Effect of Signal Peptide on the Stability and Folding Kinetics of Maltose Binding Protein†

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ABSTRACT: While the role of the signal sequence in targeting proteins to specific subcellular compartments is well characterized, there are fewer studies that characterize its effects on the stability and folding kinetics of the protein. We report a detailed characterization of the folding kinetics and thermodynamic stabilities of maltose binding protein (MBP) and its precursor form, preMBP. Isothermal GdmCl and urea denaturation as a function of temperature and thermal denaturation studies have been carried out to compare stabilities of the two proteins. preMBP was found to be destabilized by about 2−6 kcal/mol (20−40%) with respect to MBP. Rapid cleavage of the signal peptide by various proteases shows that the signal peptide is accessible in the native form of preMBP. The observed rate constant of the major slow phase in folding was decreased 5-fold in preMBP relative to MBP. The rate constants of unfolding were similar at 25 °C, but preMBP also exhibited a large burst phase change in unfolding that was absent in MBP. At 10 °C, preMBP exhibited a higher unfolding rate than MBP as well as a large burst phase. The appreciable destabilization of MBP by signal peptide is functionally relevant, because it enhances the likelihood of finding the protein in an unfolded translocation-competent form and may influence the interactions of the protein with the translocation machinery. Destabilization is likely to result from favorable interactions between the hydrophobic signal peptide and other hydrophobic regions that are exposed in the unfolded state.

A significant subset of proteins in both prokaryotes and eukaryotes needs to be exported to their site of action from the cytosol, the usual site of protein synthesis. Many of these exported proteins are synthesized in a precursor form (pre-proteins) with an additional, N-terminal amino acid extension called a signal peptide. Signal peptides consist of short stretches of nearly 15−40 amino acids. After delivery of the protein to the correct subcellular compartment, they are normally removed by specialized membrane-associated signal peptidases (1). The significance of the signal peptide in protein translocation and secretion as a target recognition motif is a well-established concept (1, 2), but its role in directly modulating the properties of the pre-proteins themselves and hence the export process is not as well characterized.

The earliest studies on signal peptides were related mainly to their direct interaction with the membrane for protein insertion into and across the membrane barrier (3−5). Park et al. first reported the involvement of the signal peptide in the folding of the precursor proteins of maltose binding protein (MBP) and ribose binding protein (RBP) (6). Subsequently, there have been very few studies that report a quantitative comparison of thermodynamic and kinetic properties of a pre-protein and the corresponding mature protein (7, 8).

Previous comparisons of precursor and mature forms of MBP have mainly focused on the folding rates of the two proteins using the relaxation time measured in the manual mixing experiments (9−11), but an exhaustive, quantitative comparison of folding kinetics of the two proteins is not available. No direct measurement of the relative stability of the precursor form and the mature forms have been carried out. Stability studies have only been reported with mutants of the mature protein which are known to refold at rates similar to the precursor form or with slow folding suppressor mutants of export defective preMBP (12). In the current work, we report a complete thermodynamic and kinetic comparison between the precursor and the mature forms of an Escherichia coli protein MBP.

MBP is a large, two domain, 370-residue periplasmic protein involved in maltose uptake and chemotaxis. It is synthesized in the cytosol as a pre-protein containing a 26-residue signal peptide at the N-terminus (preMBP). Wild type preMBP is difficult to isolate in large amounts because it is

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1 Abbreviations: MBP, maltose binding protein; preMBP, precursor MBP with signal peptide; CGH10, citrate, glycine, and HEPES buffer (10 mM each); GdmCl, guanidinium chloride; CD, circular dichroism; Tm, temperature of maximal heat capacity; ANS, 8-anilino-1-naphthalenesulfonic acid; Cm, denaturant concentration at which unfolded protein population is 50%; wt, wild type.
readily cleaved by signal peptidase in vivo to generate the mature protein. Hence, we have used a mutant of preMBP carrying a mutation in the signal peptide, A14E-preMBP, as a substitute for authentic preMBP. This mutant has already been reported to be identical to the authentic preMBP with respect to its refolding and unfolding relaxation times \((9)\) and interaction with SecB \((13)\). In the present work, isothermal GdmCl, urea denaturation, and thermal denaturation studies have been carried out to compare the stabilities of the proteins. Folding/unfolding kinetics have been monitored using circular dichroism and fluorescence measurements. This study clearly reveals that the signal peptide affects the stability as well as the folding and unfolding kinetics of MBP to a greater extent than previously appreciated.

preMBP is a substrate for the \(E.\ coli\) chaperone SecB. The cytosolic chaperone SecB binds to pre-proteins and maintains them in a translocation-competent state \((14)\). A number of studies have examined the interaction of SecB with physiological as well as model substrates \((10, 15 - 17)\). However, a detailed quantitative understanding of the relative importance of kinetic and thermodynamic factors in SecB/substrate interactions is still to be achieved. From manual mixing experiments, preMBP is known to fold more slowly than MBP \((6)\). Since SecB binding occurs in competition with the substrate folding, slower folding substrates are more likely to bind to SecB. While it is known that the signal peptide slows down the folding of MBP, the molecular basis of this effect has been unclear. The present studies suggest that the signal peptide stabilizes the unfolded state as well as the collapsed intermediate(s) and thereby decreases the refolding rate.

**EXPERIMENTAL PROCEDURES**

**Materials.** Maltose, PMSF, IPTG, ampicillin, trisodium citrate, glycine, papain, trypsin, chymotrypsin, subtilisin, proteinase K, fast flow Q-Sepharose, ANS, and SephadexG75 were from Sigma. HEPES and ultrapure GdmCl were purchased from USB. Amylose resin was obtained from NEB. All the other chemicals were from local commercial sources and were of analytical reagent quality.

**Methods, Strains, Plasmids, and Protein Purification.** The MBP-deficient strain POP6590 harboring the plasmid pMAL-P2 -MBP \((18)\) was used as a source of MBP and preMBP. The mutant A14E-preMBP was generated by mutagenesis using the Stratagene Quik Change site-directed mutagenesis protocol. MBP was purified using an osmotic shock procedure \((19)\). preMBP was purified by amyllose affinity chromatography using 10 mM maltose for elution \((20)\). Purified protein was stored at \(-70^\circ\text{C}\) and the bound maltose was removed by passing it over a PD10 (Amersham Biosciences) column in CGH10 buffer containing 3 M GdmCl at pH 7.2. Maltose removal was confirmed by refolding the protein and measuring the fluorescence emission spectrum \((21)\).

**Buffers and Solutions.** All kinetic and equilibrium experiments were carried out in the native buffer, CGH10, pH 7.2 containing 150 mM sodium chloride. MBP unfolding was carried out for not less than 2 h in native buffer containing 2 M GdmCl at room temperature. Maltose-free preMBP in 3 M GdmCl obtained from the PD10 column was used directly as unfolded preMBP for further study. Concentration of preMBP was determined from the absorbance value at 280 nm and an extinction coefficient of 66 350 M\(^{-1}\) cm\(^{-1}\) \((22)\). \(\varepsilon_280\) of MBP can be used for preMBP since the signal peptide does not contain Trp or Tyr residues. Concentration of GdmCl was determined by refractive index measurements. All buffers were filtered through 0.22 \(\mu\text{m}\) filters, and degassed buffers were used for the stopped flow kinetic experiments.

**Mass Spectroscopy and Dynamic Light Scattering (DLS).** ESI-MS was performed on a Q-TOF II machine from Micromass. DLS was performed on the refolded proteins (at a minimum concentration of 10 \(\mu\text{M}\)) on a Dyna Pro-99 instrument at 25 \(^\circ\text{C}\) (Protein Solutions Ltd.). Samples were spun at 14 000 rpm for 10 min and filtered through a 0.02 \(\mu\text{m}\) filter. Data were acquired for 3 s per data point at a sensitivity of 90%. All fluctuations in intensities greater than 15% were marked excluded and not used for the data analysis. At least 40 points were used to derive the hydrodynamic radius. Dyna LS software (Protein Solutions Ltd.) was used for the data analysis.

**Analytical Gel Filtration.** MBP and preMBP were subjected to gel filtration chromatography using an analytical Superdex-75 column (Amersham Pharmacia, column volume, \(V_i = 24 \text{ mL}\)) on a Duo Flow FPLC system (BIORAD). The column was equilibrated with CGH10 buffer, pH 7.2 containing 150 mM sodium chloride and 10 mM maltose. A total of 20 \(\mu\)g of protein in a volume of 200 \(\mu\)L was loaded on the column and eluted at a flow rate of 0.4 mL/min. The void volume \((V_o)\) for the column was estimated to be 8.7 mL using blue dextran.

**Proteolytic Digestion.** Eight micrograms each of refolded MBP and preMBP were subjected to controlled proteolysis in 1 mL of CGH10 buffer, pH 7.2 containing 150 mM sodium chloride at 37 \(^\circ\text{C}\) using the proteases, trypsin, chymotrypsin, proteinase K, papain, and subtilisin with an enzyme-to-substrate ratio of 2% (w/w), for the indicated time. The proteolyzed mixtures were TCA precipitated by incubating the samples with 10% TCA at \(-20^\circ\text{C}\) for nearly two hours followed by harvesting the precipitated protein by centrifugation at 12000 g at 4 \(^\circ\text{C}\). The pellet was washed twice with acetone, dried, and redissolved in 30 \(\mu\text{L}\) of SDS loading buffer and subjected to SDS-PAGE using a 10% gel.

**CD Measurements.** Far UV CD spectra were acquired on a JASCO J715A spectropolarimeter. A protein concentration of 0.5 \(\mu\text{M}\) was used in a 2-mm or a 5-mm path length quartz cuvette. Measurements were done at 25 \(^\circ\text{C}\) over a wavelength range of 200–250 nm at a scan speed of 50 nm/min. Measurements were also done at lower scan rate of 10 nm/ min, but the observed structural content in terms of MRE’s was found to be independent of the scan rate. Data were collected with a response time of 4 s and a bandwidth of 2 nm. Each spectrum was an average of four consecutive scans. Buffer scans were acquired under similar conditions and subtracted from the protein spectrum before analysis.

For the refolding experiments, unfolded protein was refolded in 1 mL of native buffer to a final protein concentration of 0.5 \(\mu\text{M}\) and GdmCl concentration of 0.15 M. The decrease in the CD signal was monitored at 222 nm. The dead time for manual mixing was about 10 s. Data were averaged over two traces.
A thermal melt was carried out with the refolded protein at a concentration of 0.6 μM in native buffer in a 1-cm path length quartz cuvette by monitoring the CD signal at 222 nm using a JASCO PTC-348W1 peltier device. The sample was heated from 25 to 80 °C with a heating rate of 60 °C/h. A bandwidth of 2 nm and response time of 4 s were used.

Equilibrium Unfolding Studies. A sample concentration of 0.25 μM was used for the isothermal equilibrium denaturation melts. Denaturation was carried out at various temperatures in the range of 10–42 °C, by incubating the proteins at the appropriate GdmCl or urea concentration until equilibrium was established. Fluorescence was measured on a SPEX Fluoromax3 spectrofluorimeter in a 1-cm water-jacketed cell using an excitation wavelength of 280 nm and an emission wavelength of 340 nm in case of GdmCl melts and 337 nm in case of urea melts. In the urea denaturation studies, the buffer also contained 150 mM KCl. Spectral bandwidths of 1 nm for excitation and 5 nm for emission were used. Each value was the average of three consecutive measurements with a response time of 4 s.

Equilibrium unfolding as a function of GdmCl and urea concentration were also monitored at 222 nm using CD with a protein concentration of 0.4 μM in a 5-mm path length quartz cuvette. Each measurement was taken as an average of the data collected for a minute with a response time of 1 s.

Kinetic Experiments. The kinetics of refolding at 25 °C were monitored at protein concentrations of 0.2 and 0.5 μM in the manual mixing and stopped flow studies, respectively. Unfolded protein was refolded by dilution into the native buffer. The final GdmCl concentration was 0.15 M. Rapid mixing experiments by measuring the fluorescence at 340 nm using a JASCO PTC-348WI peltier device. The sample length quartz cuvette by monitoring the CD signal at 222 nm was 0.8 mm was used. The dead-time of the instrument was 1.4 ms.

The kinetics of unfolding of the native protein in different unfolding concentrations of GdmCl was monitored in manual mixing experiments by measuring the fluorescence at 340 nm with an excitation of 280 nm at the specified temperatures. The final protein concentration was 0.2 μM.

ANS Binding Studies. ANS binding studies were carried out on the SPEX Fluoromax3 spectrofluorimeter using excitation wavelength of either 280 or 380 nm and an emission wavelength of 475 nm. The spectral bandwidths used were 1 and 5 nm for excitation and emission, respectively. Protein and ANS concentrations used were 0.2 and 20 μM, respectively. Measured kinetic rate constants with excitation at 280 nm were identical to those obtained with excitation at 380 nm. For the delayed ANS binding study, protein refolding was initiated by dilution of the unfolded protein. After variable times of incubation (15 to 300 s), ANS was added to the refolding protein, and the ANS fluorescence was measured as described above.

Data Analysis. Equilibrium Studies. The equilibrium data were analyzed in terms of a two-state transition using the linear extrapolation model (23) to obtain the parameters ΔG°, m, and Cm as described previously (24).

For both preMBP and MBP, m values from individual melts did not vary appreciably with temperature in the range of 25–42 °C in case of urea melts and 15–37 °C in case of GdmCl melts, and hence all the denaturation data for each denaturant were subjected to a global fitting procedure with a single “m” value using the multiple function nonlinear regression of SigmaPlot for Windows scientific graphing software. The equation describing a two-state unfolding transition (25) was used to fit multiple isothermal denaturation data. The folded and the unfolded baseline parameters derived from individual fits at each of the temperatures were used to generate the fraction unfolded (fu) as a function of denaturant concentration. The fu data sets were subjected to the global fit regression. In the final fitting procedure, each data set was fitted to a separate two-state equation with the slopes of the baselines and free energy of unfolding as local parameters and the m value as a shared parameter across the data sets. The m value thus obtained was a temperature independent m value.

With the assumption of ΔCp being temperature independent, the unfolding free energy (ΔG°) as a function of temperature was fit to determine the denaturation temperature (Tm) as described (25).

Thermal Melt. Unfolding as a function of temperature was monitored by observing the change in CD signal at 222 nm. The data were fit to a reversible two-state model,

$$k_{app} = \frac{N \rightarrow U}{T}$$

where N is the native protein, U is the unfolded protein, and $k_{app}$ is the apparent equilibrium constant. The data can be described by the equation (25):

$$Y_o = \{y_F + m_F T\} + \{y_U + m_U T\} \exp\left\{\frac{\Delta H_0(T_{m})}{T(T_m - 1)} + \frac{\Delta C_p (T - T_m - T \ln(T/T_m))}{RT}\right\}/\{1 + \exp\left\{\frac{\Delta H_0}{T(T_m - 1)} + \frac{\Delta C_p (T - T_m - T \ln(T/T_m))}{RT}\right\}$$

where $Y_o$ is the mean residue ellipticity measured at temperature T, $y_F$ and $y_U$ represent the intercepts and $m_F$ and $m_U$ are the slopes of the folded and unfolded baselines of the transition, respectively, $T_m$ is the midpoint of the thermal transition, $\Delta H_0(T_m)$ is the change in enthalpy at $T_m$, and $\Delta C_p$ represents the change in heat capacity.

The observed data were fit to the above equation with a nonlinear least squares program using SigmaPlot for Windows scientific graphing software.

Kinetic Studies. Changes in signal intensity as a function of time in all the kinetic experiments were fit to the equation

$$a_x + \sum a_i \exp(-k_i t)$$

where $a_x$ represents the amplitude achieved at equilibrium, $a_0$ represents the amplitude change occurring in the burst phase, and $a_i$ represents the amplitude change of the phase i occurring with an observed rate constant $k_i$.

RESULTS

General Features of preMBP. Because of cleavage of the signal peptide soon after preMBP synthesis, it was difficult to obtain authentic preMBP in large amounts. To overcome this problem, we have used an export-defective mutant of MBP carrying a mutation, namely, A14E, in the signal peptide. The presence of the A14E mutation in the signal peptide has been shown to decrease the rate of folding relative to that of MBP to approximately the same extent as
does the wild-type signal peptide (9). SecB slows the folding of MBP but blocks the refolding of preMBP. A similar blockage effect has been reported in the case of A14EpreMBP as well (13). Hence, this mutant preMBP can be used as a substitute for authentic preMBP. The A14EpreMBP mutant protein will be referred to as preMBP hereafter. ESI-MS yielded masses of 40707 (1 and 43444 (3 for MBP and preMBP, respectively. The calculated masses were 40707.2 for MBP and 43445.6 for preMBP (inclusive of a N-terminal Met residue).

preMBP was found to be aggregation prone and highly unstable. Hence, maltose bound to the protein during elution from the amylose column was removed by desalting the protein, on a PD10 column, in a GdmCl unfolded state rather than by extensive dialysis under native conditions. Maltose binding to MBP and preMBP is accompanied by fluorescence quenching and a red shift (21). Fluorescence emission spectra of the refolded protein confirmed the complete removal of maltose. Refolded preMBP was confirmed to be aggregate free by Superdex75 gel filtration chromatography (Figure 1A), absence of ANS binding (data not shown), and absence of a dependence of refolding kinetics on protein concentration (data not shown). The maximal protein concentration in the peak eluted from the gel filtration column was about 0.7 μM. Because both MBP and preMBP are carbohydrate binding proteins, they tend to elute later than expected on gel filtration columns (data not shown). To reduce the interaction between the protein and column beads, gel filtration was also carried out in the presence of 10 mM maltose. In the presence of maltose, both proteins eluted at the expected volumes of 10.6 and 10.8 mL for preMBP and MBP, respectively (Figure 1B,C). Gel filtration was also carried out under denaturing conditions in the presence of 1.5 M GdmCl. Under these conditions, preMBP and MBP had similar elution volumes of 8.8 and 8.9 mL, respectively (data not shown). DLS carried out at a protein concentration of 10 μM yielded radii of 2.9 ± 0.6 and 3.2 ± 0.1 nm for the folded MBP and preMBP, respectively, confirming the monomeric nature of these proteins.

The far UV CD spectrum of refolded preMBP was found to be similar to that of MBP in the native state, indicating that the proteins have similar structure (Figure 2). Refolded preMBP was further found to be active, and Scatchard plot analysis showed that it also binds maltose at a stoichiometry of 1:1 with an affinity similar to that of MBP or the wild-type preMBP (26) (data not shown).

Refolding of MBP and preMBP was found to be reversible under the conditions used in the study. In the case of preMBP, since the refolded protein was used, reversible folding was also confirmed by repeated refolding and
unfolding. Unfolded protein from the PD10 column was refolded and was subjected to a second round of unfolding after appropriate dilution corrections (data not shown). The rate of refolding and the relative unfolding reaction. The rate of refolding and the relative amplitudes of the folding phase were found to be unaffected by multiple rounds of refolding and unfolding after appropriate dilution corrections (data not shown). The rate of refolding was found to be independent of the protein concentration over a 10-fold concentration range. Also the amplitude of the slow folding phase was found to be linear as a function of protein concentration.

Proteolysis was carried out using trypsin, chymotrypsin, proteinase K, papain, and subtilisin at 37 °C (Figure 3). Under these conditions, the signal peptide in preMBP is rapidly cleaved. Following cleavage of the signal peptide preMBP, which now lacks the major portion of the signal peptide, and MBP are digested with similar kinetics. This suggests that in the folded state, the signal peptide is accessible to proteases and does not interact with the rest of the protein. Refolded preMBP was thus confirmed to be the wild-type precursor with respect to all the reported features of the precursor form (9, 13, 20, 27).

Isothermal Equilibrium Denaturation Studies. GdmCl Induced Unfolding. Isothermal GdmCl induced equilibrium unfolding studies were carried out at five different temperatures. Initially, each data set at a given temperature was fit locally for the parameters as described (4). The unfolding transition was also monitored using the CD transition for both the proteins. The unfolding transition is well described by a two-state unfolding model.

Table 1: Comparison of Thermodynamic Parameters for the GdmCl-induced Denaturation of MBP and preMBP as a Function of Temperature.a,b

<table>
<thead>
<tr>
<th>Temperature (°C)</th>
<th>ΔG° (MBP) (kcal/mol)</th>
<th>ΔG° (preMBP) (kcal/mol)</th>
<th>ΔΔG° (kcal/mol)</th>
<th>ΔCm (kcal/mol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>9.1 ± 0.7</td>
<td>9.2 ± 0.3</td>
<td>-0.1</td>
<td>0.9</td>
</tr>
<tr>
<td>15</td>
<td>9.2 ± 0.9</td>
<td>10.1 ± 0.4</td>
<td>-0.9</td>
<td>0.6</td>
</tr>
<tr>
<td>20</td>
<td>10.4 ± 0.3</td>
<td>10.9 ± 0.4</td>
<td>-0.5</td>
<td>0.4</td>
</tr>
<tr>
<td>25</td>
<td>16.9 ± 0.8</td>
<td>10.0 ± 0.5</td>
<td>6.9</td>
<td>0.4</td>
</tr>
<tr>
<td>30</td>
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<td>5.7</td>
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</tr>
<tr>
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<td>6.3</td>
<td>0.4</td>
</tr>
<tr>
<td>40</td>
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<td>7.3 ± 0.4</td>
<td>6.2</td>
<td>0.5</td>
</tr>
</tbody>
</table>

Data were fit to a two-state model as described in ref. 24. Error shown is the standard error from the fit. Cm values were calculated using the m values. In case of the GdmCl melts, m values of −8.9 ± 0.3 and −7.7 ± 0.3 kcal mol⁻¹ M⁻¹ for MBP and preMBP, respectively, were used. In case of the urea melts, m value of −6.1 ± 0.2 kcal mol⁻¹ M⁻¹ for preMBP and a temperature-dependent m value for the temperatures 10−20 °C and a global value of −5.2 ± 0.2 kcal mol⁻¹ M⁻¹ in the temperature range of 25−42 °C for MBP were used. Units are as follows: (°C), ΔG° (kcal/mol), ΔΔG° (kcal/mol), ΔCm (M). a ΔΔG° = ΔG°(MBP) − ΔG°(preMBP). b ΔCm = Cm(MBP) − Cm(preMBP).
be smaller in magnitude by about 1.2 ± 0.4 kcal mol⁻¹ M⁻¹ for preMBP in comparison to MBP. The m value is believed to be correlated with the surface area exposed upon unfolding (28). A lower m value for preMBP suggests that a smaller amount of surface area is exposed upon denaturation of preMBP. Because the signal peptide is solvent exposed in the native state, it suggests that the difference in the m value reflects a difference in the denatured states of the two proteins. The lower m value for preMBP may indicate that the unfolded state of preMBP is more compact and stabilized in comparison to the unfolded state of MBP.

**Urea-Induced Unfolding.** The difference in stability of preMBP and MBP was also monitored using isothermal urea-induced denaturation in the temperature range of 10–42 °C (Table 1B). In case of MBP, the m value was found to be temperature dependent. However the data in the temperature range of 25–42 °C could be fitted globally for a common m value as described above for the GdmCl melts (Figure 6). A good agreement in the m values and C_m from the individual and global fits validated the global fitting procedure in the higher temperature range. At temperatures below 25 °C, the m values for MBP from individual fits were appreciably lower than from the global fit value; hence, in this temperature range individual m values were used. Consequently, in the temperature range of 10–20 °C, MBP and preMBP appear to have similar ΔG° values. However, MBP consistently has a substantially higher C_m than preMBP even in this temperature range. We therefore believe that MBP is more stable than preMBP even in the temperature range of 10–20 °C, although we currently cannot explain the large decrease in m value observed in this temperature range. Comparison of stability of the two proteins in the temperature range of 25–42 °C where global fitting procedures are used indicates that preMBP was destabilized by about 6.3 ± 0.5 kcal/mol relative to MBP. Similar to the GdmCl denaturation studies, a lower m value (Δm = 1.6 ± 0.2 kcal mol⁻¹ M⁻¹) was observed for preMBP in the urea denaturation studies also, consistent with a greater compaction of the unfolded state of preMBP in comparison to that of MBP. Urea-mediated denaturation was also confirmed to be a two-state unfolding process for both MBP and preMBP since the denaturation profiles monitored by CD at 222 nm and fluorescence were identical (data not shown).

**Equilibrium Thermal Denaturation.** The lower stability of preMBP was also confirmed by thermal denaturation studies of both the proteins monitored by CD measurement at 222 nm (Figure 7). Thermal denaturation of MBP was reversible, but thermal denaturation of preMBP was found to be irreversible. preMBP precipitates at higher temperatures. The presence of a hydrophobic stretch of amino acids in the signal peptide leads to irreversible aggregation upon thermal unfolding, probably because of the interactions of the signal peptide with other hydrophobic regions that become exposed upon unfolding.
The $T_m$ and $\Delta H^\circ(T_m)$ for MBP were found to be in good agreement with the values obtained from the calorimetric measurements. An apparent $T_m$ was calculated from the thermal melt of preMBP by CD (Figure 7). $T_m$ for preMBP thus calculated was found to be about 8 °C lower than that of MBP, emphasizing the destabilization of preMBP with respect to MBP. As with GdmCl and urea denaturation, thermal denaturation for preMBP was also much less cooperative than for MBP.

Conformational Stability. In the case of MBP, using the thermal denaturation data, the free energy of unfolding ($\Delta G^\circ$) was calculated at various temperatures in the transition zone of thermal unfolding. These are shown in Figure 8 (filled circle symbols). These free energy values were then combined with the free energy values obtained from the global fit of the isothermal GdmCl denaturation data, shown in filled circle symbols in Figure 8, to generate the stability curve. The composite data were then fit to obtain the thermodynamic parameters over a wide temperature range, 10–65 °C. There is a small but systematic deviation of $\Delta G^\circ$ values derived from the thermal melt data from the fitted stability curve. This might be due to differences in GdmCl and thermally denatured states. Values of $T_m$, $\Delta H_m$, and $\Delta C_p$ derived from the MBP stability curve shown here are 63.4 ± 1.5 °C, 143 ± 7 kcal/mol, and 3.2 ± 0.3 kcal mol$^{-1}$ K$^{-1}$. For comparison, more accurate calorimetric values are 63.1 ± 0.1 °C, 207 ± 10 kcal/mol, and 4.7 ± 0.9 kcal mol$^{-1}$ K$^{-1}$ (18). Since the thermal denaturation of preMBP was found to be irreversible, a similar procedure of combining the GdmCl denaturation data with the thermal melt data was not carried out and only the isothermal GdmCl melt data was used to generate the stability curve for preMBP. For the preMBP stability curve, values of $T_m$, $\Delta H_m$, and $\Delta C_p$ were 55.7 ± 1.5 °C, 162 ± 11 kcal/mol, and 4.9 ± 0.6 kcal mol$^{-1}$ K$^{-1}$. From the MBP data, it is clear that values of $T_m$ from the stability curve are more accurate than the $\Delta H_m$ and $\Delta C_p$. The two $T_m$ values, calculated from the stability curve and the thermal melt (63.4 and 63.6 °C, respectively, in case of MBP and 55.7 and 55 °C, respectively, in case of preMBP) were in close agreement.

The stability curve was also generated from the isothermal urea denaturation data following a similar procedure as described above. The $T_m$ values thus obtained were 55.5 and 63.5°C for preMBP and MBP, respectively (data not shown) similar to those obtained from either GdmCl melts or the thermal melts described above.

Unfolding Kinetics. MBP and preMBP have eight tryptophan residues. As the protein unfolds, these buried residues become exposed to the solvent, and the intrinsic Trp fluorescence decreases because of solvent quenching. Unfolding kinetics were monitored by following the reduction in the fluorescence signal during the unfolding of the protein from the native state to different unfolding GdmCl concentrations at 25 and 10°C (Figure 9).

The apparent rate constant increases with an increase in the final GdmCl concentration. preMBP unfolding rate...
constants were found to be similar to those of MBP at 25 °C (Figure 9A, Table 2A). However, there was a burst phase change during the unfolding of preMBP in the dead time of the fluorescence measurement, which was not present in the case of MBP. Figure 10 shows an overlay of the kinetic unfolding traces for both MBP and preMBP, the fluorescence intensity at the endpoint (t = ∞) of the kinetic traces fall on the equilibrium unfolding baseline, indicating that the kinetic unfolding data have been measured to completion. The fluorescence intensity at the start point of the kinetic trace (t = 0) was obtained from back extrapolation of the fitted data to t = 0. In the case of MBP, the start points of the kinetic unfolding traces fall on the native baseline of the equilibrium data (Figure 10A). For preMBP, these were appreciably lower than that of the native baseline (Figure 10B).

Unfolding of preMBP was found to be faster than MBP at 10 °C (Figure 9B). Similar to the unfolding at 25 °C, burst phase changes were observed only in case of preMBP and not MBP even at 10 °C (Table 2B). Stopped flow measurements to observe unfolding could not be carried out due to the unavailability of aggregation free refolded preMBP at high concentrations. Nevertheless, the presence of a large burst phase in the manual mixing experiment indicates that a fast unfolding phase is associated with the preMBP unfolding. Hence, in contrast to the earlier report (6), signal peptide affects the unfolding as well as refolding kinetics of MBP.

Refolding Kinetics of MBP. Refolding of MBP and preMBP were monitored by CD, intrinsic Trp fluorescence, and binding of the hydrophobic dye, ANS. All the kinetic parameters are listed in Table 3.

Refolding data for MBP showed the presence of a large burst phase change, indicating the formation of a collapsed intermediate within a few milliseconds of denaturant dilution. This is followed by a major slow folding phase occurring with a rate constant of 0.03 s⁻¹. This rate constant was found to be independent of protein concentration and the probe used.
the ANS binding studies. N* rearranges slowly (0.006 s⁻¹)
contains some exposed hydrophobic surface as indicated by
secondary and tertiary structure to the native state but
for the rate constants are s⁻¹
in turn goes to I3. I3 folds slowly with a rate constant of
undergoes a minor structural rearrangement to yield I2 which
a few milliseconds. I1 has exposed hydrophobic surface and
scheme can be proposed as
was found to involve two more phases (Figure 11B).
fluorescence measurements (Figure 11A). MBP refolding
the folding pathway, refolding was examined by stopped flow
collapse occurring in the slow folding phase.
refolding process and gives a true picture of hydrophobic
This indicates that the presence of ANS does not affect the
fluorescence intensities were found to be in close agreement.
The rate constants of the two phases were found to be
independent of the time of addition of ANS. The fluorescence
intensity at the time of ANS addition was calculated from
in the ANS binding experiment indicates that in
phase, restructuring of the collapsed chain occurs with
removal of 70% of the previously bound ANS molecules.
ANS binding also revealed the presence of an additional very
slow phase during which the remaining 30% ANS molecules
are ejected from the protein. This probably involves the slow
rearrangement in the structure to attain the final native form
of the protein. ANS binding to the late kinetic intermediates
during the folding of protein was investigated by delayed
addition of ANS to the refolding protein (data not shown).
the fit.
for the measurement. Since CD and fluorescence measure-
ments yield the same rate constant, this implies that the
protein acquires both secondary and tertiary structure simul-
taneously during the slow phase. A similar rate constant
observed in the ANS binding experiment indicates that in
this phase, restructuring of the collapsed chain occurs with
removal of 70% of the previously bound ANS molecules.
fluorescence measurements and CD measurements (Figure 11B).
errors shown is the standard error from the
Table 3: Kinetic Parameters of Refolding of MBP and preMBP at
298 K, pH 7.2 Monitored Using CD Measurement at 222 nm,
Fluorescence at 340 nm, and ANS Fluorescence at 475 nm°

<table>
<thead>
<tr>
<th>kinetic param</th>
<th>CDb</th>
<th>Trp fluoresce</th>
<th>ANS fluoresceb</th>
</tr>
</thead>
<tbody>
<tr>
<td>a0</td>
<td>0.3</td>
<td>0.58</td>
<td>0.52</td>
</tr>
<tr>
<td>a∞</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>a1</td>
<td>0.03</td>
<td>0.03</td>
<td>0.00</td>
</tr>
<tr>
<td>k1</td>
<td>4.7 ± 1.3c</td>
<td>0.1</td>
<td></td>
</tr>
<tr>
<td>k2 × 10106</td>
<td>3.7 ± 0.1</td>
<td>0.33</td>
<td></td>
</tr>
<tr>
<td>a1</td>
<td>0.7</td>
<td>3 ± 0.2</td>
<td></td>
</tr>
<tr>
<td>k1 × 10107</td>
<td>5.3 ± 0.1</td>
<td>7.3 ± 0.6</td>
<td></td>
</tr>
</tbody>
</table>

° Data were fit to equations as described in the data analysis. Units
for the rate constants are s⁻¹. CD and ANS fluorescence measurements
were conducted only in the manual mixing mode. a0 and a∞ are the
burst phase change and steady-state amplitude, respectively. a0 (i = 1−4)
represents the amplitude change associated with a phase i which
occurs with a rate constant ki. Error shown is the standard error from the
fit.

FIGURE 11: Kinetics of refolding of MBP (upper trace) and preMBP
(lower trace) in 0.15 M GdmCl, pH7.2. Measurements were made
on a stopped flow mixing set up as mentioned in Experimental
Procedures. For each trace, the continuous line represents the fit to
the data. The fluorescence signal was normalized to 1 for the native
protein. (A) In case of MBP, the folding process is described by a
three- exponential equation, while for preMBP a single exponential
defines the entire folding process. Dotted line and continuous line
represent the unfolded preMBP and MBP baseline, respectively.
(B) Data observed in the first second of measurement for refolding
MBP. Dotted line, dot−dashed line, and continuous line through
the data represent the single-, double-, and triple-exponential fits
of the data, respectively (C) Data observed in the first second of
measurement for refolding preMBP. The continuous line represents
the single-exponential fit of the data.

Here I1 is a collapsed form of the protein formed within
a few milliseconds. I1 has exposed hydrophobic surface and
undergoes a minor structural rearrangement to yield I2 which
in turn goes to I3. I3 folds slowly with a rate constant of
0.03 s⁻¹ to give a native like state N*. N* has identical
secondary and tertiary structure to the native state but
contains some exposed hydrophobic surface as indicated by
the ANS binding studies. N* rearranges slowly (0.006 s⁻¹)
to give N. MBP is a large protein with several Pro residues.
Each of the intermediates indicated above are unlikely to be
single conformations, but rather are collections of different
species with similar spectroscopic properties. It should also
be emphasized that the above kinetic scheme is only one of
many that are consistent with the spectroscopic data and
others (for example, involving multiple parallel pathways
of refolding) cannot be ruled out. However, none of the conclusions of this work depend on the details of the folding kinetics.

**Refolding Kinetics of preMBP.** A burst phase change was also observed in the case of preMBP refolding similar to that of MBP. The collapsed state formed then folds with a rate constant of 0.007 s⁻¹ to form the native protein. Stopped flow measurements show that preMBP refolding occurs in a single phase (Figure 11A,C). The intermediates I2, I3, and N⁺ appear to be absent. The collapsed state I1 formed within a few milliseconds goes directly to the native state. CD, Trp, and ANS fluorescence monitored refolding all occur with a similar rate constant of approximately 0.006 s⁻¹ (Table 3). Pulsed addition of ANS to refolding preMBP showed that the binding occurred in a single phase with a rate constant independent of the time of ANS addition. Thus, the folding of preMBP appears to follow a surprisingly simple folding pathway in contrast to the complex multistep folding of MBP. The folding scheme for the preMBP refolding can be represented as

$$U \rightarrow I1 \rightarrow N$$

Relaxation times for MBP/preMBP unfolding at 25 °C were measured by manual mixing using GdmCl concentrations of 0.1–1.0 M for refolding and 1.2 and 3.0 M for the unfolding experiments. These data were in good agreement with previously measured values and are therefore not indicated.

**DISCUSSION**

Precursor proteins typically differ from the corresponding mature proteins by the presence of the signal peptide. In such cases, the differences in the properties of the two proteins can solely be attributed to the effect of the signal peptide on the protein. In case of RBP, the retarding effect of the signal peptide on the refolding rate of the precursor form of RBP and tryptophan substituted RBP has been demonstrated using fluorescence and CD measurements. The conformational stability of the precursor and the mature form were found to be similar in this case (7), but only data for a single melt at 25 °C were presented. The only other example (to our knowledge) reporting thermodynamic characterization of a pre-protein is that of a mitochondrial pre-protein of bovine pre-adrenodoxin (adx) by DSC (8). The precursor form of adx (Padx) was found to be as active as an electron transporter as the mature adx. In this case, Padx was found to show a reduced transition temperature, denaturation enthalpy, and heat capacity in comparison to the mature adx. However, the thermal denaturation of Padx was completely irreversible, complicating the interpretation of the data. Thus, there is very limited data on the effects of signal peptides on protein stability and folding.

The folding kinetics of MBP has been previously studied using manual mixing techniques. Relaxation times for the unfolding of MBP and preMBP were shown to be very similar (9, 10), while the refolding kinetics indicated a slower folding of preMBP in comparison to MBP by a factor of 3. However, folding studies have not been carried out on the millisecond time scales. In the earlier studies of unfolding kinetics, only the relaxation rates were described, and there was no discussion of burst phase amplitudes. Because the folding of MBP/preMBP are kinetically not two-state processes, a slower folding rate of preMBP does not necessarily imply reduced thermodynamic stability. Detailed thermodynamic characterization of authentic or mutant preMBP is also not available. The current work provides a detailed comparison between preMBP and MBP in terms of their thermodynamic stabilities, folding and unfolding kinetics. While earlier work had demonstrated only a difference in the refolding kinetics, the present study shows that there are changes in both refolding and unfolding kinetics and also in the thermodynamic stabilities of the two proteins.

Retention of maltose binding activity in preMBP suggests that the presence of signal peptide does not alter the formation of the maltose-binding pocket. This is quite reasonable, since in the crystal structure, the N-terminus is far away from the maltose-binding site and the signal peptide is at the N-terminus of the protein (29). Resistance of the refolded preMBP to various proteases used in the current study is consistent with the earlier proteolytic digestion studies of preMBP (27, 30, 31). The present data indicate that the refolded protein achieves a stable, monomeric tertiary conformation with an exposed signal peptide.

preMBP is destabilized by 20–40% in comparison to MBP, as indicated from the isothermal denaturation studies. Lowered stability is also evident by the decrease in the apparent melting temperature by 8 °C. This decrease was both predicted by the stability curve and confirmed by monitoring thermal unfolding using the CD signal at 222 nm. From the equilibrium data, a reduction in the m value indicates that a smaller amount of surface area is exposed upon unfolding of preMBP relative to MBP. Analyses of signal peptides from various sources show that these have diverse sequences with certain conserved features such as the presence of a positively charged N-terminus, a large central hydrophobic stretch, and a signal peptidase recognition site. In the unfolded state, it is possible that the hydrophobic region of the signal peptide interacts with other hydrophobic regions of the protein. This kind of stabilization of the unfolded state can occur preferentially in the precursor form and can thus account for a lower stability as well as a lower m value for preMBP. Gel filtration studies under denaturing conditions did not show any appreciable differences in elution volume between MBP and preMBP. However, given the conformational diversity and dynamic nature of the denatured state, it may be difficult to detect transient interactions between signal peptide and the rest of the protein using this technique.

In addition to interactions in the unfolded state, it is also possible that the signal peptide may destabilize the native state of the protein. For example, it is possible that removal of the charged amino terminus from its location in MBP to a different location in preMBP may result in protein destabilization. Future studies with chemically synthesized signal peptides will help clarify these issues. It has been previously suggested that changes in the m value upon mutation/alteration of the protein sequence may also result from the formation of equilibrium intermediates (32–34). In the present study, there is no direct evidence for the formation of equilibrium intermediates during denaturant mediated unfolding of preMBP as denaturation followed by CD and Trp fluorescence is coincident. preMBP has fewer
detectable kinetic folding intermediates than MBP but at least one burst phase unfolding intermediate. In contrast, MBP has no kinetic unfolding intermediate. Hence, for preMBP, an appreciable fraction of the protein unfolds fast within the dead time of measurement. While we cannot rule out the possibility of the presence of small amounts of an equilibrium intermediate as being the cause of the difference in the $m$ value between MBP and preMBP, we consider this unlikely. This is because the thermal unfolding studies yield $T_m$'s that are in agreement with those calculated from the stability curves, which in turn are derived from the free energy values calculated from a two-state fit. In addition, the refolding rate of the major kinetic phase is about 5-fold faster for MBP, and unlike MBP a large fraction of preMBP unfolds within the dead time of manual mixing experiments. At low temperature, in addition to the burst phase, there is an increase in the unfolding rate constants for preMBP relative to MBP. All of these data suggest that the signal peptide results in both kinetic and thermodynamic destabilization of the protein.

MBP is a periplasmic protein and is translocated via the see pathway. A completely folded protein cannot cross the membrane using this pathway and hence needs to be in a non-native translocation competent form in the cytoplasm. preMBP is the physiological substrate of SecB. The recognition site for SecB lies within the mature domain of the protein (35, 36). However, in vitro, SecB does not bind folded MBP or folded preMBP. Overexpression of cytoplasmic MBP in vivo does not interfere with the export of other periplasmic/outter membrane proteins (37). SecB slows down but does not block refolding of MBP at room temperature while it does block the refolding of preMBP (37). The interaction of SecB with preMBP and MBP is typically described by a kinetic partitioning model (13). In such a model, folding of substrate to the native state is in kinetic competition with binding to SecB, and the difference in interaction with MBP and preMBP are exclusively ascribed to differences in folding rates of the two proteins.

However, the molecular mechanism by which the signal peptide slows down the folding of the protein has remained unclear. The present experiments suggest that the hydrophobic signal peptide interacts with hydrophobic regions of the mature protein that are exposed either in the unfolded state or in collapsed intermediate(s) along the folding/unfolding pathways. Such interactions will presumably slow the folding rate and speed the unfolding rate of the protein. This interpretation is supported by the observation that denaturant $m$ values for both urea and GdmCl mediated unfolding are appreciably lower in magnitude for preMBP relative to MBP. The appreciable destabilization of MBP by signal peptide is functionally relevant, because it enhances the likelihood of finding the protein in an unfolded translocation-competent form and may influence the interactions of the protein with the translocation machinery. From the effect of the signal peptide on the unfolded state of preMBP, it can be speculated that signal peptides may have a role in stabilizing the unfolded state of the pre-proteins. This can have a physiological significance in the export process. More evidence is required to substantiate this role of signal peptides on protein stability.

From a different perspective, the effect of the signal peptide on the mature domain of MBP also indicates that one cannot neglect the effect of the presence of unstructured stretches of amino acids at the termini of proteins as in the case of fusion proteins or tagged proteins. Destabilization of the native protein due to the presence of a single unprocessed methionine residue at the N-terminus has been reported in case of goat $\alpha$-lactalbumin expressed in $E.\ coli$ (38), bovine $\alpha$-lactalbumin (39), and MBP (40). Thus, the presence of an amino acid extension in protein may not affect its native state in terms of structure or activity, but depending on the nature of the sequence these can still have an appreciable effect on the unfolded state and hence on the stability of the protein.

**REFERENCES**


