Thermodynamic Characterization of the Reversible, Two-State Unfolding of Maltose Binding Protein, a Large Two-Domain Protein†

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ABSTRACT: The folding and stability of maltose binding protein (MBP) have been investigated as a function of pH and temperature by intrinsic tryptophan fluorescence, far- and near-UV circular dichroism, and high-sensitivity differential scanning calorimetric measurements. MBP is a monomeric, two-domain protein containing 370 amino acids. The protein is stable in the pH range of 4−10.5 at 25 °C. The protein exhibits reversible, two-state, thermal and guanidine hydrochloride-mediated denaturation at neutral pH. The thermostability of MBP is maximal at pH 6, with a T_m of 64.9 °C and a ΔH_m of 259.7 kcal mol^{-1}. The linear dependence of ΔH_m on T_m was used to estimate a value of ΔC_p of 7.9 kcal mol^{-1} K^{-1} or 21.3 cal (mol of residue)$^{-1}$ K^{-1}. These values are higher than the corresponding ΔC_p's for most globular proteins studied to date. However, the extrapolated values of ΔH and ΔS (per mole of residue) at 110 °C are similar to those of other globular proteins. These data have been used to show that the temperature at which a protein undergoes cold denaturation depends primarily on the ΔC_p (per mol of residue) and that this temperature increases with an increase in ΔC_p. The predicted decrease in stability of MBP at low temperatures was experimentally confirmed by carrying out denaturant-mediated unfolding studies at neutral pH at 2 and 28 °C.

A large number of spectroscopic and calorimetric studies have been made on the thermodynamics of folding of small (approximately 60−150 amino acids long) globular proteins (Privalov, 1979; Murphy & Friere, 1992). In contrast, there are fewer studies of larger proteins, in part because these often do not exhibit reversible denaturation, especially at the higher concentrations required for calorimetric experiments. The hydrophobic effect and the loss of conformational entropy are two major factors that determine the stability of the folded state of a protein. The free energy change of stabilization due to the hydrophobic driving force is thought to be proportional to the amount of nonpolar surface area buried upon protein folding. Since most globular proteins have similar packing densities (Richards, 1977), it might be expected that larger proteins, on average, bury a larger fraction of the total accessible surface area upon folding than smaller proteins (Chothia, 1984). The number of conformations accessible to the unfolded state of the protein also increases with an increase in chain length. In contrast, the conformational flexibility of the folded state is much less dependent on chain length. Hence, the conformational entropy decrease in protein folding is a function of the chain length. The thermodynamics of folding, and in particular the specific heat capacity change (ΔC_p)[1] of protein folding, might therefore be expected to be a function of protein size. Hence, it is important to characterize the folding thermodynamics and kinetics of proteins as a function of molecular weight. MBP is a 370-amino acid, periplasmic protein of *Escherichia coli* involved in chemotaxis toward and uptake of a range of maltose sugars. MBP is a large, single-chain, monomeric protein without disulfide bonds, prosthetic groups, bound metal ions, or cofactors. The crystal structures of MBP have been determined for both the unliganded (Sharff *et al*., 1992) and the maltose-bound states (Spurlino *et al*., 1991). These factors make it an attractive system for studying protein folding thermodynamics.

MBP is a well-characterized substrate of the cytosolic chaperone SecB. SecB binds to unfolded MBP in the *E. coli* cytoplasm and keeps the protein in an unfolded translocation competent state. The interaction between SecB and MBP involves competition between folding of MBP and binding of SecB to unfolded MBP. A detailed understanding of MBP folding thermodynamics is therefore also relevant to its binding to SecB and subsequent translocation into the periplasm. (Diamond *et al*., 1995; Khisty & Randall, 1995; Collier *et al*., 1988; Weiss *et al*., 1988). Denaturant-mediated unfolding studies have shown that the unfolding of MBP at near-neutral pH in the presence of GdnHCl or urea is a two-state process (Liu *et al*., 1988; Chun *et al*., 1993). In the present work, we have characterized the stability of MBP as a function of pH and temperature by intrinsic tryptophan fluorescence and circular dichroism spectroscopy and also by high-sensitivity differential scanning calorimetric measurements (Privalov & Potekhin, 1986; Freire, 1995; Plum

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[1] Abbreviations: MBP, maltose binding protein; EDTA, ethylenediaminetetraacetic acid; CGH1, citrate, glycine, and HEPES buffer (1 mM each); CGH10, citrate, glycine, and HEPES buffer (10 mM each); $T^{10}M^2$, 10 mM Tris-HCl (pH 8.0) with 2 mM magnesium chloride; GdnHCl, guanidine hydrochloride; OSF, osmotic shock fluid; CD, circular dichroism; C_p, excess heat capacity; ΔC_p, change in excess heat capacity; T_m, temperature of maximal excess heat capacity; ΔH_m, molar unfolding enthalpy at T_m; ΔH_{vH}, van't Hoff enthalpy; $\Delta\nu$, difference in the number of protons bound to unfolded MBP and folded MBP; N and U, native and unfolded states, respectively; f_U, fraction of unfolded molecules; ΔG_U, free energy change upon protein unfolding; pre MBP, precursor MBP with leader sequence.

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& Breslauer, 1995). DSC measurements provide direct estimates of two important thermodynamic parameters, ΔH and ΔC_p, that are obtained only indirectly from spectroscopic measurements. We have shown that both the pH unfolding at room temperature and thermal unfolding at neutral pH are highly reversible.

EXPERIMENTAL PROCEDURES

Materials. Maltose, PMSF (phenylmethanesulfonyl fluoride), fast flow Q-Sepharose, and Sephadex G-75 were from Sigma (Sigma Chemical Co., St. Louis, MO,). Ampicillin and HEPES were purchased from Amersham (Amersham International plc, U.K.). Ultrapure GdnHCl from GIBCO-BRL was used without further purification. All the other chemicals were obtained from local commercial sources and were of analytical reagent quality.

Strains and Plasmid. The *E. coli* K-12 strain harboring the plasmid pPD1 was used as the source of MBP. The plasmid pPD1 containing the wild-type *E. coli malE* gene and its *E. coli* host PD28 (Duplay *et al*., 1984; Martineau *et al*., 1990) [F$^-$ *flbB*5301 *deoC*1 *ptsF*25 *rbsR* Δ(*argF-lac*)-U169 *rpsL*150 *relA*1 *thiA araD*139 Δ*malE*444 *malT*^{c1} Δ(*srlR-recA*)306::*Tn*10] were provided by M. Hofnung.

Protein Purification. Mature MBP was isolated from *E. coli* cells employing the osmotic shock procedure (Kellermann & Ferenci, 1982). Cells were grown in LB media (supplemented with 100 μg mL^{-1} ampicillin) at 37 °C with vigorous shaking and harvested at 4500*g* for 20 min when $A_{600} = 1-1.1$. The osmotic shock competent cells were washed twice with one-tenth of the culture volume of OSF I (10 mM Tris, 30 mM NaCl, and 100 μM PMSF at pH 7.2). The washed pellet was then resuspended in one-twentieth of the culture volume of OSF II (30 mM Tris, 0.1 mM EDTA, 100 μM PMSF, and 20% w/v sucrose at pH 7.2), stirred at room temperature for 20 min, and then pelleted at 8500*g* for 20 min. The cell pellet was osmotically shocked by resuspending the cells in one-tenth of the culture volume of ice-cold OSF III (100 μM $MgCl_2$ and 100 μM PMSF) and stirred at 4 °C for 20 min. The suspension was centrifuged at 11000*g* for 20 min, and the supernatant so obtained is the OSF. Tris-HCl, $MgCl_2$, and PMSF were added to final concentrations of 10 mM, 2 mM, and 100 μM, respectively, and the pH was adjusted to 8 at 4 °C. All subsequent steps were carried out at 4−7 °C, unless otherwise stated. The OSF was loaded immediately onto a fast flow Q-Sepharose anion exchange column. The unbound material was washed off, and MBP was eluted with a linear (0 to 250 mM) NaCl gradient in $T^{10}M^2$ (pH 8). MBP-containing fractions were pooled, concentrated, and further purified on a G-75 gel filtration column. MBP-containing fractions were pooled, concentrated, extensively dialyzed against double-distilled water or CGH10 (pH 7.4) for long term storage, and then stored at −70 °C. The purified protein was estimated to be 99% pure by SDS−PAGE (Laemmli, 1970). Protein bands were detected by either Coomassie blue or silver staining. The protein gave a single peak when run on an analytical HPLC gel filtration BioSep-SEC-S2000 column (pH 7.4, 1 mL min^{-1} flow rate). Estimates of the molecular weight from electrophoresis and gel filtration data were in good agreement with that calculated for MBP from the known protein sequence.

Determination of the MBP Extinction Coefficient. For a 1 mg mL^{-1} solution of MBP, different A_{280} (1 cm path length) values have been reported. These include 1.47 (Kellerman & Ferenci, 1982), 1.7 (Gehring *et al*., 1991), and 1.94 (Miller *et al*., 1983; Sparrer *et al*., 1996). Hence, the ϵ_{280} of MBP was re-estimated using the method outlined in Gill and von Hippel (1989). For a 1 mg mL^{-1} MBP solution, A_{280} was determined to be 1.46, close to the value reported by Kellerman and Ferenci (1982).

Buffers and Sample Concentrations. The buffer CGH10 was used for fluorescence, calorimetric, and near-UV CD studies. The buffer CGH1 was used for far-UV CD studies. The buffer pH was adjusted to the desired value with either HCl or NaOH and the sample pH checked both before and after the experiments. All experiments involving GdnHCl were carried out in 10 mM HEPES at pH 7.4. Sample concentrations were 2 μM for fluorescence, 16.2 μM for near-UV CD, and 1.98 μM for far-UV CD experiments.

CD Measurements. The conformational changes in MBP induced by changes in pH were monitored by means of CD spectroscopy using a JASCO 720 or a JASCO 500 spectropolarimeter. Wavelength scans from 190 to 250 nm for 1.98 μM protein were performed in a 1 mm path length rectangular quartz cuvette at 21 °C at a scan speed of 10 nm min^{-1}. Data points were collected with a response time of 8 s per point and a band width of 2 nm. Each spectrum was an average of four consecutive scans. Buffer scans were accumulated under the same conditions and subtracted from the protein spectra before further analysis. Changes in the tertiary structure of MBP due to changes in pH were followed by measuring the CD in the 250−300 nm range of a 16 μM solution using a path length of 5 mm. Each spectrum was an average of two consecutive scans. The mean residue ellipticity is calculated as follows:

$$[\Theta]_{MRE} = [(100\Theta_{obs})/(dC)]/(n-1) \text{ (for far-UV CD)} \quad (1)$$

$$[\Theta]_{MRE} = [(100\Theta_{obs})/(dC)]/n \text{ (for near-UV CD)} \quad (2)$$

where $[\Theta]_{MRE}$ is the calculated mean residue ellipticity in units of deg cm^2 dmol^{-1}, $[\Theta]_{obs}$ the observed ellipticity in units of degrees, *d* the path length in centimeters, C the molar protein concentration, and *n* the total number of amino acid residues in MBP (Duplay *et al*., 1984).

Differential Scanning Calorimetry Measurements. DSC scans of the change in excess heat capacity of MBP as a function of temperature were measured using the MC-2 ultrasensitive scanning calorimeter. DSC measurements were carried out as a function of protein concentration, scan rate, and pH. All solutions used for DSC were degassed just before loading into the calorimeter. At each pH and scan rate, a buffer vs buffer baseline run was first obtained. Immediately after this, the buffer solution was removed from the sample cell and the cell was cleaned and dried. The protein sample was prepared by mixing appropriate volumes of stock solutions of protein (in distilled water), buffer, and distilled water to obtain the solution with the desired concentration and incubated till equilibrium was achieved under the particular set of conditions. This method of sample preparation was preferred to the conventional dialysis method for the following reason. At some of the extreme pHs examined in these studies, partial unfolding, aggregation, and deamidation of the protein occur during the extended times required for dialysis of the sample. As a control, overnight dialyses were performed at pH 7.4 and 4 and DSC runs were

carried out using the dialyzed samples. DSC scans were carried out with the protein solution in the sample cell and buffer in the reference cell. The sample pH was checked both before and after the scan. The thermodynamic parameters obtained with the dialyzed samples were identical to those obtained using the sample preparation method described above. The reversibilities of the thermal transitions were checked by rescanning the sample, and the percent reversibility was calculated after buffer subtraction using the following criterion

$$\% \text{ reversibility} = 100(\Delta H_{m,rescan}/\Delta H_{m,scan}) \qquad (3)$$

Data were primarily analyzed using the software EXAM: A Two-State Thermodynamic Analysis Program (Kirchhoff, 1993). Baseline buffer vs buffer scans were first subtracted from the protein vs buffer scan. Data at all pHs were fit assuming the calorimetric transitions to be two-state. The validity of this assumption was confirmed by the good agreement between the experimental and calculated curves. In the EXAM software, it is possible to use the number of moles of macromolecule present (n) in the cell as an adjustable parameter to improve the quality of the fit. If the fitted value of n deviates significantly from the known value, it indicates a departure from two-state behavior. In all the data analyzed by us, the value of n was kept fixed and a satisfactory fit to a single two-state transition was obtained. Data were also fit using the ORIGIN DSC software provided by Microcal Inc. The parameter values obtained were very similar to those obtained using EXAM, and we have therefore reported only the values obtained using the EXAM program.

Steady-State Fluorescence Measurements. All steady-state fluorescence measurements were carried out at 21−22 °C in a JASCO FP777 spectrofluorimeter using an excitation wavelength of 280 nm and a spectral band width of 5 nm for emission and 5 or 1.5 nm for excitation. All samples were incubated till equilibria were established under the particular set of conditions before measuring the steady-state fluorescence intensities.

Binding Assay and Estimation of the Equilibrium Constant for MBP−Maltose Binding. The binding of maltose to MBP was assayed fluorimetrically by observing a red shift and quenching in the intrinsic tryptophan fluorescence of MBP upon maltose binding (Szmelcman *et al*., 1976). Increasing amounts of maltose were added to a fixed concentration of MBP in the cuvette, and the decrease in fluorescence emission intensity at 335 nm was followed as a function of the added maltose concentration till saturation was achieved. The fluorescence intensities were corrected for dilution effects, and a Scatchard analysis of the corrected fluorimetric titration data was used to estimate the binding constant, assuming single-site binding.

Equilibrium Unfolding of MBP in GdnHCl. Experiments were carried out at both 28 and 2 °C by incubating 0.24 μM MBP in appropriate final concentrations of GdnHCl in 10 mM HEPES, at pH 7.4 till equilibria were established (Liu *et al*., 1988). GdnHCl concentrations were calculated from measurements of index of refraction. The fluorescence data (excitation at 280 nm, emission at 341 nm) were analyzed in terms of a two-state transition. The fraction of protein in the unfolded state, f_U, and the equilibrium constant for unfolding in the folding transition zone, K_{app}, were determined as described in Khurana *et al*. (1995). The free energy of unfolding of MBP in the absence of denaturant was calculated by assuming that the free energy of unfolding, ΔG_U, is linearly dependent on the denaturant concentration, [D] (Schellman, 1978):

$$\Delta G_U = \Delta G_U(H_2O) + m_G[D] \qquad (4)$$

where $\Delta G_U(H_2O)$ is the free energy of unfolding at zero denaturant concentration and m_G is the slope of the plot of ΔG_U vs [D].

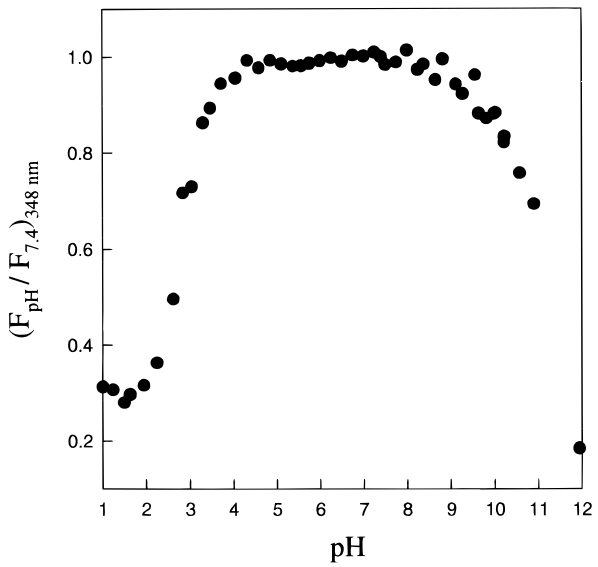

FIGURE 1: Ratio of the buffer-corrected fluorescence emission intensity of MBP at any pH to that of the native state at pH 7.4 as a function of pH. Emission was monitored at 348 nm, and excitation was at 280 nm. Each point is an average from four individual sets of experiments. The concentration of MBP was 2.07 μM.

RESULTS

Effect of pH on the Folding of MBP. The intrinsic tryptophan fluorescence of MBP has been previously used to follow the unfolding of the protein as a function of denaturant concentration at pH 7.8 and 25 °C (Liu *et al*., 1988; Chun *et al*., 1993). We have characterized the stability of MBP as a function of pH. The fluorescence emission intensity at 348 nm (the wavelength of maximal difference between the folded and unfolded forms) was monitored as a function of pH at 21−22 °C. Fluorescence intensities were normalized by calculating the ratio of the equilibrium fluorescence intensity at each pH to that at pH 7.4 and plotted as a function of pH (Figure 1). The normalized plot clearly reveals the presence of two major unfolding transitions, one in the acidic pH range of 2.5−3.5 and another in the alkaline pH range of 10−12. The acid-unfolded form exhibits a residual fluorescence, which is about 30% of the native-state fluorescence intensity. The reversibility of pH-induced unfolding was checked by jumping from a pH extreme to pH 8 and measuring the fluorescence intensity relative to that of a sample of native protein of an identical concentration maintained at pH 8.0. The refolding efficiency was estimated to be about 88% (data not shown).

Secondary and Tertiary Structure of MBP as a Function of pH and under Other Denaturing Conditions. Far-UV CD (190−250 nm) measurements serve as good reporters of the

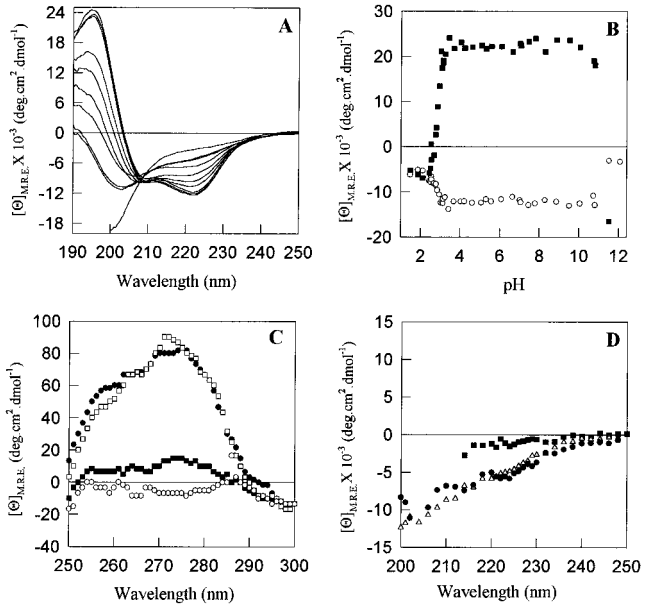

FIGURE 2: CD studies of MBP. (A) Far-UV CD as a function of pH at 21 °C. The different pH values are (from top to bottom at 200 nm) 7.14, 4.21, 3.02, 2.94, 2.84, 2.78, 2.53, 2.11, 1.86, and 12.07. (B) Mean residue ellipticities at 222 nm (○) and 197 nm (■) as a function of pH, at 21−22 °C. (C) Near-UV CD as a function of pH at 25 °C. The different pH values are (from top to bottom) 6 (□), 4 (●), 3 (■), and 2 (○). (D)Far-UV CD under the following denaturing conditions: pH 7.4 and 6 M GdnHCl (■), pH 2 (△) (both at 25 °C), and pH 7.4 and 75 °C (●).

secondary structural content of proteins under different conditions (Woody, 1995), while near-UV CD (250−300 nm) measurements are used to follow tertiary structural changes. The mean residue ellipticities of MBP in the far-UV range as a function of wavelength at different pHs are shown in Figure 2A. The presence of isodichroic points in Figure 2A suggests that the secondary structural transition at acidic pH is a two-state process. Figure 2B shows plots of the mean residue ellipticity as a function of pH at two different wavelengths of 222 and 197 nm. From pH 3 to 10.5, there is little change in protein secondary structure. However, in contrast, there is almost a complete loss of the near-UV CD signal (Figure 2C) at pH 3, suggesting that the acid-induced unfolding of the protein is non-two-state. The far-UV CD spectra of MBP under various denaturing conditions are shown in Figure 2D. The unfolded form of the protein in 6 M GdnHCl at pH 7.4 has the least secondary structure. Interestingly, the pH 2 and temperature-melted forms (T = 75 °C) appear to have some residual structure, and they exhibit similar CD profiles.

Differential Scanning Calorimetry. The energetics of MBP unfolding was characterized by examining the thermal stability of the protein under a variety of conditions employing high-sensitivity differential scanning calorimetric measurements. Figure 3 shows the buffer-corrected partial molar excess heat capacity data (open circles) for a 24.3 μM solution of MBP at pH 7.4 at a scan rate of 30 °C h^{-1}. The calculated baseline excess heat capacity of the system is indicated by a thick line. Also shown in the figure are the rescan profile (dotted curve) and a theoretical deconvolution curve (solid curve) generated from the baseline curve and the fitted parameters given in Table 1. Examination of areas under the scan and rescan reveal a high reversibility of 85%. The effects of different scan rates and protein concentrations

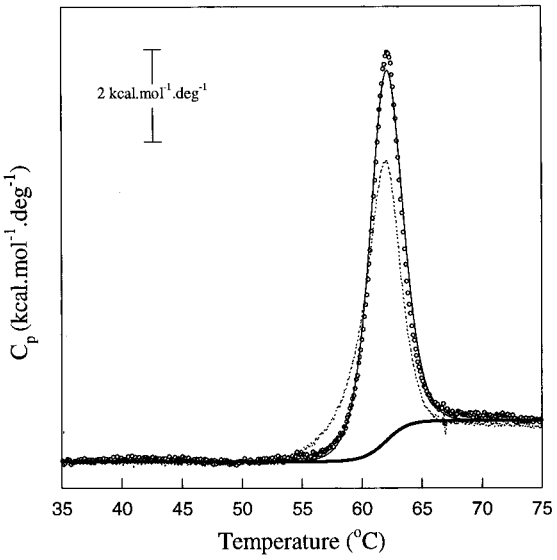

FIGURE 3: Representative DSC scan of 24 μM MBP at pH 7.4 at a scan rate of 0.5 °C min^{-1}. The data points are shown as open circles (○). The two-state fit to the data is shown as a thin solid line. The rescan data points are shown as a dotted line. and the calculated baseline for the transition is shown as a thick solid line.

Table 1: Thermodynamic Parameters for the Unfolding of MBP at pH 7.4

(a) As a Function of Protein Concentration (at a Scan Rate of 60 °C h^{-1})

concentration (mM)	T_m (°C)	ΔH_m (kcal mol^{-1})	% reversibility[a]
0.012	63.0	241.2	89
0.024	63.0	241.4	91
0.048	62.9	249.1	92

(b) As a Function of Scan Rate (at a Protein Concentration of 0.024 mM)

scan rate (°C h^{-1})	T_m (°C)	ΔH_m (kcal mol^{-1})	% reversibility[a]
10	62.1	229.7	71
30	62.0	243.9	85
60	63.0	241.4	91
90	63.4	227.5	91

[a] $(\Delta H_{m,rescan}/\Delta H_{m,scan}) \times 100$.

on the T_m, the ΔH_m, and the percent reversibility at pH 7.4 are summarized in Table 1. The data indicate that T_m and ΔH_m are relatively independent of scan rate. A scan rate of 60 °C h^{-1} and a protein concentration of 24.3 μM were decided as optimal, and all subsequent DSC studies were carried out at this concentration and scan rate. Under these conditions, the reversibility at pH 7.4 is 91%. Similar reversibilities and T_ms were obtained when the denaturation was monitored using either absorbance or fluorescence of MBP as a function of temperature (C. Ganesh and R. Varadarajan, unpublished observations). The variation in the values of ΔH_m in Table 1b was used to obtain an estimate of the error in ΔH_m of about 3% (±7.2 kcal mol^{-1}).

DSC scans were carried out as a function of pH in the pH range of 4.5−10.5, and the results are summarized in Table 2. In this pH range, CD and fluorescence spectroscopy indicate that the protein is folded at room temperature. Baseline-subtracted fits at the two pH extremes of 4.5 and 10.5 are shown in Figure 4. In all cases, the data are well fit by a two-state model. The van't Hoff enthalpy change

Table 2: Thermodynamic Parameters of MBP Unfolding as a Function of pH

pH	T_m (°C)	ΔH_m (kcal mol^{-1})	% reversibility[a]
4.5	62.3	201.2	—[b]
5.0	64.3	239.1	—[b]
6.0	64.9	259.7	—[b]
6.5[c]	63.0	248.5	35
7.0[c]	64.5	239.4	59
7.4[c]	63.0	241.4	91
8.0[c]	61.5	203.0	88
8.5[c]	60.0	173.6	97
9.0	59.0	201.2	90
9.5	57.0	179.0	89
9.9	54.9	155.6	—[b]
10.4	51.1	151.1	—[b]

[a] % reversibility is calculated as $(\Delta H_{m,rescan}/\Delta H_{m,scan}) \times 100$. [b] $\Delta H_{m,rescan}$ too low to be calculated by the two-state fit. [c] ΔC_p values calculated at these four pH values are 2.9, 5.6, 5.8, 6.6, and 10.0 kcal mol^{-1} K^{-1}, respectively. The average of these four values is 6.2 kcal mol^{-1} K^{-1}.

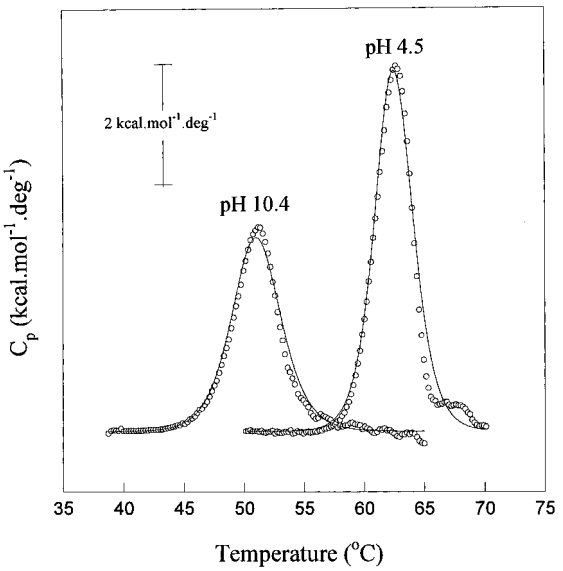

FIGURE 4: Baseline-subtracted DSC scans of 24 μM MBP at pH 4.5 and 10.43, scanned at a rate of 1 °C min^{-1}. The data points are shown as open circles (○). The two-state fit to the data is shown as a thin solid line.

$\Delta H_{\nu H}$ is normally estimated from the equation (Privalov, 1979)

$$\Delta H_{\nu H} = 4RT_m^2 C_{p(max)}/\Delta H_{cal} \quad (5)$$

where $C_{p(max)}$ is the maximal value of the excess heat capacity and ΔH_{cal} is the calorimetric enthalpy obtained by integration of the DSC curves. However, since the program EXAM carries out a two-state fit, ΔH_{cal} is equal to $\Delta H_{\nu H}$. This was confirmed by checking that $4RT_m^2C_{p(max)}$ was very close to ΔH_m^2 for all fits.

The p*I* of MBP was determined by isoelectric focusing and was 5.1, correlating well with the previously published value of 5.0 (Kellermann & Ferenci, 1982) and a theoretical estimate of 4.98, calculated from the published amino acid sequence of MBP using the software package PCGene. The reversibility of folding is low at pH values near the p*I* as the solubility of the protein is minimal at this pH. When ΔH_m is plotted as a function of T_m (Figure 5), the slope $(d\Delta H_m/dT_m)$ provides an estimate of ΔC_p. The plot is well fit with a straight line (correlation coefficient = 0.9). This

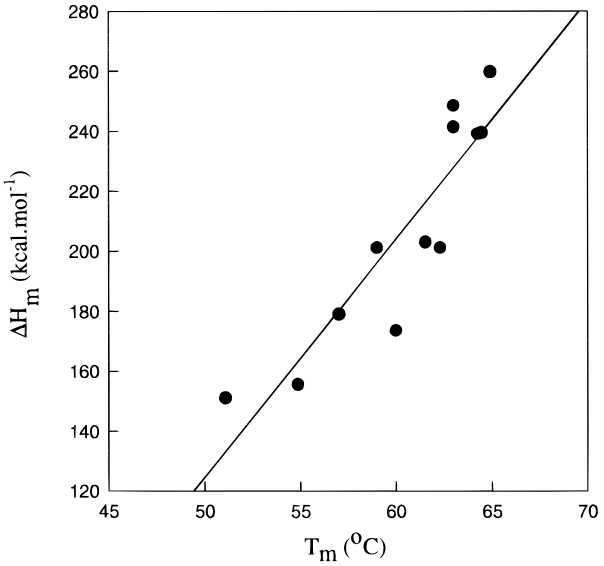

FIGURE 5: ΔC_p estimation from the linear dependence of ΔH_m on T_m. The ΔH_m and T_m values from Table 2 are fitted to a straight line. The slope yields a ΔC_p value of 7.9 ± 1.2 kcal mol^{-1} K^{-1}.

indicates that ΔC_p is independent of temperature within the temperature range of this study. The value of ΔC_p obtained from the slope of the plot is 7.9 ± 1.2 kcal mol^{-1} K^{-1}. We believe that this value of 7.9 kcal mol^{-1} K^{-1} is a better estimate of ΔC_p than the values of ΔC_p obtained from individual DSC scans as it is not subject to the large errors that arise from uncertainties in baseline determination in the latter method. Another important parameter, $\Delta\nu$, the difference in the number of protons bound to denatured and to native MBP at a given pH, can be calculated from the DSC data using the equation (Ptitsyn & Birshtein, 1969; Privalov & Ptitsyn, 1969)

$$\Delta\nu = (\Delta H_m/2.303RT_m^2)[dT_m/d(pH)] \quad (6)$$

The measurements of T_m as a function of pH were fit to a second-order polynomial function. The estimated regression coefficients from the fit were used to generate the slope $[dT_m/d(pH)]$ at different pH values. $\Delta\nu$ decreases monotonically from a maximal value of 0.8 at pH 4.5 to −1.8 at pH 10.4.

The values of T_m, ΔH_m, and ΔC_p obtained from DSC studies facilitated the estimation of free energy of unfolding of MBP at pH 7.4 as a function of temperature $[\Delta G°_U(T)]$, using the Gibbs−Helmholtz equation:

$$\Delta G°_U(T) = \Delta H_m(1 - T/T_m) + \Delta C_p\,[T - T_m - T\ln(T/T_m)] \quad (7)$$

with ΔH_m = 241.4 kcal mol^{-1}, T_m = 63 °C, and ΔC_p = 7.9 kcal mol^{-1} K^{-1} (Figure 6, solid line). In order to indicate the experimental uncertainty in the stability curves, we also show three additional stability curves (Figure 6, dashed lines) which were calculated using values of ΔH_m and ΔC_p that are within one standard deviation of the measured values of ΔH_m and ΔC_p indicated above. The temperature of maximal stability of the protein is calculated to be 34 ± 5.5 °C, with a maximal free energy of stabilization of 10.7 ± 2.2 kcal mol^{-1}. The cold denaturation temperature is predicted to be 5 ± 10 °C. MBP does not show cold denaturation at 5 °C at pH 7.4. However, cold denaturation of the protein was verified indirectly by comparing the stability of the

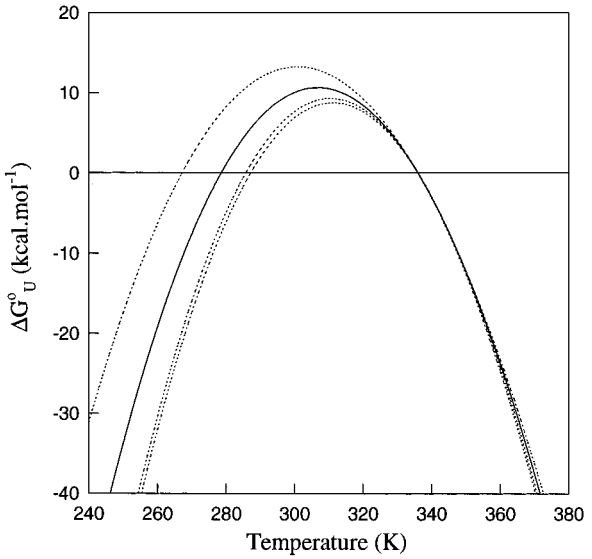

FIGURE 6: Free energy of unfolding (ΔG_U) of MBP as a function of temperature at pH 7.4 estimated from eq 7 using a value of T_m of 336.1 K. ΔG_U for the curve indicated by a solid line is estimated using a value of 7.9 kcal mol^{-1} K^{-1} for ΔC_p and 241.4 kcal mol^{-1} for ΔH_m. The effects of errors in the estimates of ΔC_p ($\pm$1.2 kcal mol^{-1} K^{-1}) and ΔH_m ($\pm$7.2 kcal mol^{-1}) on the shape of the stability curve are shown using dashed lines. The values of ΔC_p and ΔH_m used to generate the three dashed curves are as follows (in order of increasing temperature of cold denaturation): (a) ΔC_p = 6.7 kcal mol^{-1} K^{-1}, ΔH_m = 241.4 kcal mol^{-1}; (b) ΔC_p = 9.1 kcal mol^{-1} K^{-1}, ΔH_m = 248.6 kcal mol^{-1}; (c) ΔC_p = 9.1 kcal mol^{-1} K^{-1}, ΔH_m = 234.2 kcal mol^{-1}.

protein to GdnHCl denaturation at room temperature and at 2 °C as described below.

Binding of Maltose to MBP. The K_d for MBP−maltose binding at pH 7.4 (T = 21 °C) estimated by Scatchard analysis of the fluorimetric data was 0.9 ± 0.3 μM. The data were well fit by a single-site binding model (n = 1). This is in reasonable agreement with previously reported values obtained by equilibrium dialysis (2.2 μM) (Schwartz *et al*., 1976) and the concentration dependence of MBP fluorescence quenching by maltose (1 μM) (Szmelcman *et al*., 1976; Ferenci *et al*., 1986).

Denaturation of MBP by GdnHCl and Cold Denaturation of MBP. The free energy of unfolding of MBP in near-physiological conditions (pH 7.4, 28 °C) was estimated by the linear extrapolation of the GdnHCl two-state denaturation data and found to be 9.1 ± 1 kcal mol^{-1} with a C_m of 1 M and an m value of 9.2 ± 1 kcal mol^{-1} M^{-1} (Figure 7). This value is different from the previously published values for free energy change for unfolding of 12.8 kcal mol^{-1} using GdnHCl and 11 kcal mol^{-1} using urea (Diamond *et al*., 1995; Chun *et al*., 1993; Liu *et al*., 1988) but in close agreement with the value of 9.5 kcal mol^{-1} obtained recently by Betton and Hofnung (1996). The value of ΔG°_{U} at 28 °C estimated from eq 7 is 10.2 kcal mol^{-1} and is in reasonable agreement with the value obtained from GdnHCl denaturation studies. The cold denaturation of MBP at low temperatures was studied indirectly by examining the stability of MBP to GdnHCl denaturation at pH 7.4 and 2 °C. At the low temperature of 2 °C, C_m, ΔG°_{U}, and m values are reduced when compared to those at 28 °C and are about 0.75 M, 6.6 ± 0.8 kcal mol^{-1}, and 8.5 ± 1 kcal mol^{-1} M^{-1}, respectively, indicating the destabilization of MBP at lower temperatures (Figure 7).

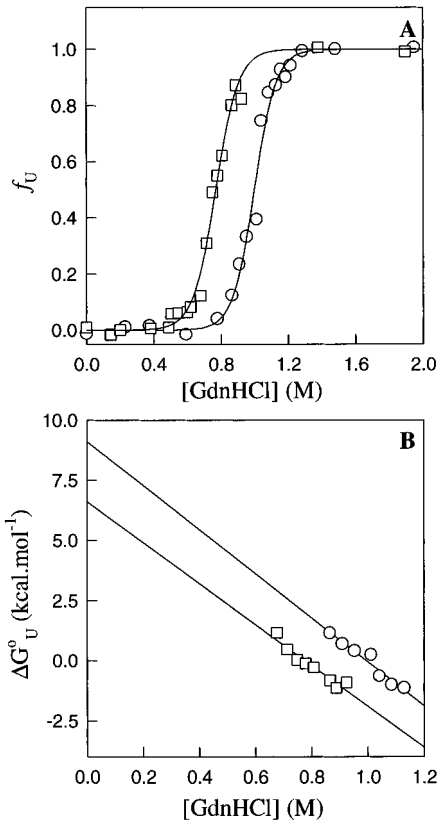

FIGURE 7: GdnHCl denaturation of MBP. (A) f_U as a function of GdnHCl concentration at 28 °C (○) and 2 °C (□). The data are fitted to a two-state denaturation process. (B) Linear extrapolation of the free energy of unfolding (ΔG_U) as a function of GdnHCl concentration at 28 °C (○) and 2 °C (□).

DISCUSSION

Thermodynamic Parameters Characterizing MBP Stability. We have characterized the stability of MBP as a function of pH at room temperature by fluorescence and CD spectroscopy. We have also characterized the thermal stability of MBP as a function of pH using DSC. At all pH values listed in Table 2, the thermal unfolding transitions could be fit using a two-state model. Moreover, within the pH range of 7−9.5, the thermal unfolding appears to be highly reversible. This is unusual for a protein the size of MBP. The free energy of unfolding of a protein can be obtained as a function of temperature, in principle, from a single DSC experiment by Gibbs−Helmholtz analysis of the DSC data for a system exhibiting reversible transitions. In practice however, it is difficult to determine the ΔC_p of unfolding accurately from a single scan. We have measured ΔH_m as a function of T_m by carrying out DSC scans at different pHs. We have estimated ΔC_p from the slope of a plot of ΔH_m vs T_m. The value of ΔC_p obtained by this method is considered a better estimate than that obtained directly as the difference between the N and U baselines in a single DSC experiment because the latter method is fraught with errors due to arbitrariness in baseline determination (Ladbury *et al*., 1994). The former method is based on the observation (Privalov & Khechinashvili, 1974) that, when corrected for ionization effects, the denaturation enthalpies of proteins were pH-independent with a pH-independent ΔC_p value over a wide pH range. Recently however, an exception was found in the case of staphylococcal nuclease, wherein ΔC_p was found to be dependent on pH (Carra *et al*., 1994). In the MBP system, we have

found that the ΔH_m vs T_m graph is well fit by a straight line over a range of T_m and pH values. This implies that ΔC_p is essentially pH- and temperature-independent over the range of T_m and pH values of this study.

Comparison of ΔC_p for MBP Unfolding with Those of Other Proteins. MBP is a 370-amino acid protein and is considerably larger in size than most proteins which display two-state reversible unfolding. It is therefore of interest to see whether it obeys the correlations between ΔC_p, ΔH, ΔS, protein size, and accessible area buried upon folding (ΔASA) that have been derived primarily from an analysis of thermodynamic data for the unfolding of smaller proteins. ΔC_p is the thermodynamic property most closely linked to the hydrophobic driving force and burial of nonpolar surface area. Recent studies (Ooi *et al*., 1987; Livingstone *et al*., 1991; Spolar *et al*., 1992; Gomez *et al.*, 1995; Myers *et al*., 1995; Khechinashvili *et al*., 1995) have attempted to correlate the value of ΔC_p with either the total accessible area (ΔASA) or the nonpolar accessible area (ΔASA_{np}) buried upon folding. According to Myers *et al*. (1995), ΔASA is linearly dependent on the number of residues (N_{res}) in the protein and ΔC_p is linearly dependent on ΔASA as follows:

$$\Delta ASA = -907 + 93N_{res} \quad (8)$$

$$\Delta C_p = -119 + 0.2\Delta ASA \quad (9)$$

Hence,

$$\Delta C_p = -300.4 + 18.6N_{res} \text{ cal mol}^{-1} \text{ K}^{-1} \quad (10)$$

Since $N_{res} = 370$ for MBP, we obtain a value of ΔC_p of 6.6 kcal mol^{-1} K^{-1} for MBP solely on the basis of its molecular size and eq 10. Myers *et al*. have also examined the correlation between *m* values obtained from GdnHCl denaturation experiments and ΔC_p values for several proteins. It was found that the two parameters are linearly related as follows:

$$\Delta C_p = -336 + 0.66m \quad (11)$$

Substituting the value of $m = 9.2$ kcal K^{-1} mol^{-1} M^{-1}, we obtain $\Delta C_p = 5.7$ kcal mol^{-1} K^{-1} which is lower than the experimentally determined value. The similarity of the *m* values observed for GdnHCl denaturation of MBP at 28 and 2 °C indicates that ΔC_p is relatively independent of temperature in this range. The experimentally determined value of 7.9 ± 1.2 kcal mol^{-1} K^{-1} for ΔC_p appears to be slightly higher than that predicted by the correlations of Myers *et al*. (1995), though this is within the 25% error estimate in the correlation (Spolar *et al*., 1992). However, it is also likely that the true ΔASA is not linearly dependent on molecular weight or N_{res} (Livingstone *et al*., 1991; Khechinashvili *et al*., 1995), and larger proteins might be expected to bury a larger fraction of ASA and ASA_{np} than smaller proteins

The estimated value of ΔC_p for MBP is 7.9 kcal mol^{-1} K^{-1} or 21.3 cal (mol of residue)$^{-1}$ K^{-1}. Both these values as well as the *m* value for GdnHCl denaturation are larger than those for most globular proteins for which thermodynamic data is available. The large molar heat capacity of MBP is partly a consequence of its high molecular weight. However, even the value of 21.3 cal (mol of residue)$^{-1}$ K^{-1} is considerably higher than the average value of 14.5 cal (mol of residue)$^{-1}$ K^{-1} observed for 45 proteins in the data set of Myers *et al*. (1995). One consequence of this large heat capacity is that MBP shows a higher than average predicted temperature of cold denaturation (Figure 6).

Comparison of ΔH and ΔS for MBP Unfolding with Those of Other Proteins. It has been noted that the $\Delta H°$ and $\Delta S°$ of denaturation (per mole of residue) of several globular proteins extrapolate to common values at temperatures of T_H and T_S, respectively, in the region of 100−112 °C (Privalov, 1979; Privalov & Gill, 1988; Murphy & Friere, 1992). According to Privalov and Gill (1988), both T_H and T_S are approximately equal to 110 °C and $\Delta H°$ and $\Delta S°$ are 1.49 kcal (mol of residue)$^{-1}$ and 4.21 cal K^{-1} (mol of residue)$^{-1}$, respectively, at 110 °C. According to Murphy and Friere (1992), the appropriate parameter values are 1.35 ± 0.11 kcal (mol of residue)$^{-1}$ at 100.5 °C for $\Delta H°$ and 4.3 ± 0.12 cal K^{-1} (mol of residue)$^{-1}$ at 112 °C for $\Delta S°$. The extrapolated values for MBP for $\Delta H°$ and $\Delta S°$ at these latter two temperatures using the measured values of ΔC_p, ΔH_m, and T_m at pH 7.4 are 1.45 kcal (mol of residue)$^{-1}$ and 4.8 cal K^{-1} (mol of residue)$^{-1}$, respectively, which are close to values seen for other proteins. If T_m is the temperature of heat denaturation of a given protein, an increase in the value of ΔC_p or a decrease in the value of ΔH_m of unfolding will result in an increase in the temperature of cold denaturation (Figure 6). Since all proteins appear to have similar values of $\Delta H°$ (per mole of residue) at about 100 °C, proteins with higher values of ΔC_p (per mole of residue) will have lower values of $\Delta H°$ (per mole of residue) at all temperatures below 100 °C (Murphy *et al*., 1990). Consequently, the cold denaturation temperature is expected to depend primarily on ΔC_p alone (not on both ΔC_p and ΔH_m) and to increase with an increase in ΔC_p.

ASA of MBP. MBP is one member of a large class of periplasmic binding proteins found in Gram-negative bacteria. These proteins are monomeric but have two distinct domains separated by a cleft. It has been previously shown by Janin (1976) that the ASA of a protein of molecular weight *M* is given by

$$ASA = 11.1M^{2/3} \quad (12)$$

We recalculated the ASAs of the proteins in the data set of Janin using the 1984 version of the program ACCESS (Lee & Richards, 1971). This uses a different set of radii from those of Janin (1976), and consequently, we obtain a slightly altered equation:

$$ASA = 10.3M^{0.68} \quad (13)$$

The ASA of MBP calculated using eq 13 is close to the experimentally observed value of 15 429 Å.[2] Thus, the fact that there are two domains does not result in an unusual value of the ASA.

Cooperativity of MBP Unfolding. The present studies show that MBP appears to unfold as a single cooperative unit, and there is no stepwise domain unfolding. Thermal as well as GdnHCl denaturation appear to be well described by a two-state model. The GdnHCl-mediated unfolding is also characterized by a high degree of cooperativity as indicated by the high *m* value for the transition. Two-state thermal unfolding has been previously observed for another monomeric, two-domain, periplasmic binding protein, arabinose binding protein (Fukada *et al*., 1983). In contrast, yeast

hexokinase, which is also a monomeric, two-domain protein that binds glucose, exhibits two peaks during thermal denaturation in the absence of glucose (Takahashi *et al*., 1981). Two other proteins with sizes similar to that of MBP have been previously characterized by DSC. These are pepsinogen (370 amino acids) and phosphoglycerate kinase (415 amino acids). Pepsinogen shows a reversible but non-two-state thermal transition (Mateo & Privalov, 1981), while phosphoglycerate kinase does not show a reversible thermal transition in the absence of denaturant. However, in the presence of 0.7 M GdnHCl, this protein shows reversible cold as well as heat denaturation (Griko *et al*., 1989).

Relevance of Present Studies to MBP−SecB Interaction Studies. MBP belongs to a class of proteins in *E. coli* which depend on the *sec* machinery for translocation across the inner membrane. The soluble cytosolic chaperone SecB binds to pre MBP and maintains the protein in an unfolded translocation competent state (Freedman, 1992; Collier *et al*., 1988; Weiss *et al*., 1988). There are two distinct views regarding the role of the leader sequence in the SecB−pre MBP interaction. According to one view, there is no specific recognition of the leader peptide by SecB. Instead, the primary effect of the leader sequence is to slow the rate of folding of pre MBP. Since SecB binds only to the unfolded protein, a reduced rate of MBP folding leads to an increase in the amount of bound MBP (Hardy & Randall, 1991; Randall *et al*., 1990). Another prevalent view is that SecB is analogous to the eukaryotic signal recognition particle, and it has been demonstrated that SecB exhibits high-affinity binding for the signal sequence region of pre MBP (Watanabe & Blobel, 1995). Additional support for this view is provided by a recent, stopped-flow study of the interaction between SecB and a model substrate which demonstrated that the rate of substrate binding was diffusion-controlled. These results suggest that the slowing of the MBP folding rate by the signal sequence would not significantly affect the amount of SecB-bound product (Fekkes *et al*., 1995). The relevance of the present studies to this issue is as follows. A method often used to study MBP−SecB interaction has been dilution of unfolded MBP into a solution containing SecB at 5 °C and monitoring of the blockage of folding of MBP by fluorescence spectroscopy (Liu *et al*., 1989). Blockage of folding is seen at low temperatures but not at room temperature. This has been thought to be due solely to the reduction in the rate of refolding of MBP at low temperatures. However, the present work shows that at 5 °C MBP is destabilized by about 2.5 kcal mol^{-1} over the 25 °C form (Figure 7). Hence, the blockage of refolding of MBP by SecB at low temperatures is not solely due to altered kinetics of refolding but may also be related to the reduced stability of the folded state at this temperature. Further studies of the thermodynamic and kinetic aspects of the pre MBP−SecB and MBP−SecB binding systems are required to clarify this point.

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NOTE ADDED IN PROOF

While this paper was under review, a related study of the thermodynamics of unfolding of a maltose binding protein variant with 16 additional C terminal amino acids was published (Novokhatny & Ingham, 1997).

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