Denaturant mediated unfolding of both native and molten globule states of maltose binding protein are accompanied by large ΔC_p 's

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Abstract

Maltose binding protein (MBP) is a large, monomeric two domain protein containing 370 amino acids. In the absence of denaturant at neutral pH, the protein is in the native state, while at pH 3.0 it forms a molten globule. The molten globule lacks a tertiary circular dichroism signal but has secondary structure similar to that of the native state. The molten globule binds 8-anilino-1-naphthalene sulfonate (ANS). The unfolding thermodynamics of MBP at both pHs were measured by carrying out a series of isothermal urea melts at temperatures ranging from 274–329 K. At 298 K, values of ΔG° , ΔC_p , and C_m were 3.1 ± 0.2 kcal mol⁻¹, 5.9 ± 0.8 kcal mol⁻¹ K⁻¹ (15.9 cal (mol-residue)⁻¹ K⁻¹), and 0.8 M, respectively, at pH 3.0 and 14.5 ± 0.4 kcal mol⁻¹, 8.3 ± 0.7 kcal mol⁻¹ K⁻¹ (22.4 kcal (mol-residue)⁻¹ K⁻¹), and 3.3 M, respectively, at pH 7.1. Guanidine hydrochloride denaturation at pH 7.1 gave values of ΔG° and ΔC_p similar to those obtained with urea. The m values for denaturation are strongly temperature dependent, in contrast to what has been previously observed for small globular proteins. The value of ΔC_p per mol-residue for the molten globule is comparable to corresponding values of ΔC_p for the unfolding of typical globular proteins and suggests that it is a highly ordered structure, unlike molten globules of many small proteins. The value of ΔC_p per mol-residue for the unfolding of the native state is among the highest currently known for any protein.

Keywords: heat capacity; molten globule; protein stability

Most data for protein folding thermodynamics come from studies of small single domain proteins. However, the magnitudes of the various driving forces for protein folding are size dependent (Ganesh et al., 1997). Hence it is desirable to characterize protein folding thermodynamics and kinetics as a function of protein size. Maltose binding protein (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). Unlike other large proteins, MBP undergoes reversible unfolding by either chemical denaturants such as urea and GdnHCl, or with temperature (Ganesh et al.,

1997). These factors make it an attractive system to study the protein folding thermodynamics of large proteins.

Molten globule states of proteins are compact states that have native-like secondary structure, more exposed hydrophobic surface than the native state, and possibly a native-like tertiary fold (Dolgikh et al., 1981; Ohgushi & Wada, 1983; Kuwajima, 1989). Such states are found both at equilibrium and as kinetic intermediates in the folding (Dolgikh et al., 1984; Arai & Kuwajima, 1996; Raschke & Marqusee, 1997). In a few cases it has been shown that the kinetic and equilibrium molten globules for a given protein have similar properties (Jennings & Wright, 1993; Ikeguchi et al., 1997). Molten globule states are stabilized by acidic pH and high salt. In several molten globules, only specific regions of the protein are structured (Baum et al., 1989; Jeng et al., 1990; Hughson et al., 1990). While it was initially thought that all molten globules lack specific tertiary interactions, recent studies (Redfield et al., 1994; Marmorino & Pielak, 1995; Morozova et al., 1995; Kay & Baldwin, 1996; Marmorino et al., 1998) have shown that specific native-like interactions can also be present in molten globules. Equilibrium molten globules are an important tool to study protein folding because they are much easier to structurally characterize than kinetic intermediates. In this paper we have characterized the thermo-

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Abbreviations: ANS, 8-anilino-1-naphthalene sulfonate; CD, circular dichroism; CGH10, 10 mM each of citrate, glycine, and HEPES buffer; EDTA, ethylenediaminetetraacetic acid; GdnHCl, guanidine hydrochloride; MBP, maltose binding protein; UV, ultraviolet.

dynamic stability of native MBP as well as a molten globule form of MBP by carrying out denaturant mediated unfolding studies as a function of temperature. The thermodynamic parameters show interesting differences relative to those of small globular proteins. The molten globule of MBP appears to be highly ordered and may represent a late stage in the folding pathway of MBP.

Results and discussion

MBP forms a molten globule at pH 3

It has previously been shown (Ganesh et al., 1997) that at pH 3 the protein shows a substantial loss of tertiary structure but has native-like secondary structure. ANS binding to MBP as a function of pH in the pH range 1–12 was monitored by fluorescence spectroscopy and the results are summarized in Figure 1, which shows that at pH 3 there is maximal binding of ANS to MBP. Above pH 4 there is no binding of ANS to MBP. Thus MBP at pH 3 appears to satisfy the criteria for a molten globule (Dolgikh et al., 1981; Ohgushi & Wada, 1983; Kuwajima, 1989; Ptitsyn, 1996). Further lowering of the pH to the acid unfolded state is accompanied by a loss of secondary structure as well as a reduction in ANS binding affinity.

The affinity of the molten globule and acid unfolded states for maltose was characterized by a fluorimetric titration as described previously (Ganesh et al., 1997). Surprisingly, the dissociation constant at pH 3 was $0.7 \mu\text{M}$ and was quite comparable to the value of $1.0 \mu\text{M}$ measured at pH 7.4 for the native state of the protein. However, there were no changes in tertiary structure as monitored by near UV-CD upon maltose binding either at pH 3 or at pH 7 (data not shown). In contrast, at pH 2.0 the affinity of MBP for maltose was too low to be detected.

Fig. 1. ANS binding studies in the pH range 1–12. ANS binding was monitored by a change in fluorescence at 472 nm with excitation at 380 nm. The fluorescence due to free ANS and free MBP were subtracted from the mixture of ANS and MBP at each recorded pH.

Fig. 2. Pepsin digestion of MBP as a function of pH: (1) molecular weight markers; (2) pH 2, MBP, 0 h, time; (3) pH 2, MBP, 8 h; (4) pH 3, MBP, 8 h; (5) pH 4, MBP, 8 h; (6) pH 2, MBP + maltose, 8 h; (7) pH 3, MBP + maltose, 8 h; and (8) pH 4, MBP + maltose, 8 h.

Limited proteolysis is another useful tool for characterizing partially folded states of proteins (Fontana et al., 1997). MBP was further characterized by pepsin digestion (Fig. 2) in the pH range 2–4. The activity of pepsin over this pH range was monitored using a control substrate, S-protein. S-protein is a proteolytic fragment of RNase A (Richards & Vithayathil, 1959) and the enzyme was found to be active over this entire pH range. At pH 2, MBP was completely digested both in the absence and presence of maltose (Fig. 2, lanes 3 and 6). At pH 3, after 8 h, the protein was digested in the absence of maltose but was partially protected in the presence of maltose (Fig. 2, lanes 4 and 7). After 4 h of digestion, in the absence of maltose, there was substantial protection at pH 3, whereas at pH 2 the protein was completely digested (data not shown). At pH 4 after 8 h, the protein was partially protected in the absence of maltose and was completely resistant to pepsin in the presence of maltose (Fig. 2, lanes 5 and 8). The increased resistance to proteolysis at pH 3 relative to pH 2 indicates the molten globule is less flexible than the acid unfolded state. The partial protection offered by maltose at pH 3 but not at pH 2 confirms that MBP binds maltose in the molten globule but not in the acid unfolded state. This suggested that the molten globule state of MBP is highly ordered, unlike most other molten globules that have been studied to date. This assertion was further confirmed by denaturation data for the MBP molten globule described below.

Denaturation studies at pH 7.1 and pH 3

The stability of MBP was monitored by carrying out isothermal chemical denaturation studies at each of the above pHs. Denaturation at both pHs was found to be reversible. At pH 7.1 both urea and GdnHCl were used to denature the protein. At pH 3 only urea denaturation was carried out. Denaturation studies with GdnHCl could not be carried out at pH 3 because the reduced stability of the protein at this pH resulted in a very short native baseline and an inadequate number of points in the transition zone. Denaturation studies were carried out as a function of temperature as described

previously (Ganesh et al., 1997). Data from a few representative denaturation melts are shown in Figure 3. At pH 7.1, denaturation was monitored by fluorescence and at pH 3.0 by far UV-CD. At both 299 K, pH 7.1 (Fig. 3B) and at 316 K, pH 7.1 (data not shown), the denaturation was monitored both by fluorescence and by far UV-CD. The coincidence of the two curves under both these conditions suggests that denaturation at neutral pH is a two state process. Thermal melts of MBP at pH 7.1 monitored by both fluorescence and far UV-CD also show coincident transitions (C. Ganesh & R. Varadarajan, unpubl. results). At pH 3.0 it was not possible to use either fluorescence or near UV-CD to monitor the unfolding, as there is little change in either of these observables upon denaturation of the molten globule. The set of denaturation curves for a given denaturant and pH were analyzed using a two state model as described in Materials and methods. At pH 3.0 the

Fig. 3. **A:** Representative GdnHCl induced denaturation curves at pH 7.1: 318 K ($\diamond$), 312 K ($\circ$), 309 K ($\square$), and 298 K ($\triangle$). The unfolding transition was monitored by a change in fluorescence at 342 nm with excitation at 280 nm. **B:** Representative urea induced denaturation curves at pH 7.1: 319 K ($\triangle$), 303 K ($\square$), 313 K (∇), and 299 K ($\circ$). The unfolding transition was also monitored by CD at 222 nm at 299 K ($\bullet$). **C:** Representative urea induced transitions at pH 3: 303 K ($\circ$), 300 K ($\square$), 296 K ($\diamond$), and 287 K ($\triangle$). The unfolding transition was monitored at 222 nm by CD.

unfolding thermodynamics was also characterized using differential scanning calorimetry (DSC). Although a cooperative transition with a T_m of 41 °C was observed, the transition was irreversible.

The values of ΔG° , m , and C_m at each temperature for all proteins are indicated in Table 1. The values of ΔG° as a function of temperature are fit to Equation 4 with T_0 equal to 298 K to obtain values of $\Delta G^\circ(T_0)$, $\Delta H^\circ(T_0)$, and ΔC_p , listed in Table 2. At both neutral and acidic pH, protein stability decreases significantly at low temperature. At each temperature, the denaturation data were analyzed to obtain the value of ΔG_D as a function of denaturant and temperature. Shown in Figure 4 are values of ΔG_D as a function of temperature for a series of different denaturant concentrations. Each stability curve in Figure 4 was fit to Equation 2 to obtain values of T_m , $\Delta H_D(T_m)$, and ΔC_{pD} . These values of $\Delta H_D(T_m)$ and ΔC_{pD} were used to calculate ΔH_D at all temperatures used for the isothermal denaturation experiments (Table 1). The calculated values of ΔH_D at each temperature were fit to Equation 1 to obtain ΔH° and ΔH_i as a function of temperature. Values of $\Delta H^\circ(T)$ were fit to Equation 3 to obtain ΔC_p . Values of ΔC_{pD} obtained from the fits in Figure 4 were also fit to Equation 1 to obtain alternate estimates of ΔC_p and ΔC_{pi} (Table 2).

Comparison of MBP unfolding thermodynamics at neutral pH with those of small globular proteins

The thermodynamic parameters from this paper were compared with those obtained earlier by calorimetry (Table 2). Three different methods of fitting the data were used to derive estimates of ΔC_p and all of these gave identical values of ΔC_p . Both chemical denaturation and calorimetric experiments resulted in similar values of ΔC_p and ΔG° at 298 K, though the calorimetric values were slightly lower than those obtained in the present chemical denaturation studies. The fact that both urea and GdnHCl denaturation gave similar values for ΔC_p and ΔG° validates the use of the linear extrapolation procedure for this system. The values of ΔC_p for MBP are much higher than those of smaller globular proteins even if ΔC_p is measured on a per mol-residue basis. The average value of ΔC_p from all the chemical denaturation studies at pH 7.1 is 8.53 kcal mol⁻¹ K⁻¹ or 23.06 kcal (mol-residue)⁻¹ K⁻¹. This is one of the highest values measured for any globular protein to date. This confirms our earlier assertion (Ganesh et al., 1997) that larger proteins are expected to have larger values of ΔC_p (per mol-residue) because they bury a larger fraction of the total surface area upon folding than smaller proteins. For comparison, thermodynamic data for three small globular proteins are summarized in Table 3.

The values of ΔH° and ΔS derived from urea and GdnHCl denaturation are somewhat different from each other. The calorimetric estimate of ΔH° at 298 K has a large associated error because the calorimetric measurements of ΔH° were made at much higher temperatures (337 K) and large extrapolations are required to calculate the value of $\Delta H^\circ(298)$.

The analysis also yielded values for m (ΔG_i), ΔC_{pi} , ΔH_i , and ΔS_i . The parameters provide insight into the differential interaction of the folded and unfolded states of MBP with denaturant. As expected, the absolute value of all these parameters is higher for GdnHCl. The m value for both urea and GdnHCl denaturation was observed to be temperature dependent (Table 2). This is in sharp contrast to what has been observed for a number of small proteins where the value of m was relatively independent of temperature (Agashe & Udgaonkar, 1995; Nicholson & Scholtz, 1996; Chiti

Table 1. Parameters obtained from isothermal denaturation studies as a function of temperature^a

Urea (pH 7.1)				GdnHCl (pH 7.1)				Urea (pH 3.0)			
<i>T</i>	ΔG°	$-m$	C_m	<i>T</i>	ΔG°	$-m$	C_m	<i>T</i>	ΔG°	$-m$	C_m
277	9.0 ± 0.4	2.8 ± 0.1	3.20	274	5.8 ± 0.6	8.0 ± 0.9	0.73	276	1.6 ± 0.3	2.0 ± 0.2	0.80
282	9.9 ± 0.8	3.2 ± 0.3	3.09	284	10.5 ± 0.6	10.7 ± 0.6	0.97	282	2.4 ± 0.4	2.2 ± 0.4	1.09
287	11.7 ± 1.2	3.5 ± 0.4	3.34	293	12.0 ± 0.6	11.3 ± 0.5	1.06	287	3.5 ± 0.3	3.1 ± 0.2	1.12
293	14.6 ± 1.2	4.5 ± 0.3	3.24	298	12.6 ± 1.4	12.2 ± 1.2	1.03	291	4.0 ± 0.4	3.7 ± 0.5	1.08
299	14.7 ± 1.2	4.5 ± 0.3	3.27	304	12.0 ± 0.8	11.6 ± 0.8	1.03	296	3.5 ± 0.3	4.1 ± 0.3	0.83
303	15.8 ± 0.9	4.7 ± 0.5	3.36	309	9.3 ± 0.8	10.8 ± 0.9	0.87	299	3.2 ± 0.5	3.9 ± 0.6	0.82
306	14.9 ± 1.3	4.6 ± 0.4	3.24	313	8.4 ± 0.4	10.0 ± 0.5	0.84	300	2.8 ± 0.4	3.7 ± 0.4	0.76
307	14.7 ± 0.7	4.7 ± 0.2	3.13	318	4.9 ± 0.4	7.1 ± 0.8	0.69	303	2.2 ± 0.3	3.4 ± 0.4	0.60
310	12.1 ± 1.3	4.1 ± 0.4	2.95								
312	12.1 ± 1.3	4.2 ± 0.5	2.88								
313	10.2 ± 1.5	3.5 ± 0.5	2.91								
319	7.2 ± 0.6	3.4 ± 0.3	2.12								
328	2.8 ± 0.5	2.5 ± 0.3	1.13								
329	2.8 ± 0.4	2.7 ± 0.3	1.04								

^aUnits are as follows: *T* (K); ΔG° (kcal mol⁻¹); m (kcal mol⁻¹ M⁻¹); C_m (M).

et al., 1998), hence ΔC_{pi} is close to zero (Table 3). One possible explanation for the temperature dependence of m could be the presence of equilibrium intermediates during denaturation. This is unlikely for the following reasons: (1) all the denaturation data in the present work fit well to a two state model, (2) transitions monitored by either CD or fluorescence at neutral pH are coincident, and (3) values of ΔG° obtained from both urea and GdnHCl denaturations are identical. Earlier DSC studies over a wide range of pH and temperature (Ganesh et al., 1997) all fit well to a two state model. Finally although the DSC at pH 3 shows an irrevers-

ible transition, the calorimetric enthalpy of the transition is identical to that obtained from the urea denaturation studies (parameters summarized in Table 2).

The observation that m is temperature dependent in the case of MBP unfolding implies that ΔC_{pi} is nonzero. This was confirmed by the data in Table 2. A recent analysis observed a correlation between the values of ΔC_p and m for several proteins (Myers et al., 1995). This was thought to arise from the fact that both ΔC_p and m are proportional to the total nonpolar surface area exposed upon folding. However, such a correlation implicitly assumes that m is

Table 2. Summary of thermodynamic parameters at 298 K for MBP unfolding

Parameter ^a	Urea (pH 3.0)	Urea (pH 7.1)	GdnHCl (pH 7.1)	DSC ^b
ΔC_p^c	5.9 ± 0.8	8.3 ± 0.7	8.6 ± 0.5	7.9 ± 1.2
ΔC_p^d	6.1 ± 0.2	8.40 ± 0.04	8.56 ± 0.01	
ΔC_p^e	6.2 ± 0.5	8.7 ± 0.2	8.59 ± 0.03	
ΔC_{pi}^e	-2.60 ± 0.06	-1.70 ± 0.03	-5.10 ± 0.03	
ΔH^{oc}	44 ± 8	12 ± 6	36 ± 4	-59 ± 53
ΔH^{of}	44.5 ± 0.6	13 ± 1	35.4 ± 0.5	
ΔG^{oc}	3.1 ± 0.2	14.5 ± 0.4	12.5 ± 0.2	9.6 ± 3.5
ΔS^{og}	140 ± 20	0 ± 20	80 ± 10	
M	3.9 ± 0.6	4.5 ± 0.33	12 ± 1	
ΔH_i^e	-5 ± 1	1.4 ± 0.4	-24.0 ± 0.6	
ΔS_i^h	20 ± 40	8 ± 4	120 ± 20	
C_m	0.8	3.3	1	

^aUnits are as follows: ΔC_p (kcal mol⁻¹ K⁻¹); ΔC_{pi} (kcal mol⁻¹ K⁻¹ M⁻¹); ΔH° , ΔG° (kcal mol⁻¹); ΔS° (cal mol⁻¹ K⁻¹); m , ΔH_i (kcal mol⁻¹ M⁻¹); ΔS_i (cal mol⁻¹ K⁻¹ M⁻¹); C_m (M).

^bData from Ganesh et al. (1997).

^cFrom ΔG° vs. T plot (Equation 4).

^dFrom ΔH° vs. T plot (Equation 3).

^eFrom ΔC_{pD} vs. $[D]$ (Equation 1).

^fFrom Equation 1.

^gUsing equation $\Delta S^\circ = (\Delta H^{\text{oc}} - \Delta G^{\text{oc}})/298$.

^hUsing equation $\Delta S_i = (\Delta H_i - m)/298$.

Fig. 4. Stability curves derived from denaturations with GdnHCl (pH 7.1), urea (pH 7.1), and urea (pH 3) at different denaturant concentrations. **A:** GdnHCl (pH 7.1): at 0.5 M (●), 0.6 M (○), 0.7 M (■), 0.8 M (□), 0.9 M (▲), and 1.0 M (△). **B:** Urea (pH 7.1) at 2.5 M (○), 2.6 M (■), 2.8 M (□), 3.0 M (▼), and 3.2 M (▽). **C:** Urea (pH 3): at 0.5 M (●), 0.6 M (○), 0.7 M (■), 0.8 M (□), 0.9 M (▲), and 1.0 M (△). The solid lines are fits of the data to Equation 2.

independent of temperature. In the case of MBP, the m value changes by as much as 40% in each of the three denaturation studies (Table 1). In fact, for urea denaturation of MBP at pH 7.1, the cold denaturation occurs primarily because of a decrease in the m value at low temperature rather than a decrease in the value of C_m . The absolute values of m and ΔC_{pi} values for interaction of denaturants with MBP were considerably higher than for smaller proteins (Tables 2, 3). The negative value of ΔC_{pi} observed in this paper is also different from the positive values of ΔC_{pi} inferred

Table 3. Thermodynamic parameters for denaturation of three small proteins^a

	CspB	HPr	Barstar
ΔC_p	0.91 ± 0.07	1.42 ± 0.09	1.49
ΔH°	14.0	15.7	2.65
ΔS°	39.7	38.2	-6.34
Denaturant	Urea	Urea	GdnHCl
ΔC_{pi}	0.024 ± 0.073	-0.040 ± 0.035	0.053 ± 0.036
m	-0.62	-1.13 ± 0.04	-2.5 ± 0.02
ΔH_l	-0.05 ± 0.17	-1.4 ± 1.3	-2.9
ΔS_l	1.9 ± 0.60	-50 ± 40	-1.7

^aData for Csp, HPr, and Barstar are from Schindler and Schmid (1996), Nicholson and Scholtz (1996), and Agashe and Udgaonkar (1995), respectively. Units are as in Table 2.

from calorimetric studies of RNase A, lysozyme, and cyt *c* (Makhatadze & Privalov, 1992). The data in Table 3 also show that barstar and CspB have small positive values of ΔC_{pi} in contrast to the small negative value seen for HPr. At the present time the origins of these apparent differences is unclear.

Analysis of thermodynamic parameters at pH 3

There have been several attempts to measure the thermodynamics associated with denaturation of molten globules. In certain cases unfolding of the molten globule is accompanied by small or zero values of ΔH° and ΔC_p (Ogasahara et al., 1993). However, different results have been obtained even for the same protein by different investigators in the case of apo α -lactalbumin (Yutani et al., 1992; Xie et al., 1993) and apomyoglobin (Griko & Privalov, 1994; Nishii et al., 1995). In the case of equine cyt *c*, thermal denaturation (Kuroda et al., 1992) and titration calorimetry (Hamada et al., 1994) gave ΔC_p values of 0.38–0.57 kcal mol⁻¹ K⁻¹ for unfolding of the salt induced molten globule. In the case of yeast iso-1-cyt *c* (Marmorino et al., 1998), unfolding of the salt induced molten globule is accompanied by a ΔC_p of 0.78 kcal mol⁻¹ K⁻¹. This is 60% of the ΔC_p for unfolding of the native protein. However, in both these proteins, the conformation of the molten globule state may be constrained by the presence of a covalently attached heme group. In addition to these small proteins, the molten globules of two large proteins β -lactamase and Gln-tRNA synthetase (Goto & Fink, 1989; Das et al., 1995) have also been characterized but the values of ΔC_p , ΔG° , and ΔH° for these proteins were not measured.

Unlike most previously studied molten globules, denaturation of the molten globule of MBP is associated with large values of ΔC_p and ΔH° . At neutral pH MBP contains two discontinuous domains. The lack of tertiary CD in the molten globule state suggests that both these domains are unfolded at pH 3. The molten globule of MBP does not require high salt or very low pH conditions. MBP has no prosthetic groups, disulfide bonds, or cofactors so it is likely that the molten globule is stabilized primarily by hydrophobic interactions. The value of ΔC_p for unfolding of the molten globule is approximately 70% of that for the native state. Since the value of ΔC_p is thought to be proportional to the total amount of nonpolar surface area exposed upon unfolding (Spolar et al., 1992; Myers et al., 1995), this suggests that the molten globule is only

30% more unfolded than the native state of the protein. Hence it is likely to be stabilized by specific tertiary contacts. The value of ΔH° at 298 K for the molten globule is more positive than the corresponding value for the native state, suggesting that the decreased ΔG° of the molten globule is dominated by an increase in the value of ΔS for unfolding of the molten globule relative to that for the native state. The value of ΔS has two components, a positive component due to the increase in chain conformational entropy upon unfolding (ΔS_{conf}) and a negative component due to the exposure of hydrophobic surface (ΔS_{hydr}). Since the molten globule is expected to be conformationally more flexible than the native state, the value of ΔS_{conf} is expected to be smaller for the molten globule. Hence the increase in the total value of ΔS must arise from a decrease in the absolute value of ΔS_{hydr} , which in turn is consistent with a decrease in the amount of nonpolar surface area exposed upon unfolding inferred from the decreased value of ΔC_p .

The large values of ΔC_p , observable cold and heat denaturation, and tight maltose binding constant for the molten globule suggest that it is a highly ordered molten globule and may represent a late stage in the folding of MBP.

Materials and methods

Strains, plasmid, and protein purification

The *E. coli* K-12 strain harboring the plasmid pPD1 was used as a 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) were provided by M. Hofnung. Mature MBP was isolated from *E. coli* cells employing an osmotic shock procedure (Ganesh et al., 1997).

ANS binding measurements

All ANS binding fluorescence experiments were carried out at 21–22 °C in a JASCO FP777 spectrofluorimeter using an excitation wavelength of 380 nm, an emission wavelength of 472 nm, and spectral bandwidths of 5 and 1.5 nm for emission and excitation, respectively. The concentration of protein used was 0.55 μ M and the ANS concentration was 100 μ M.

Proteolytic digestion experiments

Proteolytic digestions of MBP in CGH10 at pH 2, pH 3, and pH 4 were performed at 25 °C using pepsin at a protease:substrate ratio of 1:10 (by weight). Sixty micrograms of MBP were digested in 50 μ L of CGH10 buffer adjusted to the desired pH. Digests were carried out both in the absence and presence of 180 mM maltose. At intervals 10 μ L was removed from the reaction mixture and proteolysis was arrested by adding 10 μ L of 100 mM Tris, pH 8.8 and 5 μ L of SDS-PAGE loading buffer (50 mM Tris-HCl, pH 6.8 containing 2.0% SDS, 0.1% bromophenol blue, and 5% β -mercaptoethanol). The samples were heated for 5 min in boiling water and subjected to SDS-PAGE electrophoresis followed by staining with Coomassie Brilliant Blue R250. The activity of pepsin at various pHs was checked by using the S-protein fragment of RNase A as a control substrate.

Equilibrium unfolding of MBP at neutral pH

Sample concentrations were 0.5 μ M for fluorescence, 16.2 μ M for near UV-CD, and 1 μ M for far UV-CD experiments. Protein con-

centrations were estimated as described (Ganesh et al., 1997). Denaturation experiments were carried out at various temperatures in the range 1–55 °C by incubating MBP in appropriate concentrations of either GdnHCl or urea in CGH10 at pH 7.1 until equilibria were established. In the urea denaturation studies at pH 7.1, the buffer also contained 150 mM KCl. Denaturant concentrations were calculated from measurements of the index of refraction.

Equilibrium unfolding of MBP at pH 3 in urea

Experiments were conducted at various temperatures (3–30 °C) by incubating 1 μ M MBP at pH 3 in appropriate final concentrations of urea in CGH 10, pH 3.0 until equilibria were established (~3 h). Urea concentrations were calculated from measurements of index of refraction. Denaturation was monitored using far UV-CD at 222 nm, as there is little change in fluorescence upon denaturation at pH 3.

Determination of the free energy of unfolding (ΔG_D) in the transition zone

The spectroscopic data were analyzed in terms of a two state transition. The fraction of protein in the unfolded state (f_U) and the unfolding free energy (ΔG_D) as a function of denaturant concentration were determined as described (Agashe & Udgaonkar, 1995).

Temperature and denaturant dependence of thermodynamic parameters

In accordance with the linear free energy model (Schellman, 1987; Chen & Schellman, 1989), it was assumed that values of the unfolding free energy (ΔG_D), enthalpy (ΔH_D), entropy (ΔS_D), and heat capacity (ΔC_{pD}) depend linearly on the denaturant concentration. Hence,

$$\Delta X_D = \Delta X^\circ + \Delta X_i [D] \quad (1)$$

where ΔX° ($X = G, H, S, C_p$) is the value of the thermodynamic parameter X at zero molar denaturant and ΔX_i are the changes in X associated with preferential interaction of the denaturant with the denatured state relative to the native state of the protein.

The values of ΔG° and m ($m = \Delta G_i$) at a given temperature can be estimated by fitting the measured values of ΔG_D to Equation 1. The denaturant concentration C_m at which ΔG_D is zero is given by $C_m = -\Delta G^\circ/m$. If it is assumed that both ΔC_p and ΔC_{pi} and hence ΔC_{pD} are independent of temperature, it can be shown that (Agashe & Udgaonkar, 1995)

$$\Delta G_D(T) = \Delta H_D(T_m)(1 - T/T_m) + \Delta C_{pD}(T - T_m - T \ln(T/T_m)) \quad (2)$$

$$\Delta H^\circ(T) = \Delta H^\circ(T_0) + \Delta C_p(T - T_0) \quad (3)$$

$$\Delta G^\circ(T) = \Delta H^\circ(T_0) - T\Delta S^\circ(T_0) + \Delta C_p(T - T_0 - T \ln(T/T_0)) \quad (4)$$

where T_0 is some reference temperature and T_m is the denaturation temperature.

All data were analyzed using the Sigmaplot scientific graphing software.

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