Differential Scanning Calorimetry of Thermal Unfolding of the Methionine Repressor Protein (MetJ) from *Escherichia coli*†

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ABSTRACT: The thermal stability of the methionine repressor protein from *Escherichia coli* (MetJ) has been examined over a wide range of pH (pH 3.5–10) and ionic strength conditions using differential scanning calorimetry. Under reducing conditions, the transitions are fully reversible, and thermograms are characteristic of the cooperative unfolding of a globular protein with a molecular weight corresponding to the MetJ dimer, indicating that no dissociation of this dimeric protein occurs before unfolding of the polypeptide chains under most conditions. In the absence of reducing agent, repeated scans in the calorimeter show only partial reversibility, though the thermodynamic parameters derived from the first scans are comparable to those obtained under fully reversible conditions. The protein is maximally stable (T_m 58.5 °C) at about pH 6, close to the estimated isoelectric point, and stability is enhanced by increasing ionic strength in the range I = 0.01–0.4 M. The average calorimetric transition enthalpy (ΔH_m) for the dimer is 505 ± 28 kJ mol^{-1} under physiological conditions (pH 7, I = 0.125, T_m = 53.2 °C) and shows a small temperature dependence which is consistent with an apparent denaturational heat capacity change (ΔC_p) of about +8.9 kJ K^{-1} mol^{-1}. The effects of both pH and ionic strength on the transition temperature and free energy of MetJ unfolding are inconsistent with any single amino acid contribution and are more likely the result of more general electrostatic interactions, possibly including significant contributions from electrostatic repulsion between the like-charged monomers which can be modeled by a Debye-Hückel screened potential.

The methionine repressor protein (MetJ)[1] from *Escherichia coli* controls the transcription of at least six genes, including its own, involved in the biosynthesis of methionine and *S*-adenosylmethionine (SAM). SAM acts as a corepressor in this system by enhancing the affinity of MetJ for its DNA target sequences, which comprise tandem repeats of an 8 bp sequence unit, the met-box. These operator sequences lie in the 5′ promoter regions upstream of the regulated operons [for review, see Saint-Girons et al. (1988), Old et al. (1991), and Weissbach and Brot (1991)]. Crystal structures of MetJ, both with and without bound SAM, and of the protein–operator complex have recently been determined by high-resolution X-ray diffraction techniques (Rafferty et al., 1989; Somers, 1990). The repressor is a dimer of identical intertwined polypeptides of 104 amino acids each, with the approximate dimensions 30 × 30 × 50 Å (dimer M_r 2 × 11 996 = 23 992). The monomers are linked by antiparallel β-strands, which loop over one another, and by contacts between adjacent faces of α-helical regions ("B-helices"). The structure lacks the characteristic helix–turn–helix motif which is a feature of many other DNA binding proteins (Steitz, 1990). Each monomer contains a single cysteine residue, though there are no disulfide bridges in the structure.

The results of both crystallographic (Somers, 1990) and functional studies in solution (He et al., unpublished results) show that the MetJ dimer binds to operator DNA with the β-strands of the protein inserted into the major groove of the double helix. Binding of SAM, which occurs on the face opposite to this DNA–protein interface, produces only very minor structural changes in the protein, suggesting that its function as co-repressor does not depend on conformational change within the protein. Recent electrostatic calculations (Phillips and Phillips, unpublished results) suggest that SAM functions by increasing the positive electrostatic potential upon the DNA binding face via the charge carried on the tertiary sulfur atom. However, the *E. coli* tryptophan repressor, TrpR, which is also an interlinked dimer of similar molecular weight to MetJ, shows differences in the dynamics of the aporepressor and the co-repressor-bound complex (Arrowsmith et al., 1991) which may indicate the importance of such effects in DNA/protein interactions. SAM binds to isolated MetJ relatively weakly (K_d 200 μM), with an SAM/dimer stoichiometry of 2:1 (Saint-Girons et al., 1986; Rafferty et al., 1989), whereas the binding affinity is at least 10-fold stronger in the presence of operator. The effective repression complex consists of a number of repressor dimers bound in tandem array to repeating met-boxes. Such arrays are stabilized by extensive protein–protein contacts between the A-helices of neighboring dimers (Somers, 1990). Biochemical studies of operator binding in vitro reveal positive cooperativity with respect to protein concentration, presumably largely because of this protein–protein contact (Phillips et al., 1989).

Transcriptional control is the primary mechanism governing gene expression in all living things. Despite its fundamental importance, we still have only limited data on the energetics and thermodynamics of the systems involved. The *E. coli* methionine repressor represents an ideal model system in which to study such phenomena. The extended protein arrays which are formed at *met* operators are analogous to the transcriptional complexes which form in eukaryotic cells, so the data obtained with MetJ should be of widespread interest. The structures

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[1] Abbreviations: MetJ, methionine repressor protein from *E. coli*; SAM, *S*-adenosylmethionine; DSC, differential scanning calorimetry; DTT, dithiothreitol.

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of this small protein, and its complexes with DNA and co-repressor molecules, allow us to probe various questions related to the energetics and thermodynamics of protein folding and protein–nucleic acid recognition using direct microcalorimetry combined with site-directed mutagenesis and DNA synthesis techniques. We present here a detailed study of the thermal stability of wild-type MetJ over a range of pH and ionic strength conditions using differential scanning calorimetry (DSC) as a preliminary to studies of more complex systems involving DNA and co-repressor interactions.

EXPERIMENTAL PROCEDURES

Repressor protein was isolated from overproducing strains of *E. coli* essentially as described by Saint-Girons et al. (1986), except for the use of S-Sepharose in place of phosphocellulose in the second chromatographic stage and by omission of the final Sephacryl S-200 step. MetJ was easily located in crude extracts using SDS–PAGE, as described by Schägger and von Jagow (1987), and was followed during the purification to homogeneity using this technique. Purified MetJ preparations revealed a single band of molecular weight 12 000 on such gels. Samples of repressor were stored as $(NH_4)_2SO_4$ precipitates at 4 °C until use. All buffers were prepared using distilled water and with Analar-grade reagents. Wherever possible, the appropriate buffer salts were used to titrate to the required pH. Alternatively, samples of the indicated buffer concentration were titrated using concentrated HCl or KOH as required.

Samples for calorimetry were dialyzed extensively against the required buffer at 4 °C and then centrifuged at 20000*g* for 30 min before use, and aliquots of the final dialysis buffer were used for DSC reference and base-line corrections. The protein concentrations of dialyzed samples were determined from absorbance measurements using $\epsilon_{280} = 15\ 340\ M^{-1}\ cm^{-1}$ for the monomer ($A_{280} = 1.28$ for 1 mg mL^{-1}), on the basis of the amino acid composition (Gill & von Hippel, 1989; Smith et al., 1985). DSC was performed using a Microcal MC-2D instrument fitted with an EM Electronics N2a nanovolt preamplifier, at a routine scan rate of 60 °C h^{-1} unless otherwise indicated. Sample and reference solutions were degassed for approximately 1 min under vacuum with gentle stirring before being loaded, and were held under 2–3 atm N_2 pressure during DSC to inhibit degassing and bubble formation at higher temperatures. Samples were routinely rescanned at least once, after being cooled to room temperature in the DSC cell for 1 h or more, to check for reversibility. DSC scans were normalized by subtraction of appropriate control buffer data and converted to excess specific heat capacity assuming a molecular weight of 24 000 for the methionine repressor (dimer) and a cell volume calibrated as described previously (Cooper & Johnson, 1992). Data analyses were performed using Microcal Origin software, which is based on standard deconvolution procedures (Sturtevant, 1987; Privalov & Potekhin, 1986), and transitions were fit to single- or multiple-peak non-two-state thermal unfolding models, as appropriate.

RESULTS

Thermal Transition, Reversibility, and the Cooperative Unit. DSC measurements on MetJ in aqueous solution over a 15–80 °C temperature range have been carried out under different conditions to include variations in pH, ionic strength, buffer salts, protein concentration, and thermal scan rate. Repeat DSC scans were used to examine the refolding and reversibility of the process, and typical examples are illustrated in Figure 1. MetJ shows a single endothermic transition under

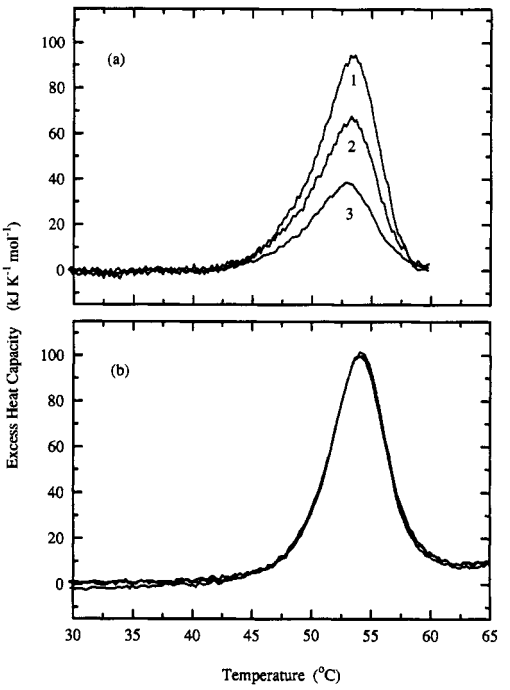

FIGURE 1: Typical heat capacity endotherms for the repeated thermal denaturation of MetJ in 25 mM potassium phosphate buffer/100 mM KCl, pH 7. Samples were scanned at 60 °C h^{-1} and cooled in situ over a period of about 1 h before rescanning under the same conditions. Excess heat capacities versus temperature, corrected by subtraction of the appropriate buffer base line and normalized with respect to protein concentration. (a) Three successive scans to 60 °C without DTT; (b) three successive scans to 65 °C, or higher, in the presence of 1 mM DTT.

all conditions, and repeated scans of the protein after cooling in situ give essentially identical data in all respects other than the magnitude of the transition. Specifically, in the absence of the reducing agent dithiothreitol (DTT), rescans normally give thermograms of similar shape and identical transition temperature (T_m), but with reduced calorimetric amplitudes, indicating only partial reversibility of the thermal unfolding process under these conditions. Detailed examination of these MetJ thermograms shows, in addition, a small but consistent distortion of the peaks, manifest as a slight asymmetry when compared to theoretical fits. This "sharpening" on the high-temperature side of the transition is not uncommon in DSC scans, and we, along with others, have noted its association with exothermic irreversible processes such as sample aggregation, de-amidation, proline isomerization, sulfhydryl oxidation, and so forth, which may occur during or after protein denaturation (Hu & Sturtevant, 1987; Johnson et al., 1991; Takahashi et al., 1981). Levels of MetJ reversibility are usually less at higher protein concentrations and at slower scan rates or in samples heated to higher final temperatures before rescanning, but under optimal conditions, roughly 70% reversibility is observed in the absence of DTT. These observations are consistent with some form of kinetic control to the irreversible process(es) associated with denaturation. The rate of onset of irreversibility is indicated in Figure 2, which shows the fraction of refolded protein (estimated from the area under the calorimetric transition) as a function of incubation time above 45 °C. Unfolding of the methionine repressor protein starts at approximately 45 °C, and samples heated no higher than this temperature exhibit complete and normal transitions on rescanning. At higher temperatures, however, the extent of reversibility (as determined from repeated DSC traces) depends on the length of time that the sample is held above this temperature as shown in Figure 2.

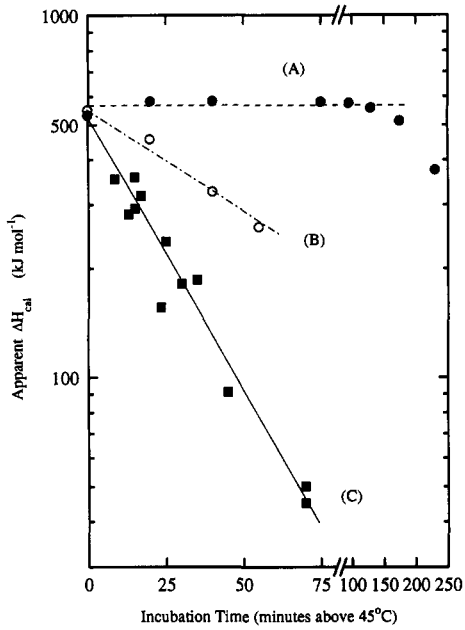

FIGURE 2: Degree of reversibility of the MetJ calorimetric transition, expressed as the integrated enthalpy (ΔH_{cal}) of the MetJ endotherm, following different incubation periods above 45 °C. Measurements were made in 25 mM potassium phosphate buffer/100 mM KCl, pH 7, in the presence of 1 (A) and 0.2 mM (B) DTT and in the absence (C) of DTT.

Data such as these fit well to a single-exponential curve, with a relaxation time of the order of 20 min at 50 °C or above. This gives some indication of the time scale involved in the irreversible process associated with the thermal transition, and suggests that, although thermal denaturation of MetJ is eventually irreversible under nonreducing conditions, these processes are sufficiently slow that the assumption of thermodynamic reversibility during the initial DSC transition, implicit in the deconvolution of the transitions in terms of standard thermodynamic models, is not too misleading an approximation in this instance.

Much stronger support for reversibility assumptions is provided by thermal unfolding experiments done under reducing conditions. Addition of increasing concentrations of the conventional disulfide reducing agent dithiothreitol (DTT) to the protein buffer mixture significantly enhances the reversibility of the MetJ transition until with 1 mM DTT, in complete contrast to the situation without DTT, the process is entirely reversible and repeatable throughout multiple heat–cool cycles in the DSC (Figures 1 and 2). Unfortunately, the presence of DTT (and other sulfhydryl protection reagents) at relatively high concentrations in calorimetric buffers does, for reasons that we do not fully understand, lead to technical difficulties due to unpredictable and occasional erratic base-line effects which can make effective data analysis difficult. Nevertheless, with the possible exception of ΔC_p effects (see below), comparison of experiments in the presence and absence of reducing agent shows no major difference in thermodynamic parameters (T_m and ΔH).

Figure 3 illustrates a typical thermogram, together with a control buffer base line, and its deconvolution in terms of a single unfolding transition model. After base-line subtraction and normalization for cell volume and protein concentration, the excess heat capacity data for MetJ fit reasonably well to either of two standard thermodynamic two-state transition models: (a) a single cooperative transition with associated heat capacity change (base-line shift) ΔC_p: or (b) a single, potentially noncooperative transition. The latter has the

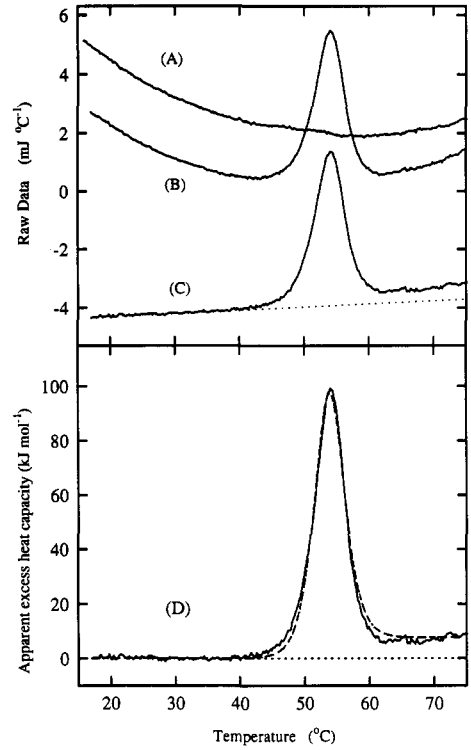

FIGURE 3: Typical DSC endotherm showing the apparent excess heat capacity for the thermal denaturation of MetJ at a protein (dimer) concentration of 42 μM in 25 mM KP_i buffer, 100 mM KCl, and 1 mM DTT, pH 7.0, scan rate 60 °C h^{-1}: (A) control buffer base line; (B) MetJ thermogram (raw data); (C) MetJ raw data after buffer base line subtraction, indicating the base-line extrapolation (dotted line) used for deconvolution; (D) deconvolution of normalized data according to a single cooperative transition model with ΔC_p, assuming a cooperative unit of 24 000 Da, with the dashed line showing the theoretical best fit.

advantage of yielding three independent parameters for each transition: (i) the midpoint temperature (T_m) of the transition; (ii) the absolute calorimetric enthalpy at T_m, derived from the integrated area beneath the DSC transition and expressed either in terms of sample mass (Δh_{cal}, in joules per gram) or, using a presumed molecular weight for the cooperative unit of the transition, as the molar enthalpy ($\Delta H_{m,cal}$, in kilojoules per mole); (iii) the van't Hoff enthalpy ($\Delta H_{m,VH}$, in kilojoules per mole) derived from the shape of the transition assuming a simple two-state model. Comparison of Δh_{cal} (or $\Delta H_{m,cal}$) with $\Delta H_{m,VH}$ can give information regarding the size of the unit undergoing thermal transition (Sturtevant, 1974). A survey of 14 MetJ DSC scans, recorded at instrumental heating rates between 10 and 90 °C h^{-1} with MetJ dimer concentrations of 29 ± 2 μM (ca. 0.7 mg mL^{-1}) in 25 mM potassium phosphate buffer/100 mM KCl, pH 7.0, showed no scan rate dependence in thermal behavior. Analysis gives a mean T_m of 53.2 ± 0.2 °C under these conditions, with Δh_{cal} = 21.0 ± 1.1 J g^{-1} corresponding to an $\Delta H_{m,cal}$ of 505 ± 28 kJ mol^{-1} for a 24 000 M_r protein. The average $\Delta H_{m,VH}$ for the same data is 547 ± 40 kJ mol^{-1}. The apparent size of the cooperative unit, given by $\Delta H_{m,VH}/\Delta h_{cal}$, is 26 050 ± 3450 g mol^{-1}, consistent with the molecular weight of the MetJ dimer. Similar estimates under reducing conditions give a value of 24 700 ± 2200 g mol^{-1}. This confirms that the MetJ dimer is very stable in solution under widely ranging conditions and apparently maintains its dimeric integrity until thermal unfolding takes place.

MetJ thermograms in the presence of DTT show evidence of a small base-line shift during the transition, consistent with the increase in heat capacity (ΔC_p) upon unfolding of the

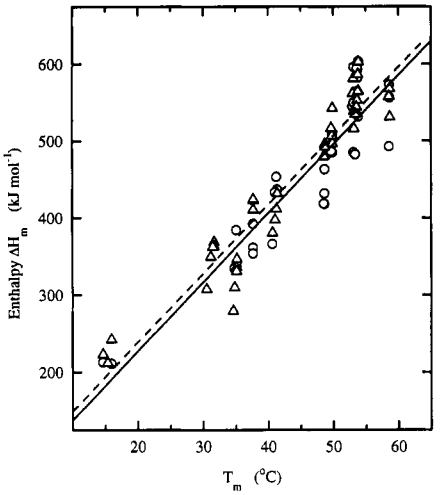

FIGURE 4: Temperature dependence of $\Delta H_{m,cal}$ (circles) and $\Delta H_{m,VH}$ (triangles) for MetJ thermal denaturation in the presence of 1 mM DTT. A range of transition temperatures was obtained by pH adjustment in phosphate, citrate, or borate buffers. Linear regression of the $\Delta H_{m,cal}$ (solid line) and $\Delta H_{m,VH}$ (dashed line) data gives slopes of 8.94 ± 0.66 and 8.93 ± 0.43 kJ mol^{-1} K^{-1} and intercepts of 48 ± 31 and 60 ± 19 kJ mol^{-1}, respectively.

polypeptide frequently seen in other proteins (Privalov & Khechinashvili, 1974; Sturtevant, 1977; Privalov, 1979; Privalov & Gill, 1988). Analysis of this effect from single DSC traces is, however, not unambiguous, and can be difficult to quantify due to apparent differences in slope of the pre- and posttransition base lines (which may reflect a temperature dependence of ΔC_p) and difficulties in choosing consistent (and nonsubjective) base-line extrapolation procedures. The analysis is further exacerbated in this case by the base-line instabilities introduced by high concentrations of DTT in the calorimeter. ΔC_p effects are rarely apparent in the absence of DTT, most likely because of slow exothermic processes associated with irreversibility in the unfolded polypeptides. Less ambiguous evidence for a ΔC_p of denaturation is given by the temperature dependence of the calorimetric enthalpy values determined from experiments at different pHs with different transition temperatures (Figure 4). The slope of this plot for either ΔH_{cal} or ΔH_{VH} indicates a ΔC_p of about 8.9 kJ mol^{-1} K^{-1}, roughly consistent with the base-line shifts observed in the original thermograms (in the presence of DTT). Linear regression of these data allows us to express the calorimetric or van't Hoff transition enthalpies (per dimer) at any T_m as

$$\Delta H_{m,cal} = 48 + 8.94(T_m - 273) \text{ kJ mol}^{-1}$$

$$\Delta H_{m,VH} = 60 + 8.93(T_m - 273) \text{ kJ mol}^{-1}$$

where T_m is the midpoint transition temperature in degrees kelvin.

Effect of pH on Thermal Stability. The thermal stability of MetJ at a concentration of 29 ± 4 μM was examined over the pH 3.5–10 range as shown in Figure 5a. A wide range of buffer salts with overlapping pH ranges was used to eliminate possible specific buffer effects. Buffer systems used included potassium phosphate, Tris, imidazole, MOPS, borate, PIPES, HEPES, EPPS, acetate, cacodylate, and citrate. Each mixture comprised 25 mM buffer salts, plus 100 mM KCl, adjusted to the appropriate pH at room temperature (22 °C). Buffer pHs are, however, temperature-dependent in most cases and will vary (decrease, mostly) during a DSC scan. For simplicity, in the data that are illustrated in Figure 5, we have corrected the pH to the value at the T_m of the transition using standard

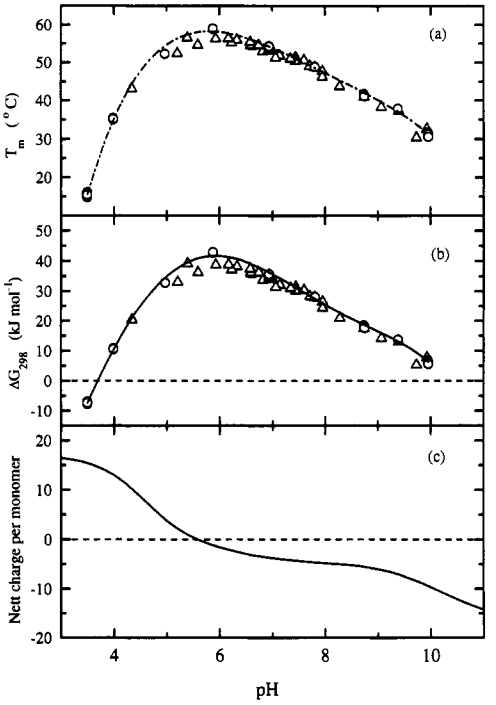

FIGURE 5: Effect of pH on the thermal stability of MetJ at DSC scan rates of 30 or 60 °C h^{-1} in 25 mM buffer/100 mM KCl. pH values are corrected to the transition temperature (T_m) using published dpH/dT values for the appropriate buffer (Cooper & Johnson, 1992). Experimental data were obtained either in the presence (circles) or in the absence (triangles) of 1 mM DTT. (a) Midpoint temperature, T_m, for the unfolding transition; (b) free energy of unfolding (ΔG_{298}) of the MetJ dimer at 25 °C; (c) theoretical hydrogen ion titration curve for the MetJ monomer using standard intrinsic pK values.

heat of buffer ionization (ΔH_I) or dpH/dT data (Cooper & Johnson, 1992; Christensen et al., 1976). These data may be transformed into standard free energies for the transition at 25 °C (ΔG_{298}, Figure 5b) using

$$\Delta G_{298} = \Delta H_{m,cal}(1 - 298/T_m) + \Delta C_p[298 - T_m - 298 \ln (298/T_m)]$$

where ΔH_m was estimated using the linear regression data (above, and Figure 4). The hypothetical hydrogen ion titration curve for MetJ, shown for comparison in Figure 5c, was estimated from the amino acid composition using average intrinsic pK_a values (Tanford, 1962).

With the single exception of cacodylate buffer systems (see below), the observed T_m of MetJ varies consistently with pH as shown in Figure 5a and shows no specific buffer or DTT effects. A consistent slightly higher T_m in phosphate buffer, compared to other buffers in the same pH region, may be ascribed to the slightly higher ionic strength in this case (see below). The maximum stability (highest T_m ca. 58 °C) occurs at about pH 6 under these conditions, decreasing by about 6 °C per pH unit above this pH, and falling more rapidly at more acidic values. This variation in stability with pH, expressed either as T_m or as ΔG_{298}, correlates reasonably well with the estimated net charge on the protein (Figure 5c) and, further, indicates that significant changes in protonation are occurring in the MetJ polypeptide during denaturation. Analysis of the T_m versus pH behavior using the model of Fukada et al. (1983) for protein unfolding with simultaneous ligand (H^+ in this case) dissociation indicates that about 1.8 protons per dimer are released during thermal denaturation in the pH 7–10 range. More generally, the model-independent thermodynamic theory of linked functions (Wyman, 1964) indicates that any pH-dependent process must, of necessity,

involve pK changes and proton uptake or release. Specifically, the pH dependence of the free energy of any process is related to the change in proton ionization ($\Delta\nu$) by $d\Delta G/dpH = 2.303RT\Delta\nu$, which, using free energy data from Figure 5b, indicates protonation changes ($\Delta\nu$) ranging from about +6 at low pH, passing through zero at the pH maximum, and falling to about −2 H^+ ions per dimer at the high-pH extreme. In other words, thermal unfolding of the MetJ dimer at low pH is accompanied by an uptake of about six protons, compared to a release of about two protons at high pH.

These protonation changes associated with unfolding will, in principle, give rise to additional heat effects due to compensating ionization of buffer molecules in the mixture (Sturtevant, 1962; Cooper & Converse, 1976) which might complicate the calorimetric observations. Fortunately, this effect is relatively insignificant using phosphate or carboxylic acid buffers, where the heats of ionization (ΔH_I) are close to zero under the conditions used here (Christensen et al., 1976), and no systematic buffer effects were seen. This is not necessarily true, however, for the ΔH of processes measured in other buffers. For example, in the case of Tris buffer (which shows the largest effect) over the pH 7–9 range, we observe average $\Delta H_{m,cal}$ values for the MetJ transition that are consistently lower (i.e., less endothermic) than seen in phosphate under otherwise identical conditions. This enthalpy difference, amounting to about 90 kJ mol^{-1} in this case, may be accounted for by the exothermic uptake by Tris of the protons released by the protein. Given the known heat of protonation of Tris (−47.44 kJ mol^{-1}; Christensen et al., 1976), this corresponds to a proton release of the order of 1.9 protons per MetJ dimer, consistent with the above estimates by other means. Similar, though smaller, effects are seen with other buffers such that, when corrected for buffer ionizations using $\Delta\nu$ in the range −1.8 to −2, the mean enthalpies for the protein transitions show no significant variation from the average values in phosphate. We may, therefore, take these values as representative of the unfolding transition under all buffer conditions.

The stability of the methionine repressor decreases markedly below pH 6, and DSC analysis of unfolding transitions is sometimes complicated by significant distortion of the thermograms, particularly in the pH 4.5–6 range, probably due to rapid aggregation of denatured protein. Indeed, unlike experiments at higher pH, samples from these scans were frequently turbid on removal from the calorimeter, and aggregation effects of this type are to be expected in the region of an isoelectric pH (estimated pI ca. 5.5 for MetJ). The T_m values should be reasonably accurate in this pH range, however, and ΔG_{298} values may be estimated as above.

Ionic Strength Effect. The effects of ionic strength on the stability of MetJ at pH 7 were investigated both by variation of phosphate buffer concentration and by addition of KCl and other salts. As shown in Figure 6, the thermal stability of MetJ is significantly enhanced by increasing ionic strength with an increase in T_m of almost 10 °C over the I = 0.01–0.4 range, approaching a plateau at higher ionic strength. Converted to free energy values (ΔG_{298}) as before, these data indicate a significant negative contribution to the stabilization free energy that can be screened out by high ionic strengths. Similar behavior was observed at a higher pH in imidazole hydrochloride buffer systems (data not shown). These results therefore indicate a general ionic strength effect rather than a specific ion stabilization interaction with the MetJ protein. The variation in unfolding free energy with ionic strength can

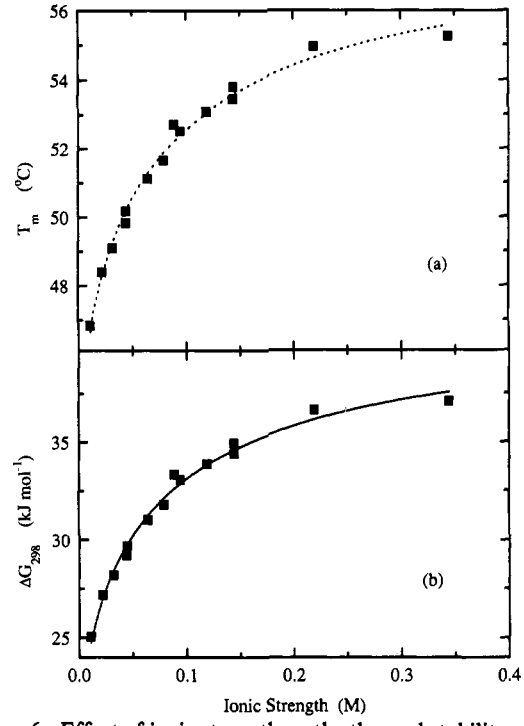

FIGURE 6: Effect of ionic strength on the thermal stability of MetJ at pH 7 in a potassium phosphate/KCl buffer system. Ionic strength (I) was varied either by changing the concentration of the phosphate buffer or by the addition of KCl to 25 mM buffer, both in the presence and in the absence of DTT, with identical results. (a) Increase in T_m with ionic strength; (b) increase in the estimated unfolding free energy at 25 °C with ionic strength. The dashed line (in panel A) is merely a guide to the eye, whereas the solid line (in panel b) is the least-squares fit function: $\Delta G_{298} = 40.1 - 22.8 \exp[-3.73(I)^{1/2}]$ kJ mol^{-1}.

be described empirically by the equation:

$$\Delta G_{298}(I) = 40.11 - 22.76 \exp(-3.73I^{1/2}) \text{ kJ mol}^{-1}$$

which has the functional form expected for a significant repulsive contribution from a screened Debye–Hückel potential between point charges (see Discussion).

Effect of Protein Concentration. The thermal stability of MetJ shows a small but reasonably consistent dependence on total protein concentration in a manner expected qualitatively for unfolding and dissociation of oligomeric systems (Takahashi & Sturtevant, 1981; Fukada et al., 1983; Manly et al., 1985; Bae et al., 1988). At pH 7 under standard conditions, T_m increases uniformly by about 1.6 °C (52.8–54.4 °C) upon increasing [MetJ] over the 10–60 μM range, spanning the normal concentration of about 30 μM used for the majority of measurements reported here. Above this level (up to [MetJ] = 130 μM), the concentration effect was less pronounced, but analysis is potentially complicated by the larger and faster irreversible effects manifest at much higher protein concentrations, especially under nonreducing conditions, which might significantly distort or truncate the transition thermograms and lead to potentially lower estimates of T_m. Nevertheless, analysis of the transitions provided no evidence of additional distortions at higher concentrations, and theoretical fits are equally good throughout the concentration range examined. Despite the small changes in T_m with concentration, but in accord with a small ΔC_p, the derived enthalpies showed no significant concentration effects, giving average values of ΔH_{cal} = 493 ± 64 kJ mol^{-1} and ΔH_{VH} = 555 ± 33 kJ mol^{-1}, respectively, which compare closely with the values obtained at a fixed repressor concentration of 29 μM but under differing scan rates, buffers, and pH.

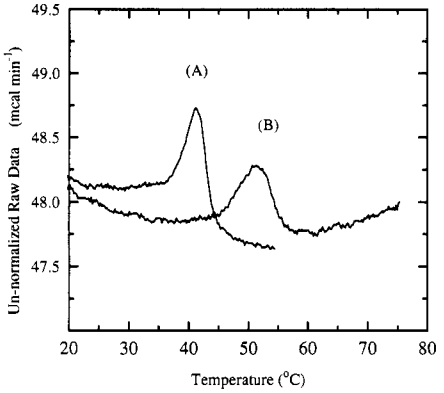

FIGURE 7: DSC thermograms for 17 μM MetJ in 25 mM cacodylate (A) or phosphate (B) buffers, pH 7, with 1 mM DTT, showing the anomalous effects of cacodylate on the methionine repressor thermal unfolding transition. Data are shown uncorrected and unnormalized.

Cacodylate Effect. Preliminary MetJ DSC experiments in cacodylate buffer, chosen because crystal growth for X-ray studies utilized this system (Rafferty et al., 1989), showed erratic and anomalous behavior compared to other buffers at the same pH. Specifically, in 10 mM sodium cacodylate buffer, pH 7–7.5, containing 1 mM DTT, the T_m was reduced by up to 10–12 °C compared to phosphate buffer under otherwise identical conditions as shown in Figure 7. The thermograms were also significantly sharper than expected for a protein of this size, and the $\Delta H_{VH}/\Delta h_{cal}$ ratios indicated cooperative unit molecular weights of 60 000 or more. This suggests that cacodylate has some specific effect on MetJ under these conditions that not only destabilizes the protein but also encourages oligomer formation. We have not yet explored this further, nor do we have any satisfactory explanation for the effect, but we note that cacodylic acid (dimethylarsenic acid) is known to be readily reduced by thiols in neutral aqueous solution to give a range of organosulfur compounds (Cullen et al., 1984) that might well prove harmful to proteins. Such reactions, either with added DTT or involving the –SH groups of the single MetJ cysteine residues, seem not unreasonable here.

DISCUSSION

The DSC studies reported here show that the dimeric methionine repressor protein undergoes a cooperative endothermic transition at about 53 °C, under physiological conditions, with an associated enthalpy change (Δh_{cal}) of about +21 J g^{-1} (5.0 cal/g) typical of the unfolding of small globular proteins. Comparison of the calorimetric and van't Hoff enthalpies derived from equilibrium analysis of DSC transitions provides useful information about the cooperative unit of this process. Here the mean ratio of ΔH_{VH} to ΔH_{cal}, obtained under reducing conditions and assuming a dimeric cooperative unfolding unit, is 1.03 (± 0.09). Considering the potential inaccuracies in extinction coefficients (probably no better than ±5%; Gill & von Hippel, 1989) and in the measurement of solution absorbances, together with traces of impurity or incorrectly folded protein that might affect estimates of ΔH_{cal}, this clearly suggests that MetJ undergoes cooperative unfolding from the dimeric state to the thermally denatured form with no significant accumulation of unfolding intermediates. This behavior is not surprising in view of the intertwined nature of the MetJ structure, and compares with the similar behavior observed for the highly interlinked *trp* repressor dimer (Bae et al., 1988). In vivo MetJ dimers form extended arrays along the DNA duplex at the *met* operator sites. The dimers are in contact in these arrays which could therefore be formed either by addition of repressor dimers to preexisting operator–MetJ complexes or by the prior association of the repressor into protein arrays which then bind to the operator sites. Although the data in cacodylate suggest evidence for formation of higher protein aggregates, the data in all other more physiologically relevant conditions show unambiguously that the dimer is the dominant form of the repressor in solution, favoring a sequential model for operator binding, although the effects of co-repressor binding must also be considered. Preliminary DSC experiments on the thermal unfolding of MetJ in the presence of SAM (unpublished results) indicate that the effect of co-repressor binding is simply to enhance the thermal stability of the complex, as expected for simple ligand binding, without affecting the state of aggregation of the protein.

In the presence of 1 mM DTT, the thermal unfolding of MetJ is highly reversible in the physiological pH range, and the process of heating and cooling may be repeated many times in the DSC without any appreciable degradation of the transition thermogram. In the absence of reducing agent, the unfolding is only partially reversible, to an extent which depends on the time spent above the denaturation temperature and with an apparent time constant of the order of 20 min. This strongly suggests that the source of irreversibility in the refolding of MetJ is oxidation of sulfhydryl groups and that other processes such as proline isomerization, deamidation, and nonspecific aggregation are not significant on this time scale (though these additional effects may become relevant if the protein is kept in the unfolded state for times in excess of 2–3 h). The MetJ dimer contains two cysteine residues, one per polypeptide, which are not cross-linked and which, although located in the B-helices forming part of the dimer interface, point away from this face and are distant from each other in the native structure. Under nonreducing conditions, it is likely, therefore, that the unfolded polypeptides might form disulfide linked dimers which would be unable to refold successfully. However, under the normally reducing conditions of the living cell, simulated here by the presence of DTT, such covalent dimers could easily dissociate. Mutants lacking Cys residues are being prepared to help identify the role of –SH groups in folding and stability of this protein.

Both pH and ionic strength effects show that electrostatic interactions play a significant role in MetJ stability. Maximum stability (T_m ca. 58.5 °C at 0.1 M ionic strength) occurs at about pH 6, which compares to an isoelectric point pI = 5.6 estimated from the amino acid composition of MetJ using intrinsic pK_a values (Tanford, 1962), and stability is markedly enhanced by increasing salt concentration. The pI estimate quoted here will, of course, only relate to the fully exposed, unfolded chain, and takes no account of the changes in pK_a of acidic and basic groups in the folded protein that must arise out of changes in solvation, interaction between groups, and so forth. In any case, there is no reason to expect maximum stability to coincide with pI except in special circumstances (Stigter & Dill, 1990). The decrease in MetJ stability at higher pH is consistent with the release of 1.8–2 protons per dimer during unfolding at pH 7, or above. While it is tempting to assume that this might arise from a single amino acid residue in each subunit with an unusual pK_a in the folded form, there is no strong evidence to support this. The reduction in ΔG_{298} of more than 15 kJ/mol of monomer over the pH range 6–10 would require a positive pK shift in an acidic residue of about 3, or more, if ascribed to anomalous protonation of a single group. Detailed examination of the crystal structure of MetJ

(Rafferty, 1989) reveals no buried charged groups or other interactions sufficient to give such pK shifts.

Explanation of the rather broad pH dependence of the stability of MetJ might lie alternatively in more general, long-range nonspecific interactions involving all charged groups in the molecule where one contribution to the folding free energy of an oligomeric protein, as opposed to monomeric systems, would arise from the overall Coulombic repulsion between identically charged monomers. Detailed calculations of the contribution of electrostatic interactions to protein conformational stability are complicated by lack of any adequate model for the unfolded state. Model calculations by Stigter and Dill (1990) suggest that, for *monomeric* globular proteins at least, the proton binding contribution to folding free energy is relatively small, except at the extremes of pH, and is unlikely to give significant pH dependence near neutrality. Experimental evidence based on site-directed mutagenesis of small monomeric proteins suggests similarly that surface or long-range charge interactions contribute little to protein stability (Dao-pin et al., 1991; Sali et al., 1991). Part of the explanation for this might be that the relatively compact nature of even the unfolded state (Stigter & Dill, 1990) might result in relatively little change in global electrostatic interactions during unfolding. However, we believe that the situation might be significantly different in oligomeric systems which involve significant charge separation during the subunit dissociation associated with unfolding. In the case of a dimer such as MetJ, and assuming that the charge distribution can be modeled by a single point charge, simple Coulomb repulsion ($\Phi_0 = q^2/4\pi\epsilon_0 Dr$) between monomer units of charge q amounts to a free energy contribution to folding of the order of $\Phi_0 = +1380q^2/Dr$ kJ mol^{-1}, where r is the mean monomer separation in angstroms and D is the effective dielectric constant. For r = 25 Å, corresponding to the approximate mean monomer separation in native MetJ, and using the approximate dielectric constant of water (D = 80), this corresponds to a repulsive ΔG contribution of about $0.7q^2$ kJ mol^{-1}. For a net monomer charge q = 5, such as might occur in the region of pH 7–8 for MetJ, this destabilizes the native protein by about 17 kJ mol^{-1} with respect to the isoelectric point. This is in reasonable order of magnitude agreement with observation (Figure 6b).

The proposed Coulombic repulsion contribution to the folding free energy is further supported by the enhanced stability of MetJ at higher ionic strengths, which is consistent with ionic screening of the electrostatic repulsion between monomers. Simple Debye–Hückel theory (Debye & Hückel, 1923; Pitzer, 1977; or any standard physical chemistry text) shows that, in the limit of low ionic strength and for point charges in solution, the above repulsive potential becomes modified by a screening term which depends on $I^{1/2}$ such that

$$\Phi = \Phi_0 \exp\{-r/[0.344(I/D)^{1/2}]\}$$

This suggests that the unfolding free energy of MetJ might be written $\Delta G_{298} = \Delta G_{298}{}^0 - \Phi$, where the first term represents the free energy in the absence of the Coulombic repulsion, Φ. Least-squares regression shows that the data (Figure 6b) can be satisfactorily represented by a function of this overall form with parameters that are not inconsistent with the known dimensions and charge of the protein. Clearly, such estimates based on a point-charge, homogeneous dielectric approximation are quite oversimplified, and more realistic calculations would require us to take into account the additional effects of nonuniform charge distribution, much lower nonisotropic effective dielectric constants as monomers approach each other, and so forth, but the magnitudes are nevertheless revealing. More detailed calculations are in progress, and a number of site-directed mutants of MetJ with altered net charges have now been prepared to test these hypotheses experimentally.

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