Thermodynamic Effects of Active-Site Ligands on the Reversible, Partial Unfolding of Dodecameric Glutamine Synthetase from *Escherichia coli*: Calorimetric Studies†

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Received June 16, 1992; Revised Manuscript Received September 17, 1992

ABSTRACT: Dodecameric glutamine synthetase (GS) from *Escherichia coli* undergoes reversible, thermally induced partial unfolding without subunit dissociation. A single endotherm for Mn·GS (±active-site ligands) in the presence of 1 mM free Mn^{2+} and 100 mM KCl at pH 7 is observed by differential scanning calorimetry (DSC). Previous deconvolutions of DSC data for Mn·GS showed only *two* two-state transitions (with similar t_m values; 51.6 ± 2 °C), and indicated that cooperative interactions link partial unfolding reactions of all subunits within the Mn·enzyme dodecamer [Ginsburg, A., & Zolkiewski, M. (1991) *Biochemistry 30,* 9421]. A net uptake of 8.0 equiv of H^+ by Mn·GS occurs during partial unfolding, as determined in the present DSC experiments conducted with four buffers having different heats of protonation at 50 °C. These data gave a value of 176 ± 12 kcal (mol of dodecamer)$^{-1}$ for ΔH_{cal} corrected for buffer protonation. L-Glutamine and L-Met-(*SR*)-sulfoximine stabilize the Mn·GS dodecamer through the free energies of ligand binding, and these were shown to be partially and totally released, respectively, from the 12 active sites at high temperature. Ligand effects on T_m values from DSC were similar to those from spectral measurements of Trp and Tyr exposures in two subunit domains. Effects of varying [ADP] on DSC profiles of Mn·GS were complex; T_m is increased by low [ADP] and decreased by >100 μM free ADP. This is due to the exposure of an additional low-affinity ADP binding site per GS subunit at high temperature with log K_1' = 4.3 and log K_2' = 3.6 at 60 °C relative to log K' = 5.5 for ADP binding at 30 °C, as determined by isothermal calorimetric and fluorescence titrations. Moreover, ΔH_{cal} at >27% saturation with ADP (corrected for ADP binding/dissociation) is ~80–100 kcal/mol more than in the absence of ligands. Changes in domain interactions could result from ADP bridging subunit contacts in the dodecamer. Each of the active-site ligands investigated here produces different effects on DSC profiles without uncoupling the extremely cooperative, partial unfolding reactions in the Mn·GS dodecamer.

Glutamine synthetase (GS)[1] is an essential enzyme in the nitrogen metabolism of enteric bacteria. The structure, function, and regulation of the enzyme from *Escherichia coli* have been extensively investigated [as reviewed by Stadtman and Ginsburg (1974) and Rhee et al. (1989)]. GS from *E. coli* is a large oligomeric protein (M_r ~622 000) consisting of 12 identical subunits arranged in 2 superimposed hexagonal rings with the 12 active sites located at the interfaces between adjacent subunits within a ring (Valentine et al., 1968; Almassy et al., 1986; Colombo & Villafranca, 1986; Yamashita et al., 1989). The activity of this enzyme is regulated by both covalent modification and noncovalent binding. GS interacts not only with substrates (L-glutamate, ammonia, and ATP) and divalent cations but also with many biosynthetic end products which act as its feedback inhibitors.

Dodecameric GS can exist in distinctly different conformations that depend on the state of metal ligation (Ginsburg, 1972). The native, active form of the enzyme has 2 equiv of Mn^{2+} bound to each active site (Hunt et al., 1975). The release of metal ions, as well as other ligands, occurs in parallel with the dissociation and unfolding of GS in 6 M guanidine hydrochloride (Maurizi & Ginsburg, 1982). The Mn·GS complex has been shown to undergo a temperature-induced, reversible transition with complete recovery of enzymatic activity and without subunit dissociation (Shrake et al., 1989). By independently monitoring perturbations in Trp and Tyr environments, the thermal transition was shown to be non-two-state and involved at least two structural domains: one containing Trp and unfolding at a temperature about 2 °C lower than the other containing Tyr residues. The magnitude of spectral changes corresponded to a net exposure of 8 of the 24 Trp and 24 of the 144 buried Tyr residues per GS dodecamer. These changes were similar, although smaller than those observed for the formation of inactive apoenzyme by Mn^{2+} removal (Hunt & Ginsburg, 1972; Shrake et al., 1989). Thus, thermally induced reactions lead to an intermediate state along the unfolding pathway. Differential scanning calorimetry (DSC) showed a single endothermic peak (0.34 cal/g with a small, positive ΔC_p)[2] centered approximately between the temperatures of half-completion of the Trp and Tyr spectral progress curves (Ginsburg & Zolkiewski, 1991). The DSC results indicate a net exposure of hydrophobic groups.

† Presented in part at the Annual Meeting of the Biophysical Society, Feb 24–28, 1991 (Zolkiewski et al., 1991), and the ASBMB/Biophysical Society Joint Meeting, Feb 9–13, 1992 (Zolkiewski & Ginsburg, 1992).

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[1] Abbreviations: GS, unadenylylated glutamine synthetase dodecamer from *Escherichia coli* containing 0.8 equiv of covalently bound 5′-AMP; Mn·GS, manganese ion complex of GS [24 Mn^{2+} bound per native GS with 1.0 free Mn^{2+} at pH 7 (Hunt & Ginsburg, 1972)]; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Pipes, 1,4-piperazinediethanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane; Tes, *N*-[tris(hydroxymethyl)methyl]-2-aminoethanesulfonic acid; MetSox, L-methionine (*SR*)-sulfoximine; DSC, differential scanning calorimetry.

The low value of the enthalpy change suggests the occurrence of some compensating heat effects during disruption of active sites, each of which contains eight antiparallel β strands—six β strands from the C-terminal subunit domain and two β strands from the N-terminal domain of an adjacent subunit (Almassy et al., 1986; Yamashita et al., 1989).

By comparing the calorimetrically measured enthalpy of unfolding with van't Hoff enthalpies from DSC and spectral studies and also by performing deconvolutions of the DSC curves, two thermodynamic domains in dodecameric Mn·GS were detected (Ginsburg & Zolkiewski, 1991). The unfolding of each domain appeared to occur as a simple 2-state process, but transformations of structural portions of all 12 subunits of the protein are involved. Another example of thermodynamic cooperativity between different polypeptide chains in an oligomeric enzyme has been found by Bhakuni et al. (1991) in studies on the pentameric B-subunit of cholera toxin.

This paper reports on a continuation of calorimetric investigations of the thermally induced, partial unfolding of Mn·GS. We have tried to gain information on the partially unfolded state and the cooperative domains by studying the thermally induced unfolding of Mn·GS in the presence of active-site ligands. Ligands which preferentially bind to one domain or span both domains can alter the intrinsic stabilities of domains as well as the interactions between domains (Brandts et al., 1989). A difference in the affinity of a ligand for the native and partially unfolded forms also changes the thermodynamic parameters of unfolding. Moreover, the magnitude of proton uptake/release during unfolding was evaluated here to obtain information on the exposure of ionizable groups during unfolding since changes in domain interactions potentially can be detected by proton effects.

Recently, Straume and Freire (1992) proposed to treat the ligand concentration in a series of DSC experiments with a macromolecule as a second independent variable for a global data analysis and applied this method to a two-state unfolding process for which a simple binding mechanism was applicable. Their approach in such cases allows for the simultaneous determination of the thermodynamic parameters for both protein unfolding and ligand binding. In concurrent studies of a more complex system (i.e., non-2-state unfolding of GS with 12, possibly interacting, ligand binding sites per macromolecule which may or may not be disrupted by partial unfolding), we chose instead to obtain ligand binding parameters at different temperatures experimentally and to apply them subsequently to the analysis of DSC data.

MATERIALS AND METHODS

Chemicals and Enzyme Solutions. ADP, L-glutamine, MetSox (~1:1 *S*:*R* ratio of isomers of L-methionine sulfoximine; Shrake et al., 1982), Hepes, Pipes, and Tes were from Sigma, and Tris was obtained from Bethesda Research Laboratories. Chemicals for the enzyme preparation and activity determinations were as described previously (Ginsburg & Zolkiewski, 1991; Hunt et al., 1975) or were of analytical grade. All aqueous solutions were prepared with distilled water that was deionized and filtered through a Millipore MilliQ2 reagent-grade system. This water also was used for rinsing all glassware and for cleaning calorimeter cells.

Unadenylylated glutamine synthetase was isolated from an overproducing *E. coli* strain (Maurizi & Ginsburg, 1986) using the purification procedure of Woolfolk et al. (1966) with minor modifications (Ginsburg & Zolkiewski, 1991). Final specific activities in the pH 7.57 γ-glutamyl transfer assay at 37 °C (Stadtman et al., 1979) were 135 ± 3 units/mg. The enzyme was stored at 4 °C as a suspension in 52% $(NH_4)_2SO_4$; protein samples were collected by centrifugation and then dialyzed at 4 °C for ~36 h against three changes of ~1000-fold volumes of buffer, which usually was composed of 50 mM Hepes/KOH, 100 mM KCl, and 1.0 mM $MnCl_2$, pH 7.30 ± 0.01 at 30 °C. For some experiments, the buffer contained 100 mM KCl, 1.0 mM $MnCl_2$, and 50 mM Pipes/KOH, 50 mM Tes/KOH, or 50 mM Tris-HCl adjusted to pH 7.3 at 30 °C. For the experiments with MetSox and Gln, the appropriate concentrations of these ligands were present in the buffer during dialysis. After dialysis, enzyme samples were clarified by centrifugation and stored at 4 °C. Protein concentrations were determined from published absorption coefficients (Ginsburg et al., 1970), $A_{280} = 0.738$ and $A_{290} = 0.385$ cm²/mg, after applying fourth-power light-scattering corrections from the absorbance at 340 nm. Concentration measurements were performed on a Perkin-Elmer Model 320 spectrometer. Samples containing ADP were prepared by diluting a stock solution of ~0.1 M ADP (stored at pH 6.8 in water at –20 °C) into the final dialysate buffer and then adding this diluted ADP to protein samples and to reference buffers. The total concentration of ADP in buffer was determined from the absorbance at 259 nm (15 400 cm^{-1} M^{-1}), and the total GS concentration with ADP present was calculated from the absorbance at 290 nm.

Differential Scanning Calorimetry (DSC). DSC experiments were performed with a MicroCal MC-2 (MicroCal Inc., Northampton, MA) calorimeter equipped with tantalum cells (sample cell: 1.2206 mL) as described previously (Ginsburg & Zolkiewski, 1991). The pH of the solutions was checked before loading into the calorimeter, and the γ-glutamyl transfer activity of the enzyme was measured before and after DSC experiments; recovery of enzymatic activity after DSC was 93–98%. DSC scans (from 15 to 67–72 °C) were performed at a rate of 45 °C/h, which had been found not to affect the equilibrium of the process investigated (Ginsburg & Zolkiewski, 1991). The concentration of Mn·GS was generally in the range of 6.5–9.5 mg/mL. DSC data were corrected for instrumental base lines obtained with dialysate buffer in the absence of protein and were normalized for protein concentration using $M_r = 622\,000$ for Mn·GS; this gave plots of excess heat capacity in units of kilocalories per degree per mole of dodecamer versus temperature (in degrees centigrade).

After correction and normalization of DSC data, heat capacity plots were analyzed as described previously (Ginsburg & Zolkiewski, 1991) using the EXAM program by W. H. Kirchhoff (U.S. Department of Energy, National Institutes of Standards and Technology). This analysis allows the number of moles of cooperative units (N) per mole of protein to be varied and gives base lines in the form of sigmoidal progress curves. The area over the base lines corresponding to the enthalpy of unfolding (ΔH_{cal}) was calculated. T_m was obtained either as the temperature at half-area or at maximum C_p over the base line (C_{pm}). Deconvolutions of DSC data were performed as described previously (Ginsburg & Zolkiewski, 1991) using the software based on the procedures developed by Freire and Biltonen (1978a,b).

Isothermal Calorimetric Titrations. The calorimetric titrations were performed with a MicroCal OMEGA reaction cell connected to the MicroCal control module and interfaced

[2] Units of calories are used in this paper; for conversion to the International System of Units (SI), 1.000 cal = 4.184 J. When temperature in degrees centigrade is given, "t" is used; $t + 273.15 = T$ (K).

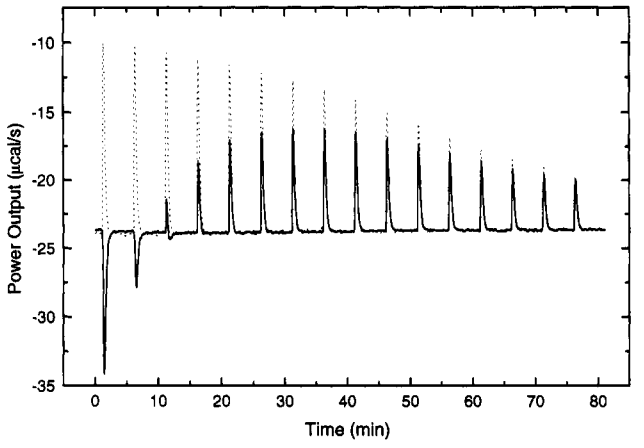

FIGURE 1: Calorimetric experiment showing the instrument output (μcal/s) vs time (min) for a titration of glutamine synthetase with ADP at 60 °C in 50 mM Hepes, 100 mM KCl, and 1.0 mM $MnCl_2$, pH 7. The data correspond to two series of 6-μL injections of 22 mM ADP into the buffer (dotted line) and into 0.093 mM Mn·GS subunit; 4.65 mg of protein/mL (solid line).

to an IBM PS 30 personal computer. The instrument design and the principles of operation and data analysis have been described by Wiseman et al. (1989). The calorimetric cells were made of Hastelloy-c alloy. The reference cell of the calorimeter was filled with distilled water, as recommended by the manufacturer. The titrating ligands were dissolved in the final dialysates, and the resulting pH was checked and found not to be affected by the ligands. The protein solutions and the buffers were thoroughly degassed before being loaded into the calorimeter. It was found that degassing the samples at approximately the temperature of the experiment led to greatly improved base-line stability. An example of a titration of Mn·GS with ADP at 60 °C is shown in Figure 1. In general, the heat of dilution was determined first by loading the dialysate into the sample cell (working volume, 1.4064 mL) and making a series of injections of the ligand solution. Then, the protein solution (concentration, 2.2–8.5 mg/mL, which corresponds to a GS subunit concentration of 0.04–0.17 mM) was loaded into the water-rinsed cell, and the same sequence of injections was repeated. Integration of the power output was performed using the software supplied with the instrument. The total heat effect for each injection was corrected for the heat of dilution. The thermodynamic parameters of binding for either one or two sets of independent binding sites were obtained by analyzing the data with the software provided by MicroCal Inc.

The cumulative heat effect (Q) during the titration process for a single set of binding sites is given by

$$Q = M_t V_0 n \nu \Delta H \quad (1)$$

and for two sets of binding sites

$$Q = M_t V_0 (n_1 \nu_1 \Delta H_1 + n_2 \nu_2 \Delta H_2) \quad (2)$$

where M_t is the macromolecule concentration in the calorimetric cell characterized by the working volume V_0, n is the number of binding sites in the given set with the binding enthalpy of ΔH, and ν is the fractional saturation of each type of sites which can be related to the apparent association constant (K') and the total ligand concentration (L_t):

$$K' = \nu/[(1-\nu)L] \quad (3)$$

and

$$L_t = L + M_t n \nu \quad (4)$$

or for two sets of noninteracting binding sites

$$K_1' = \nu_1/[(1-\nu_1)L];\ K_2' = \nu_2/[(1-\nu_2)L] \quad (5)$$

$$L_t = L + M_t(n_1\nu_1 + n_2\nu_2) \quad (6)$$

where L is the concentration of free ligand. In the data analysis, eq 1–6 are used to numerically obtain the values of n, K', and ΔH. Since the calorimetric cell is totally filled with solution during titration, the working cell volume V_0 is constant, but experimental values of the heat effects of consecutive injections of the ligand, as well as the total macromolecule and ligand concentrations after each injection, must be corrected for the volume of liquid displaced from the cell [for details, see the appendix in Lin et al. (1991)].

Fluorescence Titrations. The binding of ADP to Mn·GS was monitored by measuring the nucleotide-induced enhancement of the intrinsic tryptophanyl residue fluorescence (Maurizi & Ginsburg, 1986) with a Perkin-Elmer 650-40 fluorometer. The sample cell was kept in a jacketed four-cell holder connected to an external thermostated (±0.1 °C) water bath (Lauda K-2/R). The temperature of the sample was monitored with a digital thermometer (Omega 680) connected to a thermocouple probe immersed in a water-filled cuvette in the cell holder. The excitation and emission wavelengths were 300 and 340 nm, respectively. The initial GS subunit concentration in the titration experiment was ~3.3 μM or ~165 μg of protein/mL. Accurate injections of 1–2 μL of ADP solution were made by using Hamilton repeating dispensers.

Temperature-Induced Spectral Changes. UV spectra were recorded with Hewlett Packard Model 8450A rapid-scan, diode-array spectrophotometer with a temperature controller (Model 89100A) and temperature-controlled cuvette holders (Model 89101A) with magnetic stirrers and temperature probes. The reference cell containing the dialysate buffer was maintained at 30 °C. The temperature of the sample cell was increased in 1 °C increments in the transition region and 2 °C at the lower and higher temperatures. After the desired temperature was attained, the sample was allowed to equilibrate for 2 min, then 120 spectra were measured, and the average second-derivative spectrum [$A''(\lambda)$] was calculated. Usually, the thermal analysis was performed between 20 and 66 °C.

Thermally induced changes in tryptophanyl residue exposure were monitored by following the A''(295 nm) – A''(291 nm) peak–trough changes. Also, the r ratios (r = [A''(289 nm) – A''(285 nm)]/[A''(295 nm) – A''(291 nm)]) were calculated for exposures of tyrosyl residues (Ragone et al., 1984), although this is not a quantitative value with respect to the number of Tyr exposed per subunit (Ginsburg & Zolkiewski, 1991). Data for Trp and Tyr exposures were analyzed as previously described (Shrake et al., 1989) using a two-state equation to give $\Delta\epsilon''_{max}$ (or Δr_{max}), the midpoint temperature ($T_{0.5}$), and the van't Hoff enthalpy change (ΔH_{vH}). This analysis gave $\Delta\epsilon''_{max} = 229 \pm 21$ M subunit^{-1} cm^{-1} for the maximum change in Trp exposure in the absence and presence of MetSox or Gln and $\Delta\epsilon''_{max} = 97$ M subunit^{-1} cm^{-1} at saturating [ADP]; Δr_{max} values for Tyr exposure were as reported by Shrake et al. (1989).

RESULTS

The dodecameric structure of Mn·GS is resistant to disruption by external perturbations. The protein partially unfolds when heated, but unless the temperature exceeds ~67 °C, above which aggregation occurs, the transition is fully

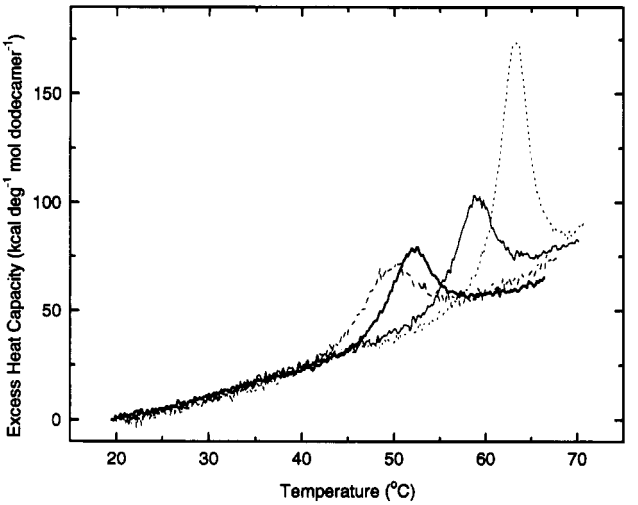

FIGURE 2: Representative DSC scans for glutamine synthetase in 50 mM Hepes, 100 mM KCl, and 1.0 mM $MnCl_2$, pH 7.3 (at 30 °C). The data were collected at a scan rate of 45 °C/h, corrected for the instrument base line, and normalized for protein concentration (see Materials and Methods). The data shown correspond to first scans of ligand-free Mn·GS (thick solid line) and of Mn·GS with saturating levels of the active-site ligands: 150 mM glutamine (thin solid line); 10 mM MetSox (dotted line); 0.37 mM ADP (dashed line).

reversible and the enzymatic activity is regained after cooling (Ginsburg & Zolkiewski, 1991). In earlier spectral studies, it was demonstrated that the temperature of transition can be affected by the presence of ligands binding to the active sites of GS (Shrake et al., 1989). This behavior also is shown by differential scanning calorimetry as illustrated in Figure 2. The data sets correspond to the first scans of GS in 50 mM Hepes, pH 7.3 at 30 °C, with 1 mM $MnCl_2$ and 100 mM KCl in the absence and presence of different active-site ligands. The data sets obtained in the presence of effectors correspond to the saturation of Mn·GS at low temperature with each of the following ligands: Gln and ADP, which are products of the biosynthetic reaction catalyzed by Mn·GS, and MetSox, which is an L-glutamate analogue bearing some features of the hypothetical transition-state molecule (Gass & Meister, 1970). As can be seen in Figure 2, the thermal unfolding reaction (±active-site ligands) produces a single peak in the excess heat capacity curve. The temperatures of unfolding (T_m) and the widths, heights, and thus the areas of the peaks are affected by the presence of high concentrations of such ligands. Under the conditions of Figure 2, Gln and MetSox have stabilizing effects (T_m increased) whereas ADP destabilizes the native protein (T_m decreased).

In the presence of ligands, the unfolding reaction is coupled to the ligand binding equilibria as shown in Scheme I where L denotes the free ligand and F (F·L) and U (U·L) are the folded and partially unfolded forms of dodecameric Mn·GS without (with) bound ligand, respectively. The stoichiometry of binding active-site ligands to the folded (F) form of Mn·GS is 12 equiv (Hunt et al., 1975; Shrake et al., 1977, 1980), and potentially ligands can bind also to the partially unfolded (U) form of Mn·GS. The thermodynamics of binding ligands to

Scheme I

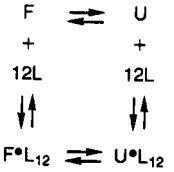

Table I: Thermodynamic Parameters for Binding Active-Site Ligands to the Native and Partially Unfolded Form of Mn·GS in 50 mM Hepes, 100 mM KCl, and 1 mM $MnCl_2$, pH 7.3 (at 30 °C)

ligand	t (°C)	log K_A'	ΔH_{bind}[a] (kcal/mol)	method[b]
MetSox	20	4.1	−12.1	1
	30	4.0 (3.8)[c]	−13.8 (−15.3)[c]	1
	40	3.7	−16.1	1
	60		~0	1, 2
Gln	30	2.1	−9.7	1[d]
	60	1.3	−12.3	1
ADP	30	5.5		3[e]
		5.5		4
		5.3	−7.5	1
		5.5	−6.8	1[f]
	60	4.1		4
		4.3	−7.1	1[g]
		3.6	−13.2	
		4.4	−6.9	1[f,g]
		3.6	−10.1	

[a] Enthalpies of binding (expressed per mole of binding site) were uncorrected for proton effects for calculations using eq 9 (Figure 7). [b] Methods: (1) calorimetry; (2) UV spectroscopy; (3) equilibrium dialysis; (4) fluorometry. [c] Gorman & Ginsburg (1982). [d] Shrake et al. (1977). [e] Value at 25 °C of Hunt et al. (1975) corrected to 30 °C (Shrake et al., 1977). [f] Pipes substituted for Hepes in the buffer. [g] Parameters obtained by fitting to the model of two independent binding sites per GS subunit.

both forms of Mn·GS therefore were investigated so that DSC data could be properly analyzed.

The temperatures of 30 and 60 °C were chosen as the reference points at which the native and the partially unfolded states of Mn·GS would be predominantly populated, respectively, under our standard conditions without added ligand. That is, only about 0.4% of the area under the DSC curve obtained in the absence of ligands extends beyond 60 °C (Figure 2). In addition, binding parameters for MetSox were obtained for other temperatures below the thermal transition.

Table I summarizes the thermodynamic parameters for binding the three active-site ligands used in this study. Agreement with literature values (when available) was good. All calorimetric titration curves at ≤40 °C were hyperbolic (noncooperative) and could be fitted to a model for a single (noninteracting) binding site per subunit within experimental error, i.e., with a standard deviation of ≤5 μcal per injection. When the value of n in eq 1 (see Materials and Methods) was allowed to vary as one of the parameters of the fit during data analysis, final n values were 0.8–1.1. The binding parameters reported in Table I were obtained when n was fixed at n = 1. Attempts to fit for a higher stoichiometry of binding led to numerically insignificant parameters for additional binding sites.

Figure 3 shows calorimetric titration data for ADP additions to two different concentrations of Mn·GS subunit and the fitted curves, which gave the ADP binding parameters at 30 °C in Table I. The different enthalpies of binding observed in Hepes and Pipes buffers (Table I) correspond to the release of ~0.3 H^+/GS subunit upon ADP binding, as calculated from buffer protonation enthalpies at 30 °C (Ginsburg et al., 1987). Previous estimates at 30 °C gave ~0 H^+ release or uptake for Gln binding (Shrake et al., 1977) and ~0.7 H^+ uptake for binding a mixture of the S and R diastereoisomers of MetSox to Mn·GS (Gorman & Ginsburg, 1982).

The binding of ligands to partially unfolded Mn·GS at 60 °C was more complex than to the folded enzyme at 30 °C. In the case of MetSox, no heat of binding at 60 °C was detected even when a large excess of MetSox (18-fold K_D' at 30 °C) was added to the protein in the calorimeter. Moreover, the addition of 10 mM MetSox to Mn·GS at 60 °C produced no

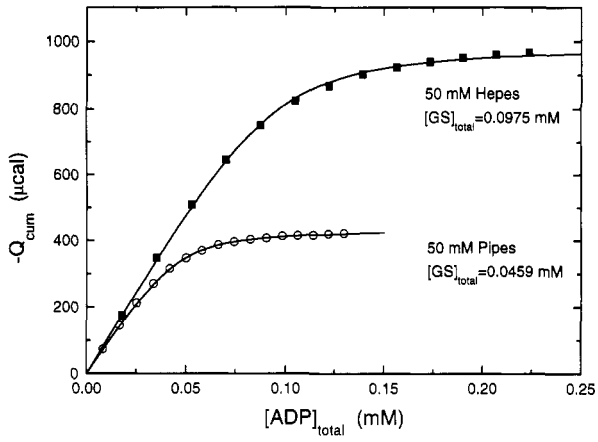

FIGURE 3: Calorimetric titrations of Mn·GS with ADP at 30 °C in 50 mM Hepes or 50 mM Pipes, 100 mM KCl, and 1.0 mM $MnCl_2$, pH 7.3, with the protein subunit concentration indicated. The data points show the total cumulative heat effect during the titration in the Microcal Omega calorimeter (see Materials and Methods). The data were fitted with the single-site binding model (solid lines), and the fitting parameters are given in Table I.

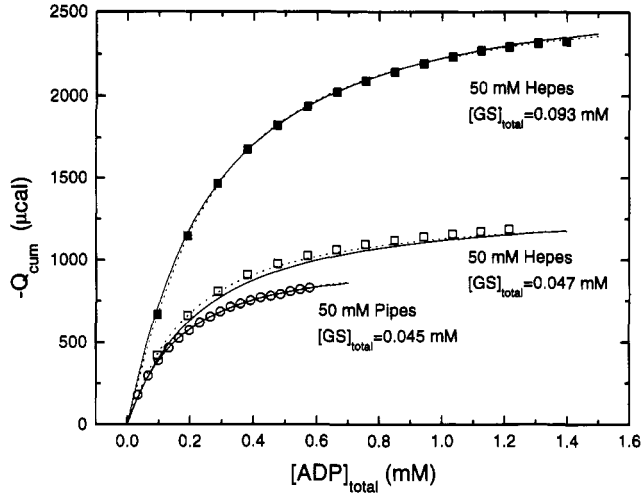

FIGURE 4: Calorimetric titrations of Mn·GS with ADP at 60 °C in 100 mM KCl, 1.0 mM $MnCl_2$, and 50 mM Hepes or 50 mM Pipes (pH 7) with the concentrations of GS subunit indicated. The data points show the cumulative heat effects during each titration experiment. Models for one binding site per subunit (solid lines) and two independent binding sites per subunit (dotted lines) were fitted to the data. Either model was fitted simultaneously to both data sets in Hepes buffer, and the resulting parameters for the two-site model are given in Table I.

spectral perturbations whereas pronounced changes in the absorption spectrum of Mn·GS in the region of Tyr and Trp absorbance (270–300 nm) are promoted by this active-site ligand at 30 °C (Shrake et al., 1980). Thus, both spectral and calorimetric results indicate that MetSox does not bind to Mn·GS at 60 °C. However, Gln does bind to one site per subunit of Mn·GS at 60 °C but with $\sim^1/_7$th the affinity observed at 30 °C. Since the effects of ADP on the thermal transition of Mn·GS were unusual (Figure 6C *below*), the binding of ADP to Mn·GS was investigated by both fluorescence and calorimetric titrations.

Binding of ADP to Mn·GS at either 30 or 60 °C produces a strong increase in the intrinsic tryptophanyl fluorescence (~2.8-fold at 340 nm). Hill plots (*not shown*) were constructed from fluorescence titration data obtained in Hepes and Pipes buffers assuming that the fractional saturation of Mn·GS with ADP is equal to $\Delta F/\Delta F_{max}$, where ΔF and ΔF_{max} are the observed and maximum fluorescence changes, respectively, and that $[ADP]_{free} = [ADP]_{total} - (\Delta F/\Delta F_{max})\text{-}[GS\ subunit]_{total}$. Plots were linear, and the slopes (n_H values) at 30 and 60 °C were $0.8_6 \pm 0.1$ and 1.4 ± 0.1, respectively. A Hill coefficient of >1 suggests positive cooperativity in binding ADP to dodecameric Mn·GS or in the fluorescence change produced by ADP binding, and/or multiple ADP binding sites at 60 °C. The binding constants from fluorescence titrations agreed well with those obtained by other methods and also indicated that the affinity of Mn·GS for ADP was ~20-fold greater at 30 °C than at 60 °C (Table I).

Figure 4 shows the calorimetric data for the titration of Mn·GS with ADP at 60 °C. The software for data analysis allows for simultaneous fitting of more than one data set which leads to more accurate values of binding parameters and provides a better test for the binding model assumed. The two data sets shown in Figure 4 that correspond to two different GS concentrations in Hepes buffer were simultaneously analyzed assuming either one or two (noninteracting) binding sites for ADP per subunit of GS (setting $n = 1$ or $n_1 = n_2 = 1$). The data are more accurately described by a model for two binding sites per subunit (SD = 12 μcal) than for a one-site model (SD = 25 μcal). The same test applied to the single set of titration data obtained in Pipes buffer at one protein concentration led to indistinguishable theoretical curves for one- and two-site models. This results from the fact that the binding constants for the two sites (Table I) differ by less than an order of magnitude and the data from a single titration at one protein concentration do not discriminate between these values and an intermediate binding constant for only one site, but with higher binding enthalpy. Thus, a global fit of calorimetric titration data for different protein concentrations improves the reliability of fitting procedures and the accuracy of binding parameters.

The binding of ADP to the higher and lower affinity sites at 60 °C was linked to the release of ~0.1 and ~1.3 H^+/GS subunit, respectively, as determined by applying the buffer protonation enthalpies at 60 °C, extrapolated from those at 30 °C (Ginsburg et al., 1987) and 50 °C (*see below*). Note that the enthalpy of binding of ADP to the higher affinity site at 60 °C is approximately the same as that measured at 30 °C.

It is important to realize that because of the differences in the binding affinities of the native and partially unfolded states, the additions of the ligands could have disturbed the unfolding equilibrium and increased the concentration of a state that had been virtually unpopulated in the absence of ligands. However, none of the ligands studied induced the unfolding transition at 30 °C since all interacted preferentially with the folded, native enzyme. For Gln binding at 60 °C, the fitting procedure enabled us to use sufficiently low ligand concentrations (≤20 mM Gln) to prevent shifting the transition temperature to the vicinity of 60 °C. The heat effects of those shifts were calculated and found negligible compared to the binding heats. The same was assumed to be the case for the binding of ADP: as shown by the data in Table II, increasing concentrations of ADP produced small increases in T_m and then T_m decreases. Consequently, ADP could not increase the population of the native state of Mn·GS during the titration experiments at 60 °C.

Table II presents the thermodynamic parameters of unfolding of GS with different concentrations of the active-site ligands. For comparison, the parameters obtained under our standard conditions without the ligands are shown (Ginsburg & Zolkiewski, 1991). In addition, the parameters for several data sets obtained in Pipes (substituted for Hepes) buffer ±

Table II: Thermodynamic Parameters for the Partial Unfolding of Mn·GS in the Presence of Active-Site Ligands[a]

ligand	ν(30 °C)	ν(60 °C)	t_m (°C)	ΔH_{cal} (kcal/mol of dodecamer)	CR
Buffer: 50 mM Hepes, 100 mM KCl, and 1.0 mM $MnCl_2$, pH 7.0 (50 °C)					
			51.6	211	1.6
MetSox	0.51	0.0	53.7	326	2.2
	0.92	0.0	57.2	431	2.3
	0.99	0.0	63.0	462	2.2
Gln	0.42	0.09	52.3	254	2.0
	0.88	0.50	56.1	259	1.7
	0.94	0.67	57.6	237	1.6
	0.96	0.75	58.7	228	1.4
ADP	0.18	0.13	52.0	230	1.9
		0.02			
	0.27	0.18	53.2	255	2.0
		0.03			
	0.50	0.31	53.0	307	2.4
		0.05			
	0.85	0.50	53.6	288	2.0
		0.09			
	0.99	0.78	48.5	231	2.0
		0.14			
Buffer: 50 mM Pipes, 100 mM KCl, and 1.0 mM $MnCl_2$, pH 7.1 (50 °C)					
			52.8	225	1.8
ADP	0.20	0.14	54.0	236	1.8
		0.03			
	0.73	0.38	55.1	350	2.5
		0.06			
	0.99	0.78	51.1	224	1.9
		0.14			

[a] For the definitions of parameters and standard deviations, see Results. Protein concentrations were 3.55–9.60 mg/mL. Values of ν(30 °C) and ν(60 °C) in the absence of ADP are for saturation of 1 site/subunit; with ADP present, ν(60 °C) values give first and second site per subunit saturations.

ADP are reported. The extents of saturation (ν) at 30 and 60 °C were calculated from the known total concentrations of ligand and protein and the binding parameters of Table I. For MetSox and Gln, the intial free ligand concentrations were the same as in final dialysates. For ADP data sets, saturation values are given also for 30 °C and the higher and lower affinity binding sites at 60 °C.

The cooperative ratio (CR) in Table II is defined as $\Delta H_{cal}/\Delta H_{vH}$, where ΔH_{vH} is the van't Hoff enthalpy calculated from $\Delta H_{vH} = 4RT_m^2C_{pm}/\Delta H_{cal}$ (Privalov & Khechinashvili, 1974). The cooperative ratio is conceptually and numerically identical to the parameter N obtained from the quasi-two-state analysis with the EXAM program used here. For all of the conditions given in Table II, $1.4 \leq CR \leq 2.5$. A deviation from the two-state mechanism toward intermolecular cooperativity is indicated by $CR < 1$, or the presence of multiple intramolecular domains is suggested by $CR > 1$. The values of T_m, ΔH_{cal}, and CR reported in Table II for MetSox and Gln are the mean values of three independent determinations. The standard deviations were 0.1–0.2 °C for T_m, 4–10 kcal/mol for ΔH_{cal}, and 0.1 for CR. For ADP, each data set corresponds to a single DSC experiment at a representative ADP concentration. For the sake of brevity, only 8 of the 11 DSC experiments performed in the presence of ADP are listed in Table II. However, all of these data are used in the calculations of ΔH^0_{cal} presented in Figure 7 *below*.

The thermal unfolding of Mn·GS was monitored also by second-derivative UV absorption spectroscopy. Data for thermally induced changes in Trp or Tyr exposure were fitted to a two-state equation (Shrake et al., 1989) to obtain the midpoint temperature ($T_{0.5}$). Changes in values of $T_{0.5}$ produced by the presence of the active-site ligands were found to follow the same patterns as T_m values observed in DSC (*see* Figure 6 and Discussion *below*).

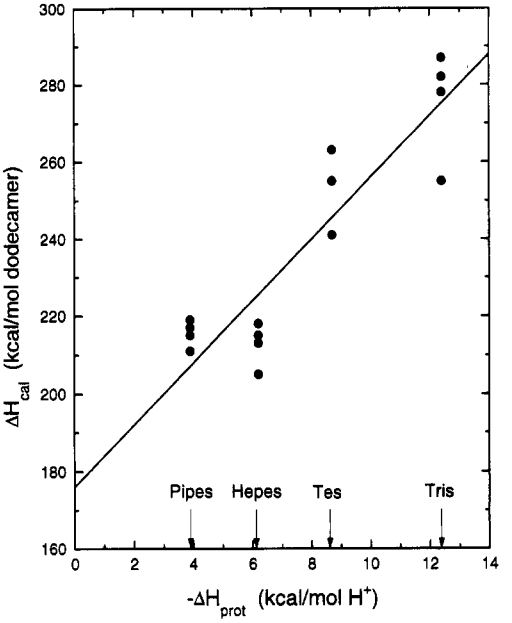

FIGURE 5: Observed enthalpy of unfolding of Mn·GS as a function of the enthalpy of deprotonation of the buffer component at 50 °C. Each experimental point corresponds to the result from a DSC scan in either 50 mM Pipes, Hepes, Tes, or Tris which contained 100 mM KCl and 1.0 mM $MnCl_2$ (adjusted to pH 7.3 at 30 °C). The enthalpy values were extrapolated to 51.6 °C (corresponding to the t_m observed in Hepes; see Results). The solid line is a linear least-squares fit to the data with the slope equal to 8.0 equiv of H^+ uptake/Mn·GS dodecamer.

The partial unfolding of the protein can involve a loosening of structures, with possible exposure of ionizable groups. If proton exchange between the biopolymer and buffer components is accompanied by a measurable enthalpy change, the calorimetrically determined enthalpy of unfolding will be influenced. In order to assess the magnitude of proton effects, DSC experiments were performed in four buffers with different enthalpies of protonation. The protonation enthalpy for each of the four buffers was measured at 50 °C in order to apply each value directly to calculate proton effects in DSC experiments with Mn·GS ($t_m \sim 51$ °C). Protonation enthalpies were determined by adding small amounts of HCl to an excess of buffer in the MicroCal Omega isothermal titrator; final results are averages from a series of 10 injections. The enthalpies of protonation at 50 °C (with SD ≤ 0.1 kcal/mol of H^+) were determined to be −3.8 kcal/mol of H^+ for Pipes, −6.1 kcal/mol of H^+ for Hepes, −8.7 kcal/mol of H^+ for Tes, and −12.3 kcal/mol of H^+ for Tris.

As the temperature increased in DSC experiments, each buffer solution reached a slightly different pH because all four buffers were adjusted to pH 7.30 at 30 °C and protonation enthalpies differ. This resulted in varying T_m values for the unfolding of Mn·GS in the different buffers, i.e., from t_m = 50.9 °C in Tes to t_m = 52.8 °C in Pipes buffer. Taking into account the differences in T_m, the measured ΔH_{cal} value in each buffer was corrected for its temperature dependence using the apparent $\Delta C_p(T_m)$ values obtained from data analysis (where ΔC_p corresponds to the shift between pre- and posttransitional base lines extrapolated to the transition T_m). The ΔC_p values, which are extremely sensitive to the quality of data below and above the transition, were 8.8 ± 2.2 kcal/(K·mol) in Pipes, 10.6 ± 0.9 kcal/(K·mol) in Hepes, 6.5 ± 1.1 kcal/(K·mol) in Tes, and 6.4 ± 0.9 kcal/(K·mol) in Tris. These values were used to calculate ΔH_{cal} at 51.6 °C (i.e., at the average T_m for the unfolding of Mn·GS in Hepes buffer), and the resulting enthalpies are shown in Figure 5. Each data

point corresponds to a single DSC experiment performed in the buffer indicated. The slope of the least-squares fit in Figure 5 gives 8.0 ± 1.9 mol of H^+ bound/mol of Mn·GS dodecamer during partial unfolding, and the intercept gives 176 ± 12 kcal/mol for ΔH_{cal} in a hypothetical buffer with $\Delta H_{prot} = 0$.

Deconvolutions of the DSC curves in the different buffers of Figure 5 revealed the same cooperative pattern of the unfolding reaction as that previously observed in Hepes buffer (Ginsburg & Zolkiewski, 1991), i.e., two thermodynamic domains unfolding as two two-state transitions. The enthalpy of unfolding for each domain was found to depend on the enthalpy of buffer protonation. However, the variation in T_m values made it impossible to quantitate the latter effects in order to assign the equivalents of H^+ uptake to either of the two domains.

DISCUSSION

The 12 active sites of GS are located at intraring interfaces between enzyme subunits arranged in 2 hexagonal rings and are formed by N- and C-terminal domains of 2 polypeptide chains (Almassy et al., 1986). If the identification of the cooperative units within the GS dodecamer is as proposed earlier (Shrake et al., 1989; Ginsburg & Zolkiewski, 1991), the active sites contain parts of both thermodynamic domains. Thus, any ligands bound to the active sites, in principle, can influence the thermodynamic stability of one or both domains as well as the interactions between them. Also, the partial unfolding process could alter the structure of the active sites to the extent that the binding of the ligands is strongly affected.

The difference in binding affinities of the ligand to the pre- and posttransitional states of the macromolecule can shift the equilibrium between these states at any given temperature and thus the T_m of transition. The shift in T_m can be related to the concentration of the ligand and the binding properties of the initial and final state by the equation (Schellman, 1975):

$$1/T_m = 1/T_m^0 - (R/\Delta H^0) \ln (\Sigma_f/\Sigma_u) \quad (7)$$

where T_m and T_m^0 are the midtemperatures of transition in the presence and absence of the ligand, respectively, ΔH^0 is the temperature-independent enthalpy of unfolding, and Σ_f and Σ_u refer to the binding polynomials of the folded and unfolded state, respectively, which in the case of n independent binding sites are given as

$$\Sigma = (1 + K_1L)(1 + K_2L)...(1 + K_nL) \quad (8)$$

where L is the concentration of free ligand and K_i is the single-site association constant.

In the derivation of eq 7, several important assumptions were made (Schellman, 1975). First, the transition was treated as a two-state process with only the folded and unfolded states populated during the course of the reaction. Second, the enthalpy and entropy of unfolding were assumed to be temperature-independent. Third, the enthalpy of ligand binding should be negligible compared to the enthalpy of unfolding, which allows treatment of the binding constants for both thermodynamic states as temperature-independent parameters. Despite these simplifications, eq 7 is more general than other approaches that assume total ligand release during unfolding (Fukada et al., 1983; Shrake & Ross, 1990).

Since ligands can bind to both folded and partially unfolded Mn·GS, we have used the formalism of eq 7 to present DSC results (Figure 6A,B). However, it should be recognized that the thermal unfolding of Mn·GS does not conform to the assumptions implicit in eq 7. Earlier studies showed that the transition is non-two-state and the measurable ΔC_p of unfolding indicates that the enthalpy is temperature-dependent (Gins-

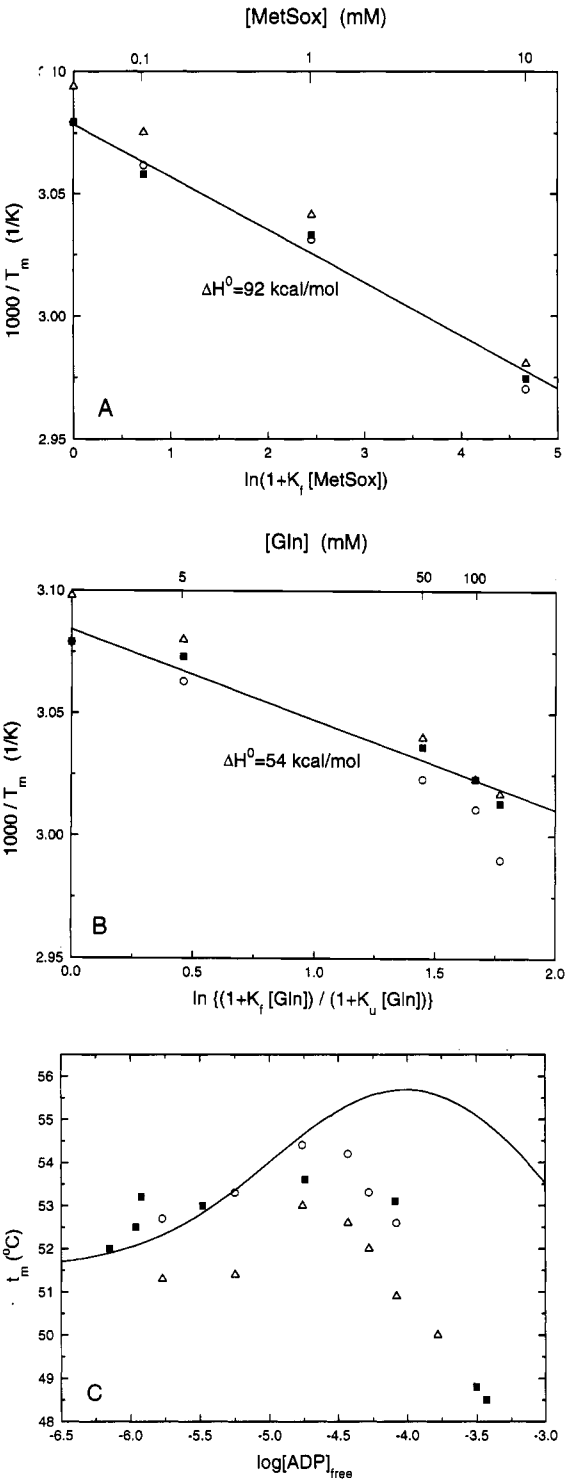

FIGURE 6: Effects of MetSox (A), Gln (B), and ADP (C) on the temperature of unfolding (T_m) of Mn·GS in 50 mM Hepes, 100 mM KCl, and 1.0 mM $MnCl_2$ buffer, pH 7.3 at 30 °C. In panels A and B, $1/T_m$ is plotted as a function of the logarithm of the ratio of binding polynomials for folded and unfolded GS (see eq 7). Panel C shows t_m as a function of free ADP concentration. The T_m data from DSC (solid squares), Trp exposure (open triangles), and Tyr exposures (open circles) are shown in panels A–C. Solid lines in panels A and B correspond to linear least-squares fits to the DSC data and in panel C to the curve simulated with eq 7 and the binding parameters from Table I. The data for MetSox and Gln (in panels A and B) are the mean values of three determinations, whereas for ADP (in panel C) each data point corresponds to a single experiment. In panel C, Tyr exposure could not be measured at the highest concentration of ADP due to interference from ADP absorbance.

burg & Zolkiewski, 1991). Moreover, the thermal transition of Mn·GS represents only a partial unfolding so that the heats of ligand dissociation can be as much as the heat of unfolding (Table II). Also, the ligand binding affinity can depend on temperature even for the same thermodynamic state of the protein, as demonstrated by the data for MetSox in Table I.

As can be seen in Figure 6A,B, the dependence of T_m on the concentrations of MetSox and Gln can be described by the linear eq 7. For these cases, the measured association constants at 30 and 60 °C for ligand binding affinities of the folded and partially unfolded states of Mn·GS, respectively, were used to calculate the binding polynomials in eq 7. For MetSox (Figure 6A), the apparent lack of binding to the partially unfolded enzyme was taken into account. The straight lines shown correspond to the least-squares fits of the data sets of calorimetric T_m values. The ΔH^0 values obtained from the slopes of these straight lines were found to be 92 ± 7 kcal/mol from the data obtained with MetSox and 54 ± 8 kcal/mol for those with Gln. Thus, the apparent stabilizing effects of MetSox and Gln on Mn·GS (increases in T_m) can be completely accounted for by the differences in the free energy of binding these ligands to the native and partially unfolded forms of the dodecameric enzyme. The temperature-independent parameter ΔH^0 obtained in the presence of MetSox approximates the values of the van't Hoff enthalpy for the thermal unfolding of Mn·GS in the absence of active-site ligands, as determined from two-state analysis of spectral progress curves for Trp and Tyr exposures (Shrake et al., 1989) and from DSC data (Ginsburg & Zolkiewski, 1991). The ΔH^0 value obtained with Gln (Figure 6B) may differ because the binding of Gln to the partially unfolded enzyme produces a different final state than in the absence of Gln.

Figure 6C shows T_m values for the partial unfolding of Mn·GS as a function of ADP concentration. The solid curve of Figure 6C is that predicted from eq 7 for 24 binding sites/dodecamer for ADP at high temperature with 2 different binding affinities (Table I). A maximum in T_m occurs as the concentration of ADP is increased. The DSC data show the same trend although experimental T_m values decrease more sharply at high [ADP] than does the calculated curve. This suggests that the binding affinity of ADP to partially unfolded Mn·GS at T_m is significantly higher than that determined at 60 °C. Both calorimetric titrations at 60 °C and the variation of T_m values in DSC experiments with varying [ADP] indicate that the partially unfolded enzyme has 2 binding sites/subunit for this nucleotide. Fluorescence titrations of Mn·GS with ADP at 30 and 60 °C also suggested that the partially unfolded enzyme bound >1 equiv of ADP/subunit since the Hill coefficient (n_H) was 1.4 at 60 °C as compared to $n_H = 0.9$ at 30 °C.

Figure 6A–C shows calorimetric T_m values as well as $T_{0.5}$ values from spectral progress curves for Trp and Tyr exposures as functions of the appropriate binding polynomials. The thermally induced changes in Trp environment occur at about 2 °C lower temperatures than those for Tyr for all the ligands studied. The presence of MetSox or Gln did not significantly influence the amplitudes of second-derivative differences used for calculating Trp and Tyr exposures. However, the molar absorbance change for Trp (but not Tyr) was dependent on the concentration of ADP, and at 0.17 mM ADP, Trp exposure was decreased ~60% due to ADP binding to partially unfolded Mn·GS (Materials and Methods). The calorimetric T_m values generally were between the $T_{0.5}$ values obtained for Trp and Tyr exposures at the same concentration of free ligand. Thus, in the presence as well as in the absence of active-site ligands, the two thermally labile subunit domains containing Trp and Tyr (Shrake et al., 1989) unfold as portions of the overall two thermodynamic domains of the dodecamer (Ginsburg & Zolkiewski, 1991).

The analysis of heat capacity data from DSC can provide insight into the mechanism and energetics of thermally induced transitions in macromolecules (Freire & Biltonen, 1978a,b). In the presence of ligands, DSC data also contain thermodynamic information concerning the ligand binding/dissociation reactions coupled to the transition of the biopolymer. A rigorous analysis of DSC data in such cases is complex and must involve studies of ligand binding reactions over a wide temperature range (both below and above the temperature of transition) so that the thermodynamic description of the macromolecule–ligand interactions as a function of temperature can be established. Unfortunately, this information is rarely accessible since frequently at temperatures above the thermal transition of a protein, irreversible aggregation makes binding studies impossible.

Ligands binding to m independent sites on the folded macromolecule and to n (noninteracting) sites on the unfolded one influence the enthalpy of unfolding in the following way:

$$\Delta H_{cal}(L) = \Delta H^0_{cal} + \sum_{i=1}^{n} \nu_i^u(L)\Delta H_i^u - \sum_{i=1}^{m} \nu_i^f(L)\Delta H_i^f \quad (9)$$

where ΔH^0_{cal} and $\Delta H_{cal}(L)$ are the enthalpy of unfolding the protein in the absence and presence of ligands, respectively, ΔH_i^f (ΔH_i^u) is the enthalpy of binding ligand to site i on the folded (unfolded) protein, and ν_i^f (ν_i^u) is the corresponding fractional saturation of sites i given by

$$\nu_i = K_i'L/(1 + K_i'L) \quad (10)$$

where K_i' is the apparent association constant for independent sites i. The parameters ΔH^0_{cal}, ΔH_i, and ν_i generally are temperature-dependent which makes eq 9 difficult to apply rigorously in real cases. Whereas the unfolding enthalpies are determined at T_m, binding parameters are measured at different temperatures so that extrapolation to T_m is necessary.

For MetSox, binding parameters measured at three temperatures below T_m were used to calculate $\Delta C_p = -200 \pm 30$ cal/(K·mol) for MetSox binding to native Mn·GS, which is about the same value as previously estimated for the binding of L-Met-(*S*)-sulfoximine to the Mn·GS·nucleotide complex at 25–30 °C (Ginsburg et al., 1987). This value was used to calculate binding constants and enthalpies of binding at T_m for three different MetSox concentrations: 1400 M^{-1} and −18.8 kcal/mol at 0.1 mM MetSox (t_m = 53.7 °C); 1000 M^{-1} and −19.5 kcal/mol at 1.0 mM MetSox (t_m = 57.2 °C); 600 M^{-1} and −20.7 kcal/mol at 10 mM MetSox (t_m = 63.0 °C). Thus, the hypothetical fractional saturation (ν_i^f) at T_m is 0.12, 0.51, and 0.86 at 0.1, 1.0, and 10 mM MetSox, respectively, and $\nu_i^u = 0$.

For Gln and ADP, it was assumed that the parameters measured at 30 and 60 °C characterize the binding properties of the native and partially unfolded GS and that their temperature dependence extrapolated to T_m can be neglected. In such cases, ligand-induced changes in the enthalpy of unfolding can be obtained from calorimetric titration curves at 30 and 60 °C after simulation for the total protein concentration used in DSC experiments.[3]

Figure 7 presents the data of the enthalpy of unfolding after the correction for ligand effects as described above. Values of ΔH^0_{cal} are now a function of temperature only and should display a behavior characterized by the temperature-inde-

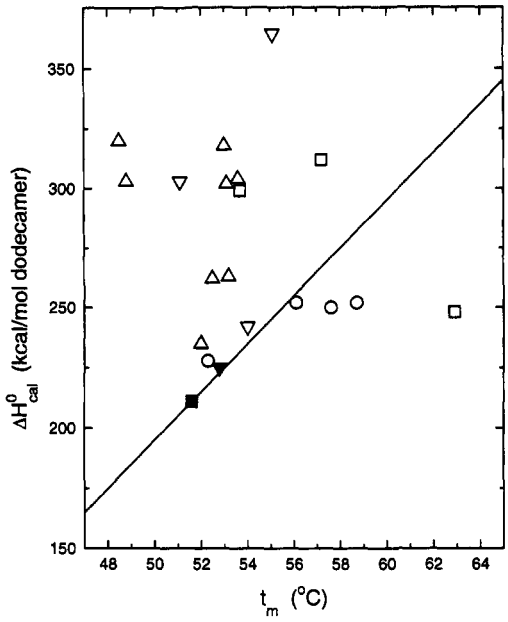

FIGURE 7: Enthalpy of unfolding of Mn·GS corrected for the enthalpies of ligand binding/dissociation (ΔH^0_{cal}) plotted as a function of the t_m of unfolding. The data point obtained in 50 mM Hepes, 100 mM KCl, and 1.0 mM $MnCl_2$, pH 7.3, at 30 °C in the absence of ligands is shown as a solid square, and the corresponding temperature-dependent function shown by the solid line is that predicted from ΔC_p = 10.1 kcal/(deg·mol) (Ginsburg & Zolkiewski, 1991). Data points obtained in the presence of MetSox (open squares), Gln (open circles), and ADP (open triangles) were approximated as described in the text. Also, inverted triangles are shown for data obtained in the absence of ligands (solid symbol) and in the presence of ADP (open symbols) in a buffer containing 50 mM Pipes. The data for MetSox and Gln are the mean values of three determinations, whereas for ADP each data point corresponds to a single DSC experiment.

pendent ΔC_p of unfolding, as has been found for many small globular proteins when variations in pH were used to change T_m values (Privalov & Khechinashvili, 1974). However, ΔC_p does vary in the presence of active-site ligands, as illustrated by the changes in the posttransitional base lines in Figure 2. Previously, ΔC_p also was found to depend on the concentration of Mn^{2+} at 1–10 mM Mn^{2+} (Ginsburg & Zolkiewski, 1991), where T_m is decreased by Mn^{2+} binding to additional sites on the partially unfolded enzyme (Shrake et al., 1989). Figure 7 shows that with MetSox, for which the most rigorous corrections were made, and with Gln, the data points are scattered around the linear function shown by the solid line in Figure 7, ΔC_p = 10.6 kcal/(mol·K) for Mn·GS (Ginsburg & Zolkiewski, 1991).

The data obtained in the presence of ADP (Figure 7) cannot be described by any simple ΔC_p function since ΔH^0_{cal} values at T_m both below and above the reference 51.6 °C are much higher than expected. This is evident at >27% saturation of Mn·GS with ADP. The higher apparent enthalpy values with ADP present are not due to proton effects since measurements in a buffer with a lower heat of protonation (Pipes) gave essentially the same results (T_m and ΔH_{cal} values) as in Hepes buffer (Figure 7). Possibly, the saturation of >2–3 subunits in the dodecamer with ADP produces an increased enthalpy of domain interactions (i.e., the additivity of the unfolding and binding enthalpies implicitly assumed in eq 9 does not hold for higher ADP concentrations). In addition, the ΔC_p value with ADP bound to the partially unfolded enzyme appears to differ from that in the absence of added ligands (Figure 2).

The studies reported here show different ways that small molecules can influence the thermodynamics of unfolding a macromolecule. The partial unfolding of Mn·GS was shown to involve a net uptake of eight protons per dodecamer. This results from an exposure of polar amino acid side chains during unfolding, and is consistent with the increased number of Mn^{2+} binding sites observed earlier (Shrake et al., 1989). MetSox does not bind to the partially unfolded form of Mn·GS and can increase T_m by as much as ~11 °C. Gln binds to the partially unfolded enzyme form, but more weakly than to folded Mn·GS. Both folded and partially unfolded Mn·GS also bind ADP, as had been suggested by spectral studies (Shrake et al., 1989). Moreover, the present studies indicate that an additional ADP binding site per subunit is exposed upon the thermal unfolding of Mn·GS. This led to an observed maximum in T_m at intermediate concentrations of ADP.

As the temperature is increased, the binding affinities of Mn·GS for ligands decrease (Table I). In the case of MetSox, the temperature dependence of K_A' at pretransition temperatures is consistent with the negative enthalpy of binding. For partially unfolded Mn·GS relative to the folded protein, association constants for Gln and ADP decrease, and binding becomes more exothermic. This indicates that the weakening of the ligand binding affinities has an entropic origin. Probably, the partially unfolded active site becomes a more open, labile structure, and the binding of a ligand, although enthalpically more favorable, greatly reduces the number of the degrees of freedom and the entropy of the system. These unfavorable entropic effects are much greater for MetSox than for Gln or ADP.

MetSox and Gln occupy the same binding site (covering the ammonia and L-glutamate sites) located in the C-terminal domain of the GS subunit. ADP spans both the C- and N-terminal domains of adjacent subunits by chelating to the n_2 metal ion binding site in the larger C-terminal domain (Hunt et al., 1975; Yamashita et al., 1989) and by binding at Lys-46 (Pinkofsky et al., 1984) near the Trp-57 loop in the smaller N-terminal domain of an adjacent subunit. Nevertheless, the thermal lability of N- and C-terminal structural domains remained virtually unchanged upon the binding of these ligands, as shown by the nearly constant difference between $T_{0.5}$ values for Trp and Tyr exposures (Figure 6A–C). The highly cooperative mechanism of partial unfolding reactions of Mn·GS, which involves only two thermodynamic domains per dodecamer, also was not much affected by these active-site ligands as judged by only small changes in the CR values reported in Table II. However, data for the enthalpy of unfolding shown in Figure 7 indicate that the binding of more than three ADP molecules to dodecameric Mn·GS influences interactions between subunits (ΔH^0_{cal} increased significantly). In addition, Trp exposure is ~60% less in the presence of ADP, and ADP binds to two sites of each partially unfolded subunit. Thus, the partially unfolded state of Mn·GS differs in the absence and presence of ADP.

[3] Calorimetric titration isotherms for Gln binding at 30 and 60 °C were simulated for $[GS]_{tot}$ = 0.18 mM subunit using the binding parameters from Table I, and these were found to cross at ~60 mM Gln. Consequently, the presence of 5 and 50 mM Gln increases and that of 100 and 150 mM Gln decreases ΔH_{cal}, although there is always net dissociation of Gln during unfolding (see ν data in Table II). In contrast, the 60 °C isotherm for ADP binding ($[GS]_{tot}$ = 0.15 mM subunit) lies below that for 30 °C, which leads to a decrease in the value of ΔH_{cal} in the presence of ADP. This result does not depend on the stoichiometry of binding at 60 °C because it is the total heat of binding to all sites which changes the apparent enthalpy of unfolding.

ACKNOWLEDGMENT

We thank Mary Rose Burnham for technical assistance during initial DSC experiments. We are grateful also to William H. Kirchhoff (U.S. Department of Energy, National Institutes of Standards and Technology) for providing us with the updated versions of the EXAM program.

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