Thermodynamics of maltose binding protein unfolding

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Abstract

The maltose binding protein (MBP or MalE) of *Escherichia coli* is the periplasmic component of the transport system for malto-oligosaccharides. It is used widely as a carrier protein for the production of recombinant fusion proteins. The melting of recombinant MBP was studied by differential scanning and titration calorimetry and fluorescence spectroscopy under different solvent conditions. MBP exhibits a single peak of heat absorption with a $\Delta H^{cal}/\Delta H^{vH}$ ratio in the range of 1.3–1.5, suggesting that the protein comprises two strongly interacting thermodynamic domains. Binding of maltose resulted in elevation of the T_m by 8–15 °C, depending on pH. The presence of ligand at neutral pH, in addition to shifting the melting process to higher temperature, caused it to become more cooperative. The $\Delta H^{cal}/\Delta H^{vH}$ ratio decreased to unity, indicating that the two domains melt together in a single two-state transition. This ligand-induced merging of the two domains appears to occur only at neutral pH, because at low pH maltose simply stabilized MBP and did not cause a decrease of the $\Delta H^{cal}/\Delta H^{vH}$ ratio. Binding of maltose to MBP is characterized by very low enthalpy changes, ~ -1 kcal/mol. The melting of MBP is accompanied by an exceptionally large change in heat capacity, 0.16 cal/K-g, which is consistent with the high amount of nonpolar surface—0.72 Å²/g—that becomes accessible to solvent in the unfolded state. The high value of ΔC_p determines a very steep ΔG versus T profile for this protein and predicts that cold denaturation should occur above freezing temperatures. Evidence for this was provided by changes in fluorescence intensity upon cooling the protein. A sigmoidal cooperative transition with a midpoint near 5 °C was observed when MBP was cooled at low pH. Analysis of the melting of several fusion proteins containing MBP illustrated the feasibility of assessing the folding integrity of recombinant products prior to separating them from the MBP carrier protein.

Keywords: calorimetry; fluorescence; heat capacity; ligand binding; maltose; maltose binding protein; nonpolar surface area; protein folding; recombinant fusion protein; thermodynamics

The maltose binding protein (MBP) of *Escherichia coli* is the periplasmic component of the transport system for malto-oligosaccharide. It belongs to the family of binding proteins found in the periplasmic space of Gram-negative bacteria. MBP, the second largest protein of this family, has relative molecular mass of 40.6 kDa and consists of 370 amino acids (Duplay et al., 1984). Its overall structure revealed by X-ray crystallography, is divided by a deep groove into two distinct globular domains (Spurlino et al., 1991). Each of the two domains is built from secondary structural elements belonging to both the amino- and carboxyl-terminal halves of the protein. Therefore, it is of interest to determine whether these two interlaced domains are able to unfold independently or whether they would cooperate in their melting. The sugar-binding site is located in the groove between the two domains of MBP. The bound maltose is buried in the groove and almost completely inaccessible to solvent. The groove is heavily populated by polar and aromatic groups, many of which are involved in extensive hydrogen bonding and van der Waals interactions with maltose (Spurlino et al., 1991).

It is known from the crystallographic data that when maltose is bound, MBP undergoes a substantial conformational change in which the angle between the two domains changes by 35 degrees with closure of the cleft (Sharff et al., 1992).

MBP is widely used as a carrier protein for the production of recombinant fusion proteins in *E. coli* (Guan et al., 1988). The presence of MBP facilitates purification by virtue of its affinity for solid-phase amylose. The protein of interest can then be liberated through enzymatic cleavage of a protease-sensitive linker region. In some cases, it is possible to address questions of interest by working directly with the fusion protein without the added step of cleavage and further purification. In either case, it is important to assess the structural integrity of the recombinant product before drawing conclusions about function, especially when negative results are obtained. Such results could be due to the genuine lack of activity in the expressed product or, alternatively, to the failure of the bacteria to produce a properly folded product. Also, the activity in the fusion protein could be affected by potential interaction between MBP and the protein of interest (Blondel et al., 1996).

In the present paper, we describe the results of an investigation of the thermodynamics of MBP unfolding and the effects of maltose on its thermodynamic behavior. We also illustrate here the

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value of differential scanning calorimetry for assessing the structural integrity of different fusion proteins.

Results

When melted in the calorimeter, MBP exhibits a single peak (Fig. 1A) with a $\Delta H^{cal}/\Delta H^{vH}$ ratio of 1.3–1.5 (Table 1) indicating that it comprises two strongly interacting domains. All of the endotherms obtained for MBP were highly reversible as judged by the fact that similar curves were produced when the samples were heated a second time (Fig. 1A, dashed curve). Binding of maltose increased the T_m by 8–14 °C, depending on pH (curve 2 and Table 1). The presence of ligand, in addition to shifting the peak to higher temperature, decreased the $\Delta H^{cal}/\Delta H^{vH}$ ratio to almost unity, indicating that the melting process becomes more cooperative when ligand is bound. This ligand-induced thermodynamic “merging” of the two domains does not occur at low pH, where maltose simply stabilizes the protein while maintaining or slightly increasing the $\Delta H^{cal}/\Delta H^{vH}$ ratio.

Fig. 1. A: Typical DSC endotherm of MBP pH 8.3 in 50 mM glycine buffer in the absence (curve 1) and presence (curve 2) of 50 mM maltose. Protein concentration was 0.7 mg/mL; scan rate, 1 K/min. After completion of scan 1, the solution was cooled in the calorimetric cells and then rescanned to obtain the dashed curve, to determine reversibility. B: DSC of MBP obtained at different pH values in 50 mM glycine buffer. Protein concentration range was 0.56–0.73 mg/mL; scan rate, 1 K/min; maltose, 50 mM. Dashed line represents the temperature dependence of C_p for the unfolded state calculated from the sequence as described in Makhatazde and Privalov (1990).

Table 1. Thermodynamic parameters of MBP unfolding with and without 50 mM maltose

Condition	T_m^a (°C)	ΔH^{cal} (kcal/mol)	ΔH^{vH} (kcal/mol)	$\Delta H^{cal}/\Delta H^{vH}$
pH 8.3	62.6	206	145	1.42
pH 8.3 + maltose	72.1 (+9.5)	226	225	1.0
pH 8.2	62.3	195	145	1.34
pH 8.2 + maltose	70.5 (+8.2)	239	210	1.14
pH 3.4	56.1	192	134	1.43
pH 3.4 + maltose	68.4 (+12.3)	281	164	1.7
pH 3.1	52.6	172	126	1.37
pH 3.1 + maltose	65.0 (+12.4)	210	143	1.47
pH 3.0	50.7	161	122	1.32
pH 3.0 + maltose	62.5 (+11.8)	201	135	1.49
pH 2.8	45.8	140	108	1.3
pH 2.8 + maltose	59.3 (+13.5)	218	136	1.6

^a T_m values are accurate to within 0.2 °C; numbers in parentheses give the shift in T_m caused by presence of maltose. Enthalpy values are accurate to within 10%.

As seen in Figure 1B, the position and magnitude of the heat-absorption peak for MBP is very sensitive to pH. Its melting temperature shifted upward by almost 30 °C upon increasing pH from 2.7 to 8.3. Below pH 2.7, MBP is completely unfolded, judging by the disappearance of the heat absorption peak and by the temperature dependence of C_p , which is very close to the theoretical one for the unfolded protein, calculated from the amino acid composition (Makhatazde & Privalov, 1990). The difference in C_p between the folded and unfolded states is unusually large. To obtain a more precise ΔC_p value, the specific melting enthalpies of MBP obtained at different solvent conditions were plotted versus the melting temperatures (Fig. 2). All the data taken together fall on a straight line which, when extrapolated to 110 °C, gives a specific enthalpy value of 13 cal/g—characteristic for typical globular proteins (Privalov, 1979). The slope of this line is equal to 0.16 cal/

Fig. 2. Specific enthalpy of melting of MBP at various solvent conditions plotted against the melting temperature. Straight line on the plot represents a fit to experimentally obtained data.

K-g, confirming that the melting of MBP is accompanied by an exceptionally large change in heat capacity. This suggests that a high fraction of its nonpolar residues are buried in the folded interior, becoming exposed upon denaturation.

In order to determine the proportion of hydrophobic groups that are buried, we calculated the accessible surface area (ASA) of nonpolar residues in the folded and unfolded states of MBP using the program Quanta. The ASA of the unfolded state was taken as the sum of the areas of each nonpolar residue in an extended Gly-X-Gly environment (Miller et al., 1987). The difference between nonpolar ASA of MBP in the folded and unfolded states turned out to be 29,580 Å² or 0.72 Å²/g. This value is one of the highest among all proteins calculated. When this value is placed on a plot of the experimentally-obtained versus calculated Δ ASA values for several globular proteins (Fig. 3), it predicts a value of ΔC_p equal to 0.163 cal/K-g; very close to that obtained from the experimental data.

The high value of ΔC_p determines a very steep ΔG versus T profile for MBP. As seen in Figure 4, each curve intercepts the x -axis twice in the positive temperature range, predicting that cold denaturation should occur above freezing temperatures at low pH. Evidence for cold denaturation was obtained by measuring changes in fluorescence intensity upon cooling the protein. The results of a representative experiment at pH 2.7 are presented in Figure 5. The MBP sample was divided into two portions. One half was used for thermal denaturation and the second to monitor the fluorescence changes upon cooling. As predicted, two sigmoidal transitions are observed, one near 40°C, corresponding to thermal denaturation, the other near 5°C, corresponding to cold denaturation. Although the amplitude of the cold transition is less than that of the thermal transition, this could be due to differential effects of temperature on the fluorescence of the native and denatured states as depicted by the thin lines.

Fig. 3. Experimentally determined changes in heat capacity plotted versus the calculated change in solvent-accessible nonpolar surface area upon unfolding. Data represented by the open circles were taken from Novokhatny and Ingham (1994). Straight line represents a best fit of the open circles only. Filled circle represents the Δ ASA value calculated from the known structure of MBP, which predicts the ΔC_p value of 0.163 cal/K-g.

Fig. 4. Temperature dependencies of ΔG for MBP at pH 3.0 and 3.4, calculated using Equation 1 and a value of 0.16 cal/g for ΔC_p .

In order to measure the heat associated with maltose binding, we employed mixing calorimetry. A representative picture of a mixing experiment is shown in Figure 6 and the results of several different experiments are summarized in Table 2. Despite the big changes in stability of MBP upon binding with maltose, the heat effect of this reaction turned out to be very small, ~ -1 kcal/mol. This is quite different from the ligand binding of another periplasmic sugar binding protein, arabinose binding protein, whose binding reaction with arabinose is characterized by a substantial enthalpy change of -15.3 ± 0.5 kcal/mol (Fukada & Sturtevant, 1983).

Fig. 5. Fluorescence-detected heat- and cold-induced denaturation transitions of MBP. The protein, at 0.1 mg/mL in 50 mM Gly, pH 2.7, was divided into two portions. One was cooled and the other heated at ~ 1 K/min while monitoring the fluorescence intensity at 345 nm with excitation at 280 nm. Thin lines were drawn by hand to simulate the apparent temperature dependence of the fluorescence intensities of the Native and Denatured states.

Fig. 6. Typical mixing calorimeter curves obtained at 25 °C in 0.02 M tris(hydroxymethyl)-aminomethane, pH 7.4, containing 0.15 M NaCl. Initial concentration of MBP was 54 μ M and of maltose, 100 mM. Exotherms of 10 mixes at a mixing ratio of 1:1 were averaged to produce all curves on the plot except the net curve, which was obtained by subtracting the maltose-TBS curve and MBP-TBS curve from the MBP-maltose curve. Data from additional experiments are summarized in Table 2.

As was mentioned before, MBP is used widely as a carrier protein for the production of recombinant fusion proteins in *E. coli*. To explore the value of scanning calorimetry for assessing the structural integrity of recombinant products at the fusion protein stage, we studied several fusion proteins at different solvent conditions. Figure 7 shows the DSC curve obtained at pH 3.2 with a fusion protein MBP-Hep2, which contains MBP and the 30 kDa heparin binding "Hep-2" fragment of fibronectin (Novokhatny et al., 1992b). It consists of two prominent peaks. The first, centered near 45 °C, is due to the melting of the MBP component, and the second, near 70 °C, to the melting of the Hep-2 moiety. These assignments are based on curves obtained with the separate components, also shown in Figure 7. The individual components are slightly more stable than their counterparts in the fusion protein,

Table 2. Enthalpy of association of maltose binding protein with maltose under different solvent conditions and different mixing ratios

Mixing ratio	[MBP] (μ M)	[Maltose] (mM)	ΔH (kcal/mol)
50 mM Tris, pH 7.4 + 100 mM NaCl			
1:1	11	10	-0.3
0.33:1	5.5	5	-0.5
3:1	16.5	15	0.1
20 mM Tris, pH 7.4 + 150 mM NaCl			
1:1	27	50	-0.9
0.33:1	13.5	25	-2.5
3:1	40.5	75	-1.4
50 mM Gly, pH 3.5			
1:1	5.5	10	-0.3
0.33:1	2.7	5	-0.5
3:1	8.1	15	-0.3

perhaps because of a destabilizing interaction between the partners, as reported in another system (Blondel et al., 1996). The deconvolution analysis of the fusion protein curve revealed three transitions in the Hep-2 peak whose thermodynamic parameters are similar to those obtained previously with the natural Hep-2 fragment, which comprises three independently folded type III domains (Novokhatny et al., 1992b).

A second example, a fusion protein between MBP and Type III domain 13 of fibronectin, is shown in Figure 8. Panel A illustrates that addition of maltose does not affect the melting properties of domain III₁₃. The ability to shift the peak for MBP by addition of ligand could be useful to better resolve the transition of interest. Alternatively, if the recombinant protein is sufficiently stable at low pH, the MBP peak can be eliminated entirely as shown for MBP-III₁₃ in Figure 8B. This allows unambiguous assessment of the thermodynamic parameters of the domain of interest, as shown at the bottom of the panel.

Finally, we were able to observe the melting of type I finger domain 6 of fibronectin fused with MBP (Fig. 9). The finger domains are rather small, containing only about 45 amino acids, but some of them are quite stable (Novokhatny & Ingham, 1994). The enthalpy, although much smaller than that of MBP, was still measurable, about 30 kcal/mol. Thus, these results clearly indicate that the flexible thermodynamic behavior of MBP allows successful characterization of the folding status of proteins of interest at the fusion protein stage.

Discussion

The results presented here indicate that maltose binding protein consists of two thermodynamic domains that interact with each other, especially at neutral pH, in the presence of ligand where the calorimetric to van't Hoff enthalpy ratio is close to unity. Even in the absence of ligand, this ratio is much lower than the value of ~ 2 , which is expected for two noninteracting domains of similar size melting at similar temperature (Privalov, 1982). These results

Fig. 7. DSC profile of melting of the MBP-Hep2 fusion protein at pH 3.2 along with the melting profiles of the isolated proteins. Protein concentration range, 1.12–1.67 mg/mL; scan rate, 1 K/min. The high-temperature peak for the fusion protein was deconvoluted into three transitions with enthalpies and T_m s given in the inset.

Fig. 8. DSC profiles of melting of the MBP-III13 fusion protein at pH 3.0 with and without 50 mM maltose (**A**) and at pH 2.7 (**B**). Protein concentration range, 1.12–1.67 mg/mL; scan rate, 1 K/min. In **B**, the excess heat capacity curve (noisy line) is shown on the bottom along with the best fit of this function to a two-state transition (smooth line).

are consistent with what is known about the crystal structure of this protein, which exhibits two distinct lobes. If we assume that these lobes correspond to the two thermodynamic domains, then it is easy to see how it would be difficult for them to melt independently because the polypeptide chain weaves back and forth between them, traversing the interface three times. Maltose binds in a cleft between the two domains, causing them to close around the ligand and increasing the number of salt links and electrostatic and hydrophobic bonds between them (Sharff et al., 1992). This increased interaction between the domains may contribute to the increased cooperativity of their melting at neutral pH. Some of the negatively charged groups that participate in the domain–domain interactions would be protonated at low pH, where the ligand-induced shift in T_m is even greater but no increase in cooperativity occurs. This suggests a role for these titratable groups in the ligand-induced increase in cooperativity between the melting of the two domains at neutral pH.

The observed stabilization of MBP by maltose is similar to that which occurs with two other sugar binding proteins, arabinose binding protein and hexokinase (Spurlino et al., 1991). However,

Fig. 9. DSC profile of the melting of fusion protein containing MBP and the sixth type I domain of fibronectin in 50 mM glycine, pH 3.4. The transition near 90°C is fit to a single two-state transition.

the almost negligible enthalpy observed here for binding of maltose to MBP was unexpected considering that the binding of arabinose to its binding protein, which is structurally similar to MBP, is accompanied by a substantial enthalpy change of -15.3 ± 0.5 kcal/mol (Fukada & Sturtevant, 1983). On the other hand, the binding of D-glucose to hexokinase also gave very small values for the enthalpy changes, -0.73 ± 0.9 kcal/mol (Takahashi et al., 1981). Thus, in spite of the structural similarities of these three systems (Spurlino et al., 1991), they differ significantly in the thermodynamics of their ligand binding.

The melting of MBP is associated with an unusually high change in heat capacity. The experimentally determined value of 0.16 cal/K-g was in excellent agreement with the value deduced from the calculated change in exposure of nonpolar surface upon unfolding. This value not only places MBP in a record zone, but also leads to the prediction that cold denaturation should occur above 0°C. Fluorescence measurements confirmed that this was the case. To our knowledge, this is one of the few examples when the process of cold denaturation can be observed at above freezing temperatures without any denaturants. The denaturants are usually used to boost the ΔC_p value of a protein and by this means to shift the cold denaturation peak closer to measurable temperatures (Privalov, 1990).

Finally, the experiments with fusion proteins demonstrate the feasibility of assessing the structural integrity of the protein of interest prior to cleavage and isolation. The ability to shift the T_m of MBP by manipulation of pH and ligand concentration increases the chances of finding conditions where the melting of MBP and the protein of interest are well resolved. That maltose appears to bind MBP even at low pH (Table 1) and even in the presence of at least 2 M urea (data not shown) suggests that purification of fusion proteins can be accomplished under a wide range of solvent conditions.

Material and methods

Proteins and chemicals

MBP and fusion proteins were produced in *E. coli* using the pMAL-p2 expression system (New England Biolabs, Inc.) as described (Matsuka et al., 1994; Busby et al., 1995). This system

generates fusion proteins comprising the desired product separated from the carrier MBP by a segment containing the cleavage site for factor Xa. Three constructs were used: MBP fused with the 30-kDa Hep2 fragment of fibronectin, MBP fused with type III domain 13 of fibronectin, and MBP fused with fibronectin type I domain 6. Isolated MBP and Hep2 were produced by digestion of the corresponding fusion protein with factor Xa at room temperature overnight and purified on amylose- and heparin-sepharose, respectively (Busby et al., 1995). The resulting recombinant MBP has a sequence identical to the natural protein, except that four residues on the C-terminus are replaced by a 20-amino acid sequence containing the linker region followed by a factor Xa cleavage site (NS-SSNNNNNNNNNLGIEGR). Factor Xa, D-maltose, porcine mucosal heparin, Tris, glycine, and molecular biological reagents were obtained from Sigma. All other chemicals were of reagent grade or higher.

Differential scanning calorimetry

Differential scanning calorimetry measurements were made as previously described with a DASM-4M instrument or with updated DASM-1 calorimeter (Biopribor, Pouschino, Russia) at a scan rate of 1 °C/min and protein concentrations between 0.4 and 2.0 mg/mL. Melting temperatures, calorimetric and van't Hoff enthalpies were determined using software provided by Dr. V. Filimonov (Institute of Protein Research, Pouschino, Russia) as described previously (Novokhatny et al., 1992b; Novokhatny & Ingham, 1994). Deconvolution analysis was performed with the same software. The relative error of the total enthalpy values is estimated at $\pm 10\%$ and that of the T_m values at ± 0.2 °C.

Temperature dependencies of the changes in free energy (ΔG) and entropy (ΔS) of MBP unfolding were calculated using the formula:

$$\Delta G = \Delta H_0 - T\Delta S_0 + \Delta C_p[T - T_0 - T \ln(T/T_0)] \quad (1)$$

$$\Delta S = \Delta S_0 + \Delta C_p \ln(T/T_0) \quad (2)$$

where ΔH_0 and ΔS_0 are the enthalpy and entropy of unfolding evaluated at the reference temperature T_0 .

Analyses of the structures of MBP (Spurlino et al., 1991; PDB code 2MBP) and other proteins deposited in the Brookhaven Protein Data Bank were done on an IRIS Indigo computer workstation (Silicon Graphics, Inc.) using QUANTA 4.0 software (Molecular Simulation, Inc.).

Mixing calorimetry

Mixing calorimetry experiments were done with a DSFS-100 stopped-flow microcalorimeter (Commonwealth Technology, Inc.) at 25 °C (Mudd & Berger, 1988). MBP was mixed with several saturating concentrations of maltose to register the heat liberated or consumed upon binding. The reported enthalpy value was obtained by averaging 5–10 runs at each mixing ratio and correcting for the heat obtained when either component alone was mixed with buffer. The final MBP concentrations were in the range of 1.0–2.2 mg/mL.

Fluorescence measurements

Fluorescence measurements of thermal unfolding were performed by monitoring the fluorescence intensity at 345 nm with excitation at 280 nm in an SLM 8000-C fluorometer (Novokhatny et al., 1992a). Temperature was controlled with a circulating water bath programmed to raise or lower the temperature at ~ 1 °C per min. Protein concentrations were ~ 0.1 mg/mL.

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