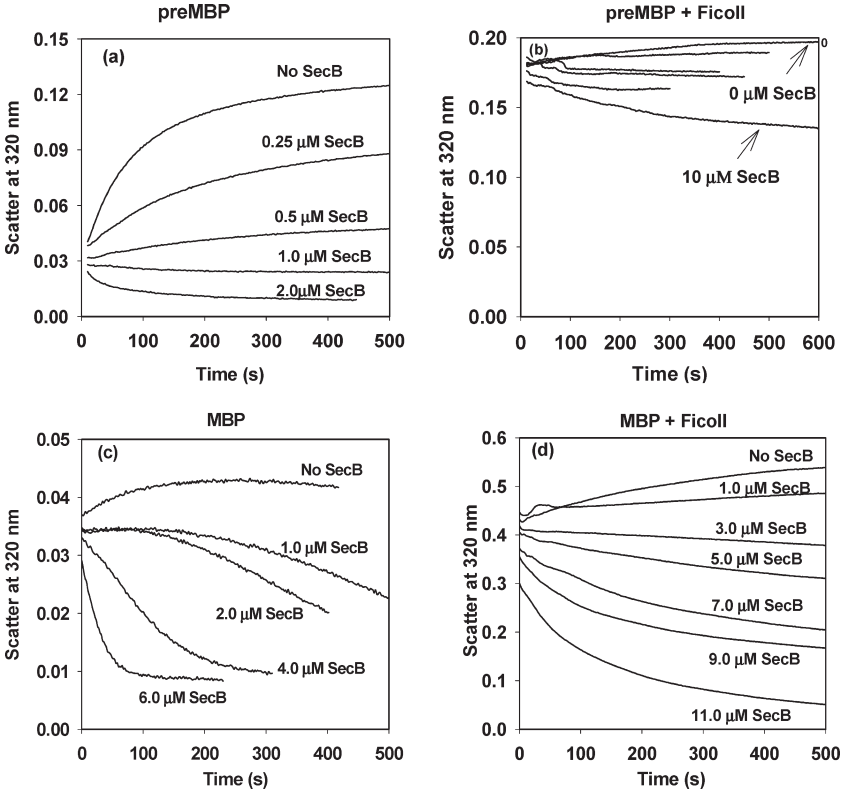

Figure 4. Kinetics of aggregation/disaggregation during refolding of preMBP and MBP as a function of the SecB concentration at 298 K. Refolding of 1 μM preMBP in the absence (a) and presence (b) of 30% Ficoll-70. Refolding of 5.0 μM MBP in the absence (c) and presence (d) of 30% Ficoll-70. In Figure 3b, SecB concentrations are (from top to bottom) 0, 0.4, 1.2, 2, 5, and 10 μM.

enhanced the aggregation of both MBP and preMBP during refolding and the extent of light scattering was much higher in Ficoll solutions than in uncrowded solutions for both proteins (compare Figure 1e with f, g, with h).

However, when 30% Ficoll-70 was added to solutions of native MBP and preMBP, there was no visible aggregation. The CD spectra of MBP in the presence and absence of Ficoll-70 were similar (Figure 3a). However, the CD spectrum of preMBP in Ficoll-70 showed reduced ellipticity (Figure 3b). Although we have not established the reason for this, it is possible that the crowding agent alters the structure of the signal peptide or the interaction of the signal peptide with the rest of the protein. The thermal stability of MBP was marginally higher in the presence of Ficoll-70 (Figure 3, T_m's of 335 ± 0.3 and 336 ± 0.7 K), and the cooperativity of unfolding was similar. As observed earlier,[36] the thermal unfolding of MBP was reversible. For preMBP, as reported previously,[34] thermal unfolding in the absence of Ficoll-70 was completely irreversible with an apparent T_m of 329 ± 0.3 K and the formation of a visible precipitate. In the presence of Ficoll-70, the apparent T_m was 329 ± 0.9 K, and the cooperativity of the transition was significantly decreased. This may be due to rapid aggregation upon unfolding, which would decrease the concentration of the monomeric unfolded form and thereby promote further unfolding of native protein. When chemically denatured preMBP/MBP was refolded as a function of temperature, the extent of aggregation was also found to be proportional to the temperature of refolding (data not shown).

Effect of SecB and Crowding on the Refolding of preMBP. SecB is a molecular chaperone that has previously been shown to prevent the reversible aggregation of MBP that occurs when it is refolded at concentrations greater than 2 μM.[40] PreMBP is the physiological substrate for SecB, and we therefore investigated the effect of SecB on the aggregation of preMBP in both crowded and uncrowded solutions. Figure 4 a,b shows the optical scattering at 320 nm as a function of the time for refolding preMBP. Denatured 10 μM preMBP in 2.0 M GdnHCl was diluted to a final concentration of 1 μM in refolding buffer with or without Ficoll-70. Varying concentrations of SecB were included in the refolding buffer to study the effect of SecB on the aggregation process. In the absence of added SecB, the extent of light scattering increases with time up to the 10 min interval for which it was monitored both in crowded and uncrowded solutions. The extent of aggregation is considerably enhanced in crowded solutions. Figure 5 shows the light-scattering intensity at 320 nm after 200 s of refolding preMBP and MBP as a function of the concentration of added SecB. In uncrowded solution, 1 mol equiv of SecB (1 μM) could largely block the aggregation of refolding preMBP (Figure 5a). The lack of optical scattering also indicated the absence of aggregated protein. When refolding was carried out in buffer containing 30% Ficoll-70, even a 10-fold molar excess of SecB could not block the aggregation of preMBP. When these refolding reactions were examined after 48 h, there was still a considerable amount of visible precipitate. The ability of SecB to block the aggregation of refolding preMBP was also examined at different temperatures. The results are shown in Figure 5b. As the temperature was increased, higher concentrations of SecB were required to prevent preMBP aggregation.

Effect of SecB and Crowding on the Refolding of MBP. MBP refolding was carried out as a function of SecB concentration in the absence and presence of 30% Ficoll-70. Studies were carried out at three MBP concentrations of 1, 2, and 5 μM. The extent of aggregation increased with increasing protein concentration, and the data for 5 μM MBP are shown in Figure 4c,d. In the absence of Ficoll-70, SecB not only could block aggregation but also could promote the disaggregation of preformed aggregates (Figure 4c). Even in crowded solution, SecB could block the aggregation and promote the disaggregation of refolding MBP.

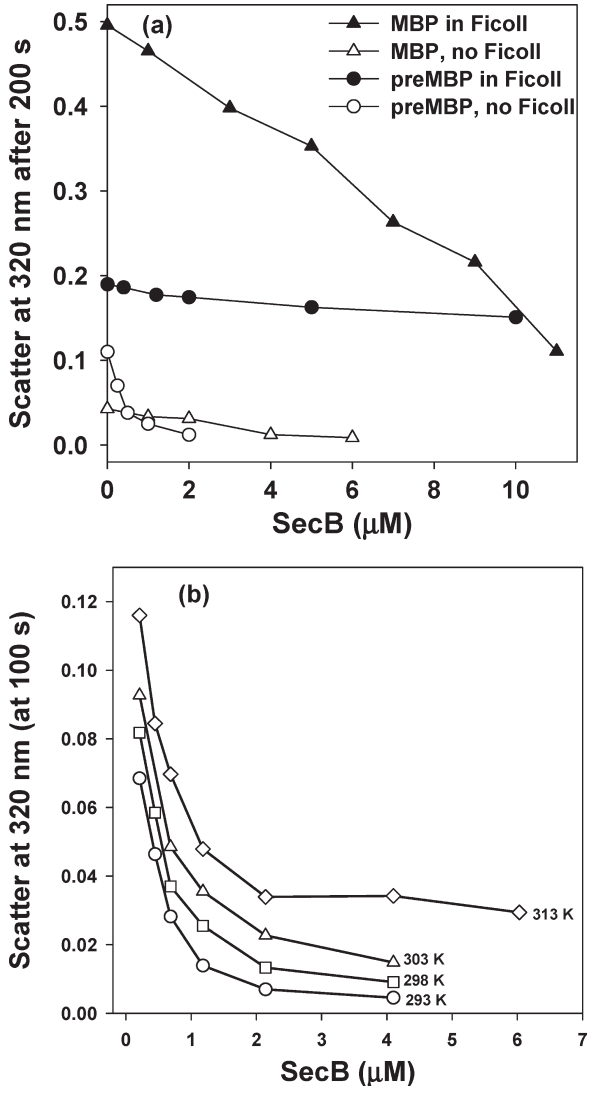

Figure 5. Effect of SecB and temperature on protein aggregation. (a) Effect of varying concentrations of SecB on the aggregation of 5.0 μM refolding MBP (triangles) and 1.0 μM refolding preMBP (circles) in CGH-10 buffer at pH 7.2 in the presence (filled) and absence (open) of 30% Ficoll-70. The extent of aggregation after 200 s of refolding in the presence of different concentrations of SecB was monitored by measuring the scattered light intensity at 320 nm. (b) Light-scattering intensity 100 s after the initiation of refolding of 1.0 μM preMBP in the presence of varying concentrations of SecB at different temperatures. The temperatures were 293 (○), 298 (□), 303 (△), and 313 K (◇).

However, this required much higher SecB concentrations than in uncrowded solutions (Figure 4d). No precipitate was detected in these reactions after 24 h, indicating that completed disaggregation had occurred.

Preformation of the SecB/Substrate Complex Prevents preMBP and MBP Aggregation. Solutions containing 30% Ficoll-70 are very viscous. As described above, in the presence of Ficoll-70, SecB was unable to block the aggregation of refolding preMBP and MBP completely. We wanted to examine whether this observation resulted from improper mixing in viscous solutions. From our earlier studies, it was known that SecB does not undergo unfolding at a GdnHCl concentration of 1.0 M.[72] We therefore first formed a complex between denatured MBP/preMBP and SecB in refolding buffer containing 1.0 M GdnHCl. This was rapidly diluted 5-fold into refolding buffer containing 30% Ficoll-70. Figure 6 shows the light-scattering intensity as a function of time for preMBP (a) and MBP (b). It can be seen that aggregation during refolding can be abolished even in crowded solutions if the denatured protein/SecB complex was formed before the addition of the crowding agent.

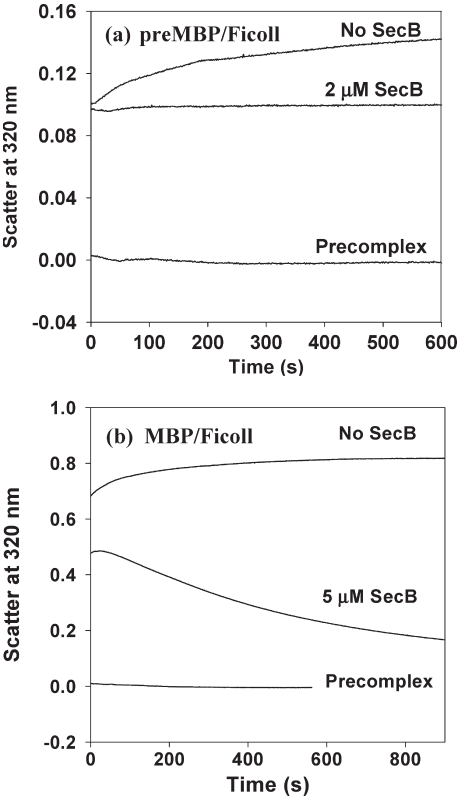

Figure 6. Effect of SecB on the aggregation of 1.0 μM preMBP (a) and 5.0 μM MBP (b) in crowded solution containing 30% Ficoll-70. Top curve: No SecB. Middle curve: SecB included in the refolding buffer. Bottom curve: SecB is first complexed to denatured MBP/preMBP in the absence of the crowding agent, and the complex is transferred to refolding buffer containing 30% Ficoll-70.

After 5 h of refolding, the solution was centrifuged to remove protein aggregates, and maltose was added at a final concentration of 10 mM to the supernatant. The fluorescence spectrum was measured and compared with the spectrum of native preMBP/MBP to quantify the amount of refolded protein in the supernatant. The results are shown in Figure 7. The red shift of the spectra and quenching of fluorescence intensity indicate that the protein in the supernatant is maltose-bound for both preMBP (Figure 7a) and MBP (Figure 7b). Hence, refolded protein in the supernatant is correctly folded and free from SecB because it can bind maltose. Supernatants from reactions with more than 1 mol equiv of SecB over the refolding protein concentration gave spectra that overlap each other and are very similar to those of native protein in the presence of maltose, indicating that all of the protein is refolded in them. Hence, molar equivalents of SecB can block MBP/preMBP aggregation even in crowded solutions. This experiment also proves that preMBP is fully recovered in its native form when refolded in the presence of SecB, even in crowded solutions. It has been reported by us earlier that the refolded MBP obtained after spontaneous disaggregation is similar to the native form as deduced from far-UV CD, native gel electrophoresis mobility, and fluorescence spectroscopy.[40]

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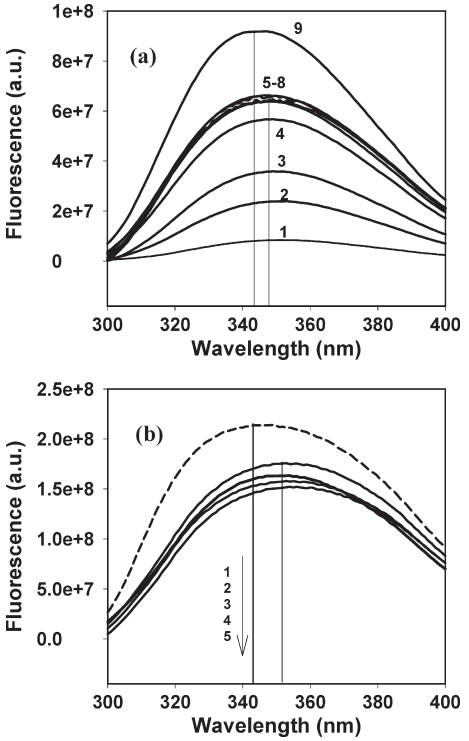

Figure 7. Fluorescence studies of maltose binding to preMBP (a) and MBP (b) when refolded in the presence of 30% Ficoll-70 and varying concentrations of SecB. (a) Fluorescence spectra of preMBP in the presence of 10 mM maltose present in the supernatant when 1 μM preMBP was precomplexed and refolded in the absence (1) and presence of 0.5 (2), 1.0 (3), 1.5 (4), 2.0 (5), 3.0 (6), 4.0 (7), and 5.0 μM (8) SecB. Spectrum 9 is that of free 1.0 μM preMBP in the absence of maltose. (b) MBP (5.0 μM) in the absence of maltose (1) and in the presence of 10 mM maltose (2−5) in decreasing order of intensity. They correspond to MBP present in the supernatant when 5 μM MBP was precomplexed and refolded in the absence (3) and presence of 2.0 (2), 5.0 (4), and 7.0 μM SecB (5). The red shift and quenching of the fluorescence emission are indicative of maltose binding. All spectra were corrected for SecB fluorescence as described previously.[33]

Structural Characterization of Aggregates. Congo red and thioflavin T (ThT) are two dyes that are commonly used to detect amyloid fibril formation. When preMBP aggregates were incubated for 30 min with Congo red, the absorption difference spectrum [(dye + preMBP) − (free dye)] shows negative and positive peaks at around 490 and 540 nm, respectively (Figure 8a,b). Thioflavin T was also found to bind to preMBP aggregates, as indicated by fluorescence emission spectra. When excited at 450 nm, aggregate-bound thioflavin T showed an increase in fluorescence emission with a peak at 485 nm. The changes in spectral characteristics of the two dyes when bound to preMBP aggregates indicate the amyloid-like nature of the aggregates. This was further confirmed with electron microscopy. Figure 8c shows two representative SEM images of preMBP aggregates. The extended nature of preMBP aggregates is evident from the images, and the observed thickness of the elongated structures was ∼1−5 μm, with the lengths in great excess over the thickness. In several images, the surface of the fiberlike aggregates appeared twisted and ropelike (data not shown). The overall nature of the aggregates was comparable under various conditions (±Ficoll, ThT, and Congo red; data not shown). Characterization of the MBP aggregates has been reported previously.[40] These were granular in nature and did not bind Congo red or thioflavin T to any significant extent. Hence, the presence of signal peptide profoundly affects the kinetics of the aggregation process as well as the nature of the aggregate.

Discussion

Following protein synthesis, a nascent unfolded protein chain can either fold into its stable native form or follow a nonproductive pathway to aggregation. Chaperones are known to bind to proteins in their non-native conformations and assist them to reach their native form.[8] Exported proteins are usually synthesized in their precursor form with an amino terminal signal sequence that contains an essential hydrophobic domain for the translocation process. The hydrophobic region of the signal peptide can potentially enhance the aggregation of such exported proteins after synthesis.

PreMBP, the precursor form of MBP, contains the N-terminal 26-residue MalE signal peptide with a 15-residue hydrophobic stretch. The present studies demonstrate that the signal peptide promotes the aggregation of refolding preMBP and results in the formation of amyloid-like precipitates. Refolding kinetics of preMBP at submicromolar concentrations in aqueous solutions show that refolding is a single-phase process involving a burst-phase intermediate (I_1) folding to form the native protein (N)[34] with a rate constant of 0.007 s^{-1}.

$$U \rightarrow I_1 \rightarrow N$$

Burst-phase intermediate I_1 with surface-exposed hydrophobic pockets binds hydrophobic dye ANS and is likely to be aggregation-prone. A similar intermediate is formed during MBP refolding. SecB, a cytosolic chaperone, functions to target a subset of secretory proteins to the Sec translocon by binding to non-native unstructured polypeptides. In the case of MBP export in *E. coli*, SecB interacts with preMBP in the cytoplasm and transports it to SecA, the next component of the secretory pathway. In vitro studies of the interaction of SecB with preMBP[33] indicate that the refolding of preMBP is completely blocked on the minute time scale by a molar excess of SecB. A SecB/preMBP complex can be isolated by gel filtration. Stopped-flow fluorescence data indicate that SecB binds to intermediate I_1 along the folding pathway of preMBP. Binding of SecB to refolding MBP/preMBP in buffer containing ANS leads to the displacement of bound ANS from MBP/preMBP. These observations suggest that SecB prevents MBP/preMBP aggregation by binding to the exposed hydrophobic surface on folding intermediates, thereby avoiding the unfavorable interactions that would otherwise lead to the aggregation of the protein.

SecB has previously been shown to form stable complexes with and prevent the aggregation of its physiological substrates, proOmpA[44] and prePhoE[73] in vitro in aqueous solution. SecB was also found to prevent denatured luciferase from aggregating.[74] In the above three cases, bound substrate appeared to form a stable complex with SecB. There was no evidence of substrate release in the active functional form in the absence of any additional chaperones or cofactors. In contrast, both MBP and preMBP are released from SecB in the folded and active form in dilute as well as crowded solutions.

We have previously shown that SecB can disaggregate preformed soluble aggregates of the insulin B-chain by a thermodynamic coupling mechanism.[46] The fact that SecB can completely

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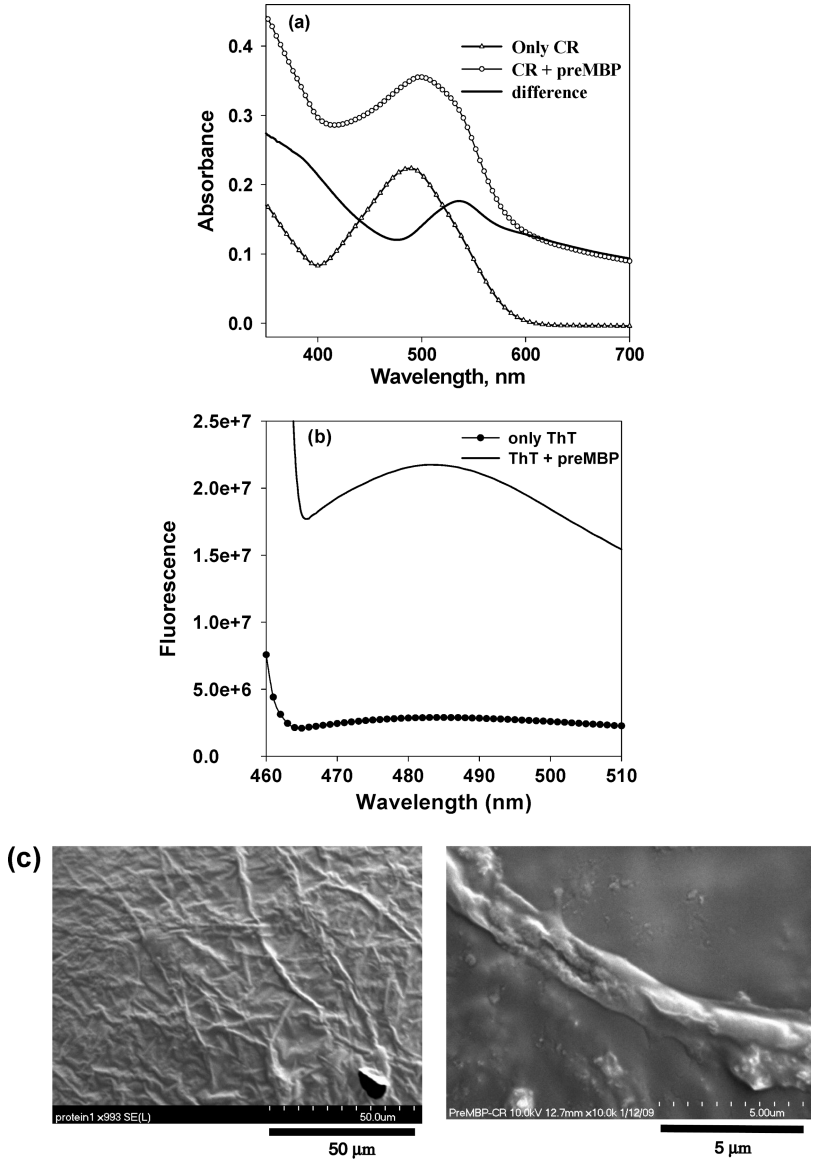

Figure 8. preMBP aggregates are amyloid-like. (a) Congo red binding. Triangles represent the spectrum of free Congo red in buffer, and circles represent the corresponding spectrum after binding to the preMBP aggregates, whereas the solid line is the difference spectrum. (b) ThT binding. The circles and the solid line represent the spectra for the free ThT and preMBP aggregate-bound ThT in buffer. (c) SEM images of preMBP aggregates at 10^3 (left) and 10^4 (right) magnifications. Sizes may be inferred from the scale bars.

prevent preMBP aggregation indicates that the prevention of preprotein aggregation can be a major role of SecB in vivo. Whether this is through repeated cycles of binding and release or whether appreciable folding occurs while bound to the chaperone remains to be elucidated. A proteomic analysis of a SecB deletion strain showed increased aggregation of both secretory and cytosolic proteins.[75] SecB overexpression suppresses the temperature-sensitive and aggregation-prone phenotypes of a strain lacking the DnaK/DnaJ and trigger factor chaperones.[76] These observations further support the role of SecB as a generalized chaperone in addition to its specific role in protein export. Recently, we have experimentally determined all of the kinetic parameters involved in the interaction of SecB with refolding MBP and preMBP.[33] PreMBP cannot fold while bound to SecB. However, in the presence of a molar excess of SecB, the amount of free (uncomplexed) protein (in the unfolded/ intermediate state) is negligible throughout the folding process. Virtually all substrate is either complexed to SecB or refolded to the native state. This decrease in the concentration of free aggregation-prone folding intermediate in the presence of SecB is the likely explanation of how SecB is able to block the aggregation of refolding preMBP and MBP even in crowded solution. In contrast to the MBP aggregates that are small, granular, and nonamyloid in nature,[40] preMBP aggregates formed during refolding are amyloid in nature. The physiological relevance of putative amyloid fibril formation of preMBP is unclear, but this is an example of spontaneous and very fast amyloid fibril formation at ambient temperature and pH without the need for any seeding. The formation of amyloid fibers in other proteins typically requires seeding and long incubation times. This is therefore an interesting model system for studying amyloid formation and its inhibition. It is of interest that addition of the short but hydrophobic signal peptide dramatically changes the nature of the aggregate, even for such a large protein. This is true even though isolated signal

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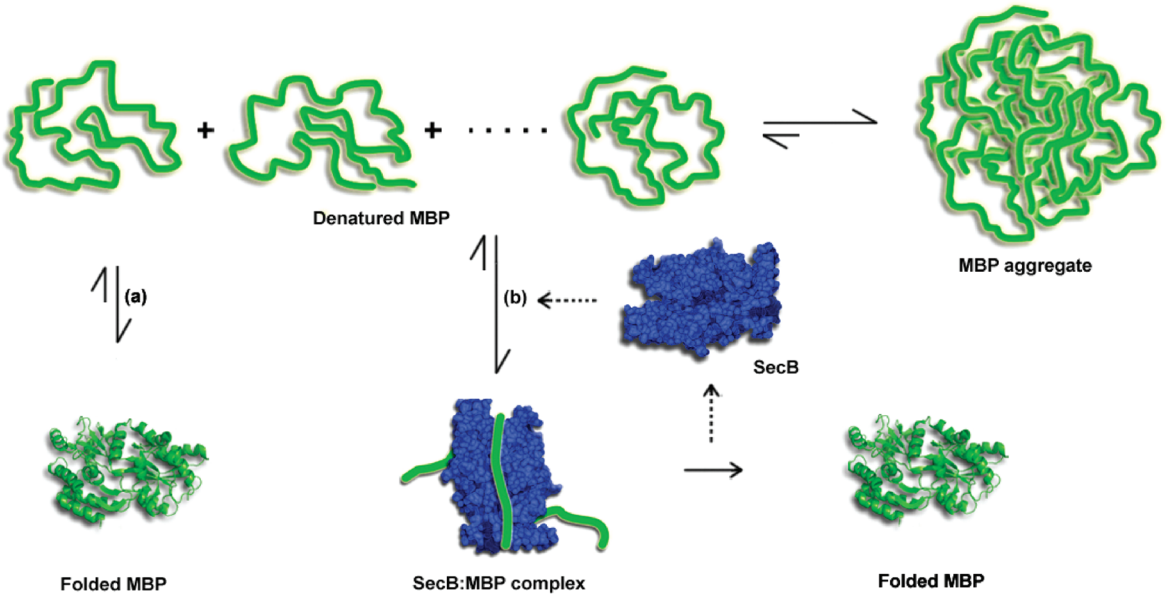

Figure 9. Model for SecB-mediated disaggregation of MBP. (a) The unfolded monomer can fold to the native state. (b) SecB binds to small amounts of monomeric unfolded MBP that are in equilibrium with the aggregate. This reduces the amount of monomeric unfolded MBP; consequently, some of the aggregate is converted to unfolded monomer via thermodynamic partitioning. MBP is also able to fold to some degree while bound to SecB.[33]

peptides are known to form helices, not sheets, at least when inserted into membranes.[77,78]

Although several chaperones have been shown to prevent protein aggregation, the disaggregation of preformed aggregates by chaperones is much less common. Protein disaggregation can be carried out by the Hsp104/ClpB chaperones[79,80] often in collaboration with Hsp70/Dna K chaperone systems. The detailed mechanism is not known. However, it requires energy input and presumably involves the extraction of individual polypeptide chains from the aggregate and forced unfolding by Hsp104/ClpB. In this and earlier work,[40,46] we show that in some cases the disaggregation of macroscopic aggregates can be facilitated by the chaperone SecB in a process that does not require any energy input or additional cofactors/chaperones. In the case of soluble aggregates of the insulin B-chain, it was shown that SecB promoted the dissociation of aggregates by binding to the small amount of monomeric B chain in equilibrium with the aggregate.[50] Consistent with this thermodynamic coupling mechanism, the apparent rate constant for disaggregation was independent of SecB concentration and presumably reflected the SecB-independent dissociation of insulin B monomer from the aggregate. In the present study, we observe that SecB is able to reduce the aggregation of both preMBP and MBP. However, it facilitates disaggregation only for MBP aggregates in both uncrowded and crowded solutions. A schematic depiction of how SecB might facilitate protein disaggregation is shown in Figure 9. SecB binds to small amounts of unfolded MBP that are in equilibrium with aggregated MBP. Once bound to SecB, MBP is protected from further aggregation. It can partially fold while bound to SecB[33] or alternatively may dissociate from SecB and refold to the native state in aqueous solution. Binding of unfolded MBP to SecB decreases the concentration of monomeric, unfolded MBP and promotes further dissociation of MBP aggregates by thermodynamic partitioning. In contrast, preMBP forms amyloid-like aggregates that do not dissociate spontaneously. In this case, the amount of monomeric unfolded preMBP is likely substantially below the K_d for SecB binding (which is about 4.5 nM[33]), and SecB is unable to catalyze the dissociation of amyloid fibrils of preMBP. In crowded solutions, as expected, the aggregation of MBP and preMBP is greatly enhanced. However, SecB is able to catalyze the dissociation of MBP aggregates presumably by a similar mechanism to that proposed above. The rate of disaggregation increases with increasing SecB concentration. SecB cannot cause the disaggregation of preMBP in crowded or uncrowded solution. However, preformation of an SecB/preMBP complex completely suppresses aggregation during refolding because the concentration of free refolding chains is below the threshold required for aggregation. This in turn is because most of the preMBP is bound to SecB.[33] This study clearly demonstrates that a simple chaperone such as SecB can prevent aggregation in crowded solution just by lowering the concentration of free unfolded chains. This is relevant to intracellular protein folding and protein translocation, both of which occur under very crowded conditions. More detailed kinetic and spectroscopic studies are required to confirm the proposed mechanism of disaggregation and to determine whether SecB binds to the macroscopic aggregates or only to monomeric/multimeric soluble species present in solution.

Concluding Remarks. We have shown that both signal peptide and crowding can dramatically effect the partitioning between aggregation and folding for maltose binding protein. The signal peptide induces the rapid formation of amyloid-like aggregates during preMBP refolding. Chaperone SecB can prevent aggregation by binding to aggregation-prone intermediates with exposed hydrophobic surface. It promotes the proper folding of MBP and preMBP even in crowded solution, probably by lowering the concentration of free folding intermediates to below the threshold required for aggregation. Refolding MBP chains exhibit the unusual property of forming macroscopic aggregates that spontaneously dissociate to yield

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properly folded functional protein. SecB facilitates the disaggregation process. The experiments suggest that in addition to its role in protein export SecB also prevents the aggregation of substrate precursor proteins. Such preproteins contain hydrophobic signal peptides that are likely to promote aggregation, especially in the crowded, intracellular environment. Several issues need to be addressed in future studies. The kinetic rate constants of substrate/SecB binding and release need to be measured in crowded solutions as a function of crowding concentration using methodology similar to that described previously for dilute solutions.[33] The mechanisms involved in spontaneous and SecB-mediated disaggregation as well as the formation of amyloid-like aggregates mediated by signal peptide need to be characterized. Finally, the generality of these phenomena should be examined first with other periplasmic binding proteins and then with proteins of other folds.

Abbreviations

MBP, maltose-binding protein; preMBP, precursor maltose binding protein with signal peptide; CGH-10, citrate 10 mM, glycine 10 mM, and HEPES 10 mM; ThT, thioflavin T; GdnHCl, guanidine hydrochloride; SEM, scanning electron microscope.

 DOI: 10.1021/la900198h