Differential Scanning Calorimetry Study of Reversible, Partial Unfolding Transitions in Dodecameric Glutamine Synthetase from *Escherichia coli*†

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ABSTRACT: Partial unfolding of dodecameric glutamine synthetase (GS) from *Escherichia coli* has been studied by differential scanning calorimetry (DSC). A single endotherm (t_m = 51.6 ± 0.1 °C and ΔH_{cal} = 211 ± 4 kcal/mol of enzyme) was observed in DSC experiments with Mn·GS in the presence of 1.0 mM free Mn^{2+} and 100 mM KCl at pH 7. The dodecameric structure of Mn·GS was retained throughout heating cycles, and thermal transitions were reversible as shown by rescans [with 6–18 mg of GS (M_r 622 000) from 15 to 68 °C at 20–60 °C/h] and by >93% recovery of activity. A cooperative ratio $\Delta H_{cal}/\Delta H_{vH}$ of 1.6 ± 0.1 and deconvolution analysis show two cooperative units (two-state transitions): t_1 = 50.4 and t_2 = 51.7 °C; the ratio of the relative sizes of thermally labile domains is ~1:2 as judged by $\Delta H_2/\Delta H_1 \cong 2$. However, the thermally induced overall enthalpy change (0.34 cal/g) for GS dodecamer is only 5–10% of that for thermal unfolding of small globular proteins at 50 °C. The t_1 and t_2 values from deconvolutions of DSC data agree with $t_{0.5}$ values previously calculated from spectral measurements of temperature-induced exposures of ~0.7 of 2 Trp and ~2 of 17 Tyr per subunit, respectively [Shrake et al. (1989) *Biochemistry 28*, 6281–6294], over a 14 °C temperature range using both stabilizing and destabilizing conditions for Mn·GS. No uncoupling of Trp and Tyr exposures or of cooperative units in DSC experiments with Mn·GS occurred in the presence of either 150 mM Gln (t_m = 58.6 °C) or 10 mM free [Mn^{2+}] (t_m = 43.9 °C). Thus, cooperative interactions apparently link partial unfolding reactions of all subunits within the GS dodecamer so that only two two-state transitions are observed.

In enteric bacteria, glutamine synthetase is the key enzyme in nitrogen metabolism, and its activity (in synthesizing L-glutamine from L-glutamate, ammonia, and ATP) is strictly regulated both genetically and metabolically (Stadtman & Ginsburg, 1974; Rhee et al., 1989). Glutamine synthetase (GS)[1] from *Escherichia coli* is a large metalloenzyme (M_r ~622 000) with 12 identical subunits arranged in 2 face-to-face hexagonal rings (Valentine et al., 1968; Ginsburg, 1972; Colombo & Villafranca, 1986; Yamashita et al., 1989). An atomic model of GS at 3.5 Å now is available from the sophisticated X-ray diffraction studies of David Eisenberg and co-workers (Almassy et al., 1986; Yamashita et al., 1989). These studies have confirmed that the 12 subunits of GS are arranged in 2 layers of 6 and, furthermore, have shown that 12 active sites are formed at heterologous interfaces between adjacent subunits within a ring. Each active site is formed by 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. The larger C-terminal domain of each subunit contains the two active-site metal ions, n_1 and n_2 (Hunt et al., 1975), as well as the binding site for L-glutamate (L-glutamine). ATP spans both domains by binding near Lys-47 (Pinkofsky et al., 1984) and Mn^{2+} at the n_2 site (Hunt et al., 1975; Villafranca et al., 1976). The N-terminal domain, which is fairly exposed (Yamashita et al., 1989), contains the Trp-57 loop (and no tyrosyl residues). The X-ray structural analysis also shows that the stability of contacts between subunits in opposing rings mainly is provided by the carboxy terminus helical "thong" which inserts into a hydrophobic pocket formed by two neighboring subunits on the opposite ring. In addition, both intra- and inter-ring hydrogen-bonded β-sheet interactions between subunits have been identified (Yamashita et al., 1989).

Scanning calorimetry can provide information on cooperative domain structure and domain interactions in proteins (Privalov, 1979, 1982; Sturtevant, 1987; Brandts et al., 1989; Ramsay & Freire, 1990). We have used this approach in the present study to investigate partial unfolding reactions in dodecameric GS. Evidence for temperature-induced, reversible transitions of two domains in GS previously was obtained by monitoring Trp and Tyr exposures spectrally (Shrake et al., 1989). Thermal transitions were found to involve the exposures of ~0.7 Trp of the 2 Trp/subunit and ~2 of the 17 Tyr/subunit; each spectral progress curve conformed to a two-state model of partial unfolding, and Trp exposure was found to occur at 2–3 °C lower temperatures than that of Tyr. These studies yielded $t_{0.5}$ and ΔH_{vH} values for thermally induced changes in Trp and Tyr exposures, but no information was obtained on the sizes of the units undergoing partial unfolding. The DSC results reported here indicate that cooperative interactions link partial unfolding reactions of all enzyme subunits.

Materials and Methods

Chemicals and Enzyme Solutions. ADP, L-glutamine, α-ketoglutarate, and Hepes were purchased from Sigma, and

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[1] Abbreviations: GS, unadenylylated glutamine synthetase from *Escherichia coli* containing 0.8 equiv of covalently bound 5′-AMP/dodecamer; Mn·GS, manganese ion complex of GS; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; DSC, differential scanning calorimetry.

Chelex 100 (100–200 mesh) was from Bio-Rad. Preparations of metal ion free L-glutamine, stock solutions of ADP, and standardized Mn^{2+} were as described by Hunt et al. (1975); stock solutions of 1 M imidazole (Eastman Kodak) were immediately titrated with HCl to pH 7.3 and then were lightly treated with activated charcoal to decolorize and filtered. All aqueous solutions were made 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 the calorimeter cells. All other chemicals were of analytical grade.

Unadenylylated glutamine synthetase with an average of 0.8 equiv of covalently bound 5′-adenylate groups per dodecamer [as determined by assay (Stadtman et al., 1979) and UV spectral (Ginsburg et al., 1970) methods] was isolated from an overproducing *E. coli* strain (Maurizi & Ginsburg, 1986). The purification procedure of Woolfolk et al. (1966) was used with 10 mM α-ketoglutarate added to the extraction buffer at pH 7 to inhibit adenylylation (Stadtman & Ginsburg, 1974) and with a buffer containing 50 mM imidazole hydrochloride, 50 mM KCl, and 10 mM $MnCl_2$, pH 7.3, in purification steps. Final specific activities in the γ-glutamyl transfer assay at pH 7.57 and 37 °C were 135 ± 3 units/mg. The purified enzyme was stored at 4 °C as a suspension (~10 mg/mL) in 52% $(NH_4)_2SO_4$ containing 5 mM Mn^{2+} and 5 or 25 mM imidazole, pH 7.3; protein was collected as needed by centrifugation and 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 $MnCl_2$, adjusted to pH 7.30 ± 0.01 at 30.0 °C (ΔpH/°C = −0.015). For some experiments, the enzyme was dialyzed as above against buffers containing 50 mM Hepes/KOH, 100 mM KCl, and 3.0 or 10.0 mM $MnCl_2$, or 1.0 mM $MnCl_2$ + 150 mM L-glutamine, adjusted to pH 7.30 ± 0.01 at 30 °C. [Measurements of pH were as described by Shrake et al. (1989).] After dialysis, enzyme solutions were clarified by centrifugation and stored at 4 °C; protein concentrations were determined from published absorption coefficients (Ginsburg et al., 1970): A_{280nm} = 0.738 and A_{290nm} = 0.385 cm²/mg after applying fourth power light-scattering corrections from the absorbance at 340 nm. Protein dilutions were made with the final dialysate in each case. For stock enzyme solutions, two to four protein concentration determinations were made and averaged (SD ≤ 3%); a molecular weight of 622000 was used to calculate the moles of dodecamer [M_r = 621 384 without taking into account bound water (Colombo & Villafranca, 1986; Yamashita et al., 1989)]. Glutamine synthetase activity was determined by the γ-glutamyl transfer, pH 7.57, method with saturating substrates (Stadtman et al., 1979) before and after DSC experiments.

Chicken egg white lysozyme (Sigma, grade 1) was dissolved and dialyzed as above against 0.1 M glycine hydrochloride buffers at pH 2.6 or 2.7 for testing the MicroCal-2 differential scanning calorimeter. For calculation of lysozyme concentrations, the specific absorbance value of Bjurulf and Wadsö (1972) of A_{280nm} = 2.65 cm²/mg (determined at pH 7 in 0.1 M potassium phosphate) and M_r = 14312 were used. Bovine pancreatic α-chymotrypsinogen A (Sigma), ~0.7% protein (w/v) in HCl at pH 2.2, also was used for calibrations.

Spectrophotometric Measurements. Spectra were recorded with either a Perkin-Elmer Model 320 spectrometer or a Hewlett Packard Model 8450A with a dedicated temperature controller (Model 89100A) and an attached Peltier junction temperature-controlled cuvette holder (Model 89101A) with a magnetic stirrer and temperature probe. The latter instrument was used for obtaining second-derivative spectra in measurements of thermally induced changes in tryptophanyl and tyrosyl residue exposures as previously described (Shrake et al., 1989). Changes in the second-derivative peak–trough at ~295–291 nm reflect changes in the environment of tryptophanyl residues which are independent of changes in Tyr exposure. Alternatively, Trp exposure was measured by temperature-induced direct absorbance changes (~295–290 nm) as described in Shrake et al. (1989). The *r* ratios from second-derivative spectra (Ragone et al., 1984) as calculated in Shrake et al. (1989) for measurements of Tyr exposure have been found recently to be influenced by the environments of Trp, especially when the Tyr:Trp ratio is >3 (unpublished data). Consequently, the average number of Tyr exposed in the thermally induced, partial unfolding of glutamine synthetase is uncertain.

Differential Scanning Calorimetry (DSC). DSC was performed with a MicroCal MC-2 ultrasensitive calorimeter (MicroCal, Inc., Northampton, MA) equipped with tantalum cells, interfaced with an IBM PS 30 personal computer through an A/D converter (DT 2801), and connected to a Haake F3 programmable external water bath. Data acquisition and data analysis software (DA-2) were provided by the manufacturer. The capacity of the sample cell was 1.2206 mL. Solutions were degassed for 30 min at ambient temperature in a test tube connected to a Gast pump (Thomas Scientific Co.) with magnetic stirring and then loaded with a 2.5-mL gas-tight Hamilton Syringe (MicroCal, Inc.) under vacuum. The instrument was run under excess N_2 pressure (~8 psi). The dialysate buffer was loaded into both reference and sample cells, and after a base-line scan had been conducted over the temperature range to be used for the protein (typically 15–68 °C), the contents of the sample cell were gently removed by aspirating with a Teflon needle (26 gauge) under house vacuum and then refilled with the dialyzed protein solution (see above). After completion of generally two up-scans (with a 60-min equilibration at 15 °C between scans and final cooling at 15 °C), the protein solution was removed the following day for assay, and the calorimeter sample cell was cleaned by washing with ~1 L of a hot (~80 °C) solution of ~5% DAWN (commercial dishwasher detergent), followed by copious rinses (~5 L) of water (heated to ~80 °C in a microwave oven) using the cell washing assembly of MicroCal, Inc. After the reference cell was washed with ~3 L of hot water and the overflow areas were rinsed, both reference and sample cells were dried under house vacuum by aspirating through Teflon needles inserted into the cells (~20 min).

The DA-2 software for temperature readout was calibrated (and adjusted if necessary) as follows: for up-scans, the sealed, pure hydrocarbons (t_m = 28.2 °C and t_m = 73.8 and 75.9 °C) obtained from MicroCal, Inc., were used as directed; for down-scans, the thermal unfolding of α-chymotrypsin at pH 2.2 was used, and the t_m value for the down-scan was matched to that of the up-scan (t_m = 46.3 °C). Electrical calibrations during up-scans of water (90 °C/h) were performed about every 3 months, and these were within ≤1% of theoretical values.

The thermally induced unfolding of lysozyme was used to test the MC-2 instrument and data analysis procedures (see below). Dialyzed lysozyme (4.78 mg/mL) in 0.1 M glycine hydrochloride buffer at pH 2.6 scanned at 10, 30, 45, and 60 °C/h in separate DSC experiments gave the following thermodynamic parameters (where $t_m = T_m - 273.15$): t_m = 63.07 ± 0.2 °C, ΔH_{cal} = 107 ± 2 kcal/mol, and ΔH_{vH} = 106 ± 1 kcal/mol. The latter two values are 4.3 and 3.4% higher,

respectively, than those obtained by Schwarz (1989) using 0.1–0.2 M glycine hydrochloride, the Hart 7707 differential scanning calorimeter, and the same analysis procedure, and are about the same as those reported by Privalov and Khechinashvili (1974). In addition, dialyzed lysozyme in 0.1 M glycine hydrochloride buffer at pH 2.7 was scanned at 45 °C/h at 3.49 (in two different MC-2 instruments), 1.16, and 0.70 mg/mL, giving t_m = 63.4 ± 0.2 °C, ΔH_{cal} = 111 ± 1 kcal/mol, and ΔH_{vH} = 109 ± 1 kcal/mol; the latter two values are 8.0 and 6.4% higher, respectively, than those calculated from the linear dependence of ΔH_{cal} and ΔH_{vH} on T_m published by Schwarz (1989).

Data Analysis. DSC data [after subtraction of the dialysate buffer base line and transformation of C_p to the units calories per degree per mole using DA-2 software][2] were analyzed by the EXAM program of W. H. Kirchhoff (U.S. Department of Energy, Thermodynamics Division, National Institute of Standards and Technology, Gaithersburg, MD). This analysis gives least-squares fits of the data to a two-state model including base lines:

$$C_p = (1 - \alpha)[B_a + B'_a(T - T_m)] + N\left(\frac{\Delta H(T)^2}{RT^2}\right)F(\alpha) + \alpha[B_b + B'_b(T - T_m)] \quad (1)$$

following procedures described by Schwarz and Kirchhoff (1988). In this expression, N is the number of moles of cooperative units per mole of protein in the calorimeter cell and can be varied as one of the parameters of the fit or held fixed at some predetermined value. For unit stoichiometry in which the number of moles of denatured species is the same as the number of moles of native protein, $F(\alpha) = \alpha(1 - \alpha)$ where α is the molar fraction of the reaction product and is related to the equilibrium constant by

$$K = \frac{\alpha}{1 - \alpha} = \exp(-\Delta G/RT) \quad (2)$$

In conducting the iterative least-squares fit, the temperature dependence of ΔH is taken to be

$$\Delta H(T) = \Delta H_m + \Delta C_{p_m}(T - T_m) \quad (3)$$

and that of ΔG to be

$$-\frac{\Delta G}{T} = \left(\frac{\Delta H_m}{T_m}\right)\left(\frac{T - T_m}{T}\right) + \Delta C_{p_m}\left(\ln\frac{T}{T_m} - \frac{T - T_m}{T}\right) \quad (4)$$

Also, the pretransition asymptotic base line, $B_a + B'_a(T - T_m)$, and posttransition asymptotic base line, $B_b + B'_b(T - T_m)$, of eq 1 are determined independently with linear least-squares fits of the low-temperature (usually <43 °C) and high-temperature (usually >61 °C) data, respectively. The transition parameters of the endotherm ΔH_m, T_m, N, and ΔC_{p_m} are determined by an iterative, nonlinear fit of eq 1 using analytical derivatives and holding the base-line parameters fixed. The $1 - \alpha$ and α coefficients of the base-line terms in eq 1 provide an overall sigmoidal base line throughout the transition region. The deviation of N from unity provides information on the cooperativity of the denaturation as discussed below.

For deconvolutions, the CPCALC program of E. M. Freire and colleagues (Biocalorimetry Center, Department of Biology, The Johns Hopkins University, Baltimore, MD) was used for subtraction of the sigmoidal transition base line generated in the EXAM program (see above), for shortening data files, and for checking area measurements. The data file from CPCALC then was entered into the deconvolution programs of the DA-2 software, which are based on the analysis procedures of Freire and Biltonen (1978a,b) either for multiple independent two-state transitions or for sequential two-state transitions.

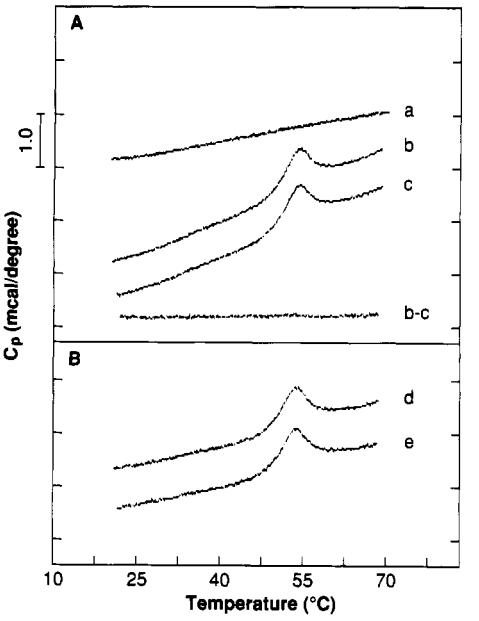

FIGURE 1: Representative DSC experiment with glutamine synthetase (12.08 mg of GS; 19.43 nmol of dodecamer) which had been dialyzed at 4 °C against a buffer containing 50 mM Hepes/KOH, 100 mM KCl, and 1.0 mM $MnCl_2$, pH 7.0 (at 50 °C). Raw (unsmoothed) data files (normalized to millicalories per degree) are shown for C_p (arbitrary scale) vs temperature (degrees centigrade) from the MicroCal-2 differential scanning calorimeter. Scans from 15 to 68 °C (collecting output every 15 s) at a scan rate of 45 °C/h gave the data in (A) for (a) dialysate buffer vs the same buffer, (b) GS vs dialysate buffer (first scan) and after 60 min at 15 °C, (c) a repeat of (b) for a second scan of GS vs buffer. A direct subtraction of the data file of (c) from that of (b) gave (b–c) in (A). In (B), the data of (d) and (e) were obtained after adjusting and subtracting the buffer base line (a) from (b) and (c) in (A), respectively.

Results and Discussion

Differential scanning calorimetry (DSC) is used here to characterize thermally induced partial unfolding reactions of dodecameric glutamine synthetase (GS). Reversible, temperature-induced two-state transitions were identified previously in spectral measurements of aromatic residue exposures, and analysis of these data gave estimates of van't Hoff enthalpy changes for partial unfolding of two domains of GS (Shrake et al., 1989). However, the number of cooperative units per mole of dodecamer was unknown. In contrast, the number of moles of GS placed in the calorimeter cell is certain, and the number of cooperative units per mole theoretically can be determined. The measured excess heat capacity can be expressed in the units of kilocalories per degree per mole of dodecamer since GS does not dissociate under the conditions of DSC experiments, as monitored by light scattering (Shrake et al., 1989). Also, aggregation of dodecameric GS occurs slowly at high temperatures, and is apparent only at >67 °C, which is at least 6 °C above observed T_m values (see below). The thermal stability of the dodecameric structure accounts for the complete reversibility of temperature-induced transitions and, in addition, explains why thermodynamic parameters are independent of protein concentration.

Figure 1A shows raw data files (normalized to millicalories per degree) from a representative DSC experiment with GS

[2] Units of calories are used in this paper; for conversion to the International System of Units (SI), 1.000 cal = 4.184 J.

(9.90 mg/mL) in the presence of 1.0 mM free $MnCl_2$ and 100 mM KCl at pH 7. A base line (a) was established first by heating the dialysate buffer in both reference and sample cells at 45 °C/h. After being cooled, the buffer in the sample cell was replaced with the dialyzed enzyme solution and equilibrated at 15 °C for ~90 min. Then, GS was heated from 15 to 68 °C at 45 °C/h twice, with a 60-min equilibration at 15 °C between scans b and c. Following the second protein scan, the cell contents were cooled to 15–25 °C overnight before removal of the protein sample for enzyme activity assays. Subtraction of the base line (a) from the first and second scans in Figure 1A gave scans d and e, respectively, shown in Figure 1B.

Several features of Figure 1 merit comment. A single endotherm is observed on heating glutamine synthetase in the presence of 1 mM free $MnCl_2$. This thermal transition is reversible, as evidenced by an identical endotherm being observed in the first and second scans. Subtraction of the two data files for the first and second scans in Figure 1A gave a horizontal line at nearly zero C_p [(b) − (c) in Figure 1A]. In addition, a down-scan at −25 °C/h after an up-scan at 45 °C/h gave the same ΔH_{cal} value (±5 kcal/mol). Also, the enzyme was >93% active after the second scan, and the recovered enzyme solution was at most only slightly hazy. Although the excess heat capacity is only about 2–3 mcal/K, it can be observed that the signal to noise ratio is excellent (with a maximum noise level of ~25 μcal/K). The response of the MicroCal-2 instrument also was rapid compared to the scan rate (with a thermal lag of ~13 s during data collection).

In order to demonstrate that the thermal reactions were at equilibrium throughout the heating cycle, glutamine synthetase also was scanned under the conditions of Figure 1 at rates of 20 and 60 °C/h. The same thermodynamic parameters were obtained from scan rates of 20 and 60 °C/h as at 45 °C/h (see Table I below), which indicates that the system was at equilibrium throughout even the fastest scan rate. Furthermore, temperature-induced UV spectral changes arising from the partial unfolding and refolding of glutamine synthetase (Shrake et al., 1989) occur rapidly so that kinetic factors would not be expected to influence DSC results.[3]

Figure 2 illustrates the analysis of the data in scan d of Figure 1B. In Figure 2A, a two-state model of unfolding was assumed for a sigmoidal base line and a least-squares fit of the DSC profile, holding the number of moles of cooperative units per mole of dodecamer (N in eq 1) equal to unity. The fit (solid curve in Figure 2A) was quite poor [with $\sigma = \pm 3.16$ kcal/(K·mol) for the standard deviation of the fit compared to the estimated random scatter of the data, ~0.2 kcal/(K·mol)]. In Figure 2B, the linear pre- and posttransitional base lines were retained while the number of moles of cooperative units per mole (N) was allowed to vary for computer simulation of the excess heat capacity and the fit improved considerably [with $\sigma = \pm 0.92$ kcal/(K·mol)]. For the fit to the data shown in Figure 2B, the value of N is 1.6, which indicates a significant departure from a simple two-state process.

In both panels A and B of Figure 2, the pre- and posttransitional base lines were linearly fitted (eq 1; Materials and Methods) below and above the markers at 43.07 and 60.77 °C, respectively, and these were extrapolated as dotted lines into the transition region. Extrapolation of the base lines to

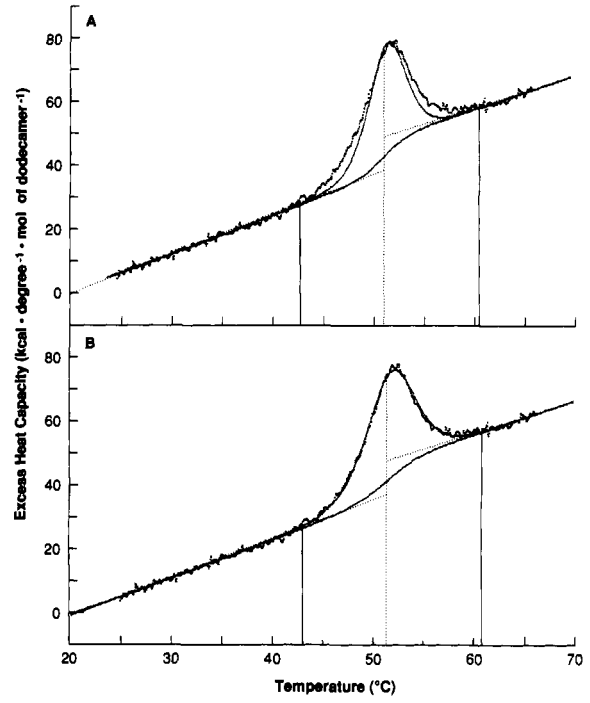

FIGURE 2: Analysis of DSC data for the partial unfolding of dodecameric GS. The data are from (d) in Figure 1B after transformation of C_p data to units of kilocalorie per degree per mole of dodecamer. Pre- and posttransitional base lines were linearly fitted below and above the vertical markers at 43.07 and 60.77 °C, respectively, and a sigmoidal transitional base line was generated for a nonlinear least-squares fit of the DSC profile (solid curve) assuming a two-state model of unfolding in the EXAM program (see Materials and Methods). In (A), the moles of cooperative units per mole of GS was held constant at $N = 1.0$. In (B), the value of N was allowed to vary, and for the fit (solid curve) shown in (B), $N = 1.6$ (see text).

the temperature at the maximum excess heat shows a positive increase from the pre- to the posttransitional base line, which indicates an increase (*at constant pressure*) in the heat capacity (ΔC_p) of the solution on protein unfolding. From nine separate DSC experiments, first scans of 6.9–18.4 mg of GS in the presence of 1 mM free $MnCl_2$ under the conditions of Figure 1 gave $\Delta C_p = 10.1 \pm 2.6$ kcal/(K·mol). Privalov and Khechinashvili (1974) observed that the change in heat capacity of a protein on denaturation is independent of the transition temperature (T_m) but that ΔC_p is not the same for different proteins. Indeed, the value of ΔC_p for GS depends on the concentration of Mn^{2+} (Table IV), presumably because varying extents of effector binding to high-temperature forms create different partially unfolded protein species.

Linear pre- and posttransitional base lines in Figure 2A,B were connected by a sigmoidal function, which is computed from the fractional area (α) under the transition profile at a given temperature in the EXAM program (see Data Analysis and eq 1 under Materials and Methods). This is thermodynamically correct for a two-state transition (Schwarz & Kirchhoff, 1988) in which α is the molar fraction of the denatured species and $\alpha = 0.5$ at the transition temperature (T_m). We assume that the sigmoidal base line in Figure 2B also represents a good approximation of the true base line for non-two-state processes with similar T_m values (see below). A similar approach has been used by others (Ramsay & Freire, 1990; Shriver & Kamath, 1990). The sigmoidal transitional base line of Figure 2B gives $\Delta H_{cal} = 209$ kcal/mol for the endotherm. Similarly fitted sigmoidal transitional base lines were used for calculating areas to obtain all ΔH_{cal} values reported here. The reproducibility in area measurements for DSC experiments with samples from the same protein solution

[3] Recently, Dr. Mark T. Fisher performed temperature jump experiments with glutamine synthetase and measured $t_{1/2}$ values of ~15 and ~7 s for first-order UV spectral changes at 52 °C from temperature increases and decreases, respectively (personal communication).

Table I: DSC Results for Glutamine Synthetase in the Presence of 1.0 mM Free $MnCl_2$ at pH 7.0[a]

GS[b] (mg)	GS act.[c] (units/mg)	heating rate (°C/h)	scan no.[d]	t_m (at C_p^{max})[e] (°C)	ΔH_{cal} (kcal/mol)	N
6.94	122	45	1	51.3 ± 0.3	184 ± 1	1.7 ± 0.1
			2	51.3	184	
18.24	122	45	1	51.5 ± 0.1	183 ± 6	1.6 ± 0.1
			2	51.5 ± 0.1	179 ± 5	1.6 ± 0.1
12.08	136	45	1	51.6 ± 0.1	211 ± 4	1.6 ± 0.1
			2	51.6 ± 0.1	210 ± 3	1.7 ± 0.1
12.08	136	20	1	51.5	216	1.8
			2	51.4	203	1.5
10.83	136	60	1	51.7[f]	210[f]	1.7
			2	51.7	204	1.7

[a] Dialysis buffer contained 50 mM Hepes/KOH, 100 mM KCl, and 1.0 mM $MnCl_2$, pH 7.0 (at 50 °C). DSC data were analyzed as illustrated in Figure 2B (where N is varied as one of the parameters of the fit, eq 1), and results are expressed as the mean ± standard deviation (SD) for three determinations when separate experiments were performed. [b] In a working cell volume of 1.2206 mL. [c] Initial specific activity of the enzyme preparation; recovery of activity was 93–98% after the second scan. [d] Scans were from 15 to 68 °C with equilibrations for 60 min at 15 °C between scans. [e] $t_m = T_m - 273.15$. [f] After correction of the data for the 13-s response of the instrument [see eq 1 of Schwarz and Kirchhoff (1988)], essentially the same parameters were obtained: t_m = 51.7 °C and ΔH_{cal} = 207 kcal/mol.

Table II: Deconvolutions of the Endotherm for the Partial Unfolding of GS in the Presence of 1.0 mM Free $MnCl_2$ at pH 7, Assuming either Independent (Random) or Sequential Two-State Transitions[a]

t_1 (°C)	ΔH_1 (kcal/mol)	t_2 (°C)	ΔH_2 (kcal/mol)	SD of fit [kcal/(K·mol)]
49.2 (49.5)	63 (76)	51.5 (51.5)	140 (120)	1.57 (1.50)
50.6 (50.4)	63 (79)	51.7 (51.7)	139 (114)	1.16 (1.07)
51.3 (50.6)	67 (75)	51.8 (51.6)	154 (133)	0.93 (0.95)
50.7 (49.8)	90 (90)	51.7 (51.9)	139 (129)	1.64 (1.42)
50.0 (50.6)	63 (79)	51.9 (51.7)	157 (130)	1.24 (1.14)
mean: 50.4 (50.2)	69 (80)	51.7 (51.7)	146 (125)	
SD: ±0.8 (±0.5)	±12 (±6)	±0.2 (±0.2)	±9 (±8)	

[a] Entries in the table are in the same order as in Table I for DSC experiments with glutamine synthetase in 50 mM Hepes/KOH, 100 mM KCl, and 1.0 mM $MnCl_2$, pH 7.0 (at 50 °C). Nonparenthetical values are the parameters of a random model (independent transitions), and parenthetical values are the parameters of a sequential model; $\Delta H_{vH} = \Delta H_{cal}$ in either model. The standard deviation (SD) of each fit as well as that for the mean of each of the five data sets is given.

is shown by the standard deviations in values of ΔH_{cal} in Tables I and IV.

Privalov and Khechinashvili (1974) defined a cooperativity ratio CR = $\Delta H_{cal}/\Delta H_{vH}$ in which the van't Hoff enthalpy change (ΔH_{vH}) is estimated from

$$\Delta H_{vH} = 4.00RT_m^2C_{p_m}/\Delta H_{cal} \quad (5)$$

where R = 1.987 cal/(mol·K), the ratio $C_{p_m}/\Delta H_{cal}$ is the maximum peak height to the area of the transition over the sigmoidal base line, and T_m is the temperature (in degrees kelvin) at C_{p_m}. By comparing this expression with eq 1, it can be shown that the estimate of ΔH_{vH} is also an estimate of ΔH_m and that CR can be more simply expressed as CR = N, where N is the number of moles of cooperative units per mole of protein in the calorimeter cell and is obtained from the least-squares fit of eq 1 to the data as illustrated in Figure 2B. For an ideal two-state unfolding transition of a biopolymer (folded ⇌ unfolded), CR = 1.0; a value of CR less than 1 suggests intermolecular cooperativity as, for example, gel to liquid-crystal transition in membranes or protein aggregation. A value of CR greater than 1 suggests overlapping independent two-state transitions or sequential two-state transitions in the thermal unfolding of a multidomain protein. For example, CR is 2.0 or ~1.4 for two independent or two sequential two-state transitions, respectively, with the same T_m and ΔH_m values in a multidomain biopolymer. For the analysis in Figure 2B, CR = 1.6, which suggests two or more domains.

Table I gives t_m, ΔH_{cal}, and N values from a series of DSC experiments (analyzed as illustrated in Figure 2B) with two different preparations of glutamine synthetase in the presence of 1.0 mM free $MnCl_2$, pH 7.0 (at 50 °C). An enzyme preparation which had been stored for 7 years had lost 10% activity, and ΔH_{cal} values were ~13% lower than with a more recent GS preparation of 136 units/mg specific activity (ΔH_{cal} = 211 kcal/mol).[4] However, values of t_m were similar (51.5 ± 0.2 °C), and the same results were found over a 2.6-fold range in protein concentration. In all cases, essentially the same parameters were obtained in second scans, and enzyme activity recovery after each second scan was 94–98% (data not shown). Also, Table I shows that the results were independent of scan rate from 20 to 60 °C/h.

Deconvolutions of DSC data were performed for the first scans listed in Table I. After calculation of the sigmoidal transitional base line for each endotherm by using the parameters from a two-state fit in which N was allowed to vary (as in Figure 2B), this was subtracted from the DSC data (ΔC_p = 0) before deconvolution programs were entered (see Materials and Methods). Either a random model for multiple, independent two-state transitions or a sequential model for sequential two-state transitions was assumed for deconvolution analysis (Freire & Biltonen, 1978a,b). A minimum of two two-state transitions was required for fits of each data set, and the fits had nearly the same standard deviation [≤1.6 kcal/(K·mol)] for either the random or the sequential model (Table II). In fitting either model to the data, each cooperative unit is assumed to undergo an ideal two-state transition from a folded to a partially unfolded form.

Figure 3 shows deconvolution analysis of the DSC data of Figure 2B, using random and sequential models for fitting procedures. The solid lines show the two two-state transitions and their sum [σ = ±0.93 kcal/(K·mol) for the overall fit]

[4] Inactivating oxidative damage to glutamine synthetase has been shown to occur during storage (Levine, 1983), and apparently inactivation affects the amount of heat observed during the temperature-induced, partial unfolding of the enzyme. This probably results from the destruction of metal ion binding sites by oxidation of His-269 residues (Farber & Levine, 1986).

Table III: Stabilization/Destabilization of Dodecameric GS at pH 7.0:[a] Comparison of Calorimetric and Spectral Parameters

	DSC results		Trp exposure[b]		Tyr exposure[b]	
$[Mn^{2+}]_{free}$ present (mM)	t_m (°C)	ΔH_{cal} (kcal/mol)	$t_{0.5}$ (°C)	ΔH_{vH} (kcal/mol)	$t_{0.5}$ (°C)	ΔH_{vH} (kcal/mol)
1.0	51.5	183	50.0	92	51.6	105
3.0	48.4	203	46.3	85	48.6	109
10.0	43.9	212	41.7	96	43.6	106
1.0 + 150 mM Gln	58.2	178	59.9	94		
1.0 + 150 mM Gln[c]	58.6	227	58.3	81	61.7	70

[a] The enzyme (122 units/mg and 5.2–15.0 mg/mL) was dialyzed against buffer containing 50 mM Hepes/KOH, 100 mM KCl, and the concentration of Mn^{2+} (±150 mM Gln) indicated; the pH of the buffer was adjusted to 7.0 at 50 °C (ΔpH/°C) = −0.015). DSC data were analyzed by using eq 1 (see Materials and Methods) as illustrated in Figure 2B for first scans only, and DSC parameters are given as the mean of three to four determinations with ΔH_{cal} expressed in kilocalories per mole of dodecamer (SD of the means are given in Table IV). [b] Spectral values, except for those entered in the fifth row, are from Shrake et al. (1989); $t_{0.5}$ values are the midpoints of spectral progress curves, and a random model for independent transitions was assumed. [c] The enzyme preparation used to obtain these data was 11% more active (136 units/mg); scans were 15–73 °C at 45 °C/h, after which 97% of GS activity was recovered.

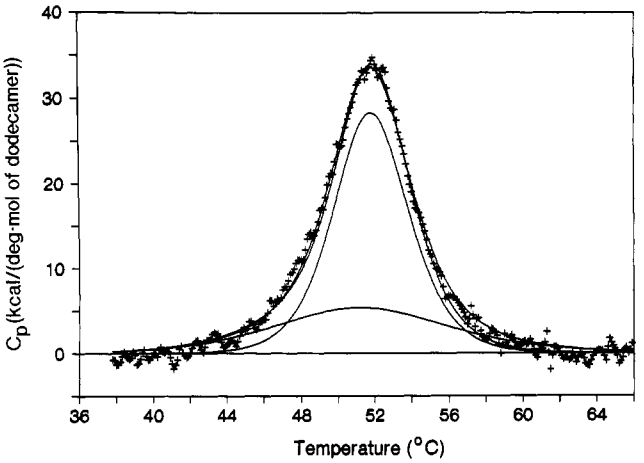

FIGURE 3: Deconvolution analysis of DSC data for the partial unfolding of dodecameric GS in the presence of 1 mM free Mn^{2+}. The data are from Figure 2B after subtraction of the sigmoidal transitional base line (with $\Delta C_p = 0$ for the base line shown) and shortening the data file. A random model for multiple, independent two-state transitions was fitted to the DSC data and gave the two two-state transitions and their sum shown by the solid curves. A model for sequential two-state transitions also was fitted to the data and gave a solid curve which is very similar to that obtained for the overall fit of the random model. Parameters of the fits and the standard deviation of each fit are given in the third row of Table II.

for the random model and also the overall fit for two sequential two-state transitions [σ = ±0.95 kcal/(K·mol)]. The fit for the sequential model gives a somewhat sharper profile than that for the random model. The parameters of the fits in Figure 3 are given in the third row of Table II and are very similar to corresponding values obtained from deconvolutions of four other DSC data files. Observe that the parameters t_1 and t_2 differ by <2 °C and $\Delta H_1 \ll \Delta H_2$ for either two random or two sequential two-state transitions (Table II). Since the fits were good and were approximately the same for either model (as illustrated in Figure 3), the deconvolution results of Table II do not discriminate between random and sequential mechanisms for the thermally induced partial unfolding of Mn·GS.

In spectral studies of the temperature-induced partial unfolding of dodecameric GS, it was observed that increasing concentrations of free Mn^{2+} from 0.3 to 10.0 mM destabilized the enzyme (i.e., decreased $t_{0.5}$ values) whereas the presence of the substrate L-glutamine increased $t_{0.5}$ values (Shrake et al., 1989). DSC experiments were performed with GS in the presence of 1, 3, or 10 mM free $MnCl_2$ and in 1 mM free Mn^{2+} + 150 mM L-glutamine in order to investigate whether the same destabilizing and stabilizing effects are observed calorimetrically. Table III gives t_m and ΔH_{cal} mean values for first up-scans from three to four DSC experiments and, for comparison, lists average values of $t_{0.5}$ and ΔH_{vH} determined previously for Trp and Tyr exposures assuming a random model for the partial unfolding of two domains of GS (Shrake et al., 1989). There is a striking similarity in t_m and $t_{0.5}$ values from DSC and spectral studies, respectively, over a 15 °C range of t_m values. This substantial range in t_m values results from t_m being *decreased* 7.6 °C by increasing $[Mn^{2+}]$ from 1 to 10 mM and t_m being *increased* ~7 °C by the presence of 150 mM Gln with 1 mM Mn^{2+} (Table III). The destabilizing effect of Mn^{2+} is attributed to Mn^{2+} binding to additional exposed sites on the partially unfolded enzyme (Shrake et al., 1989), whereas the stabilizing effect of L-glutamine results from the preferential binding of this ligand to the folded, low-temperature enzyme form.[5]

The observation that $t_{0.5}$ values differ for Trp and Tyr exposures led Shrake et al. (1989) to propose that different domains of GS were involved in thermally induced unfolding reactions. It is remarkable that the measured ΔH_{cal} value for each of the different conditions of Table III approximately equals the corresponding sum of the ΔH_{vH} values for random Trp and Tyr residue exposures. [For a sequential partial unfolding of the Tyr domain after that of Trp (which is a model that also could be fitted to spectral data), the sum of ΔH_{vH} values equals ~140 kcal/mol.] Moreover, the deconvolution analysis of the DSC endotherm shows two two-state transitions (Table II) with approximately the same parameters as those obtained for Trp and Tyr exposures.

DSC curves were simulated from the parameters obtained from spectral data for GS in the presence of 1.0 mM free Mn^{2+} at pH 7.0 (50 °C). Either a model for two independent two-state transitions or a model for two sequential two-state transitions was used for the simulation. In fitting spectral progress curves for the random and sequential models, the total ΔH_{vH} is 197 and 140 kcal/mol, respectively (Shrake et al., 1989). Figure 4 shows the simulated DSC curves for the two models from spectral parameters together with the fitted curve for DSC data (after subtraction of the fitted sigmoidal transitional base line; see above) obtained with the same enzyme preparation under identical conditions. Although the cooperative ratio of the actual DSC endotherm is closer to that for the sequential model, the total heat (187 kcal/mol of dodecamer) is better approximated by the random model.

Table IV summarizes the thermodynamic parameters obtained for thermally induced, partial unfolding of dodecameric

[5] Studies in progress show that L-glutamine binds with ~10-fold lower affinity to the high-temperature, partially unfolded enzyme than to the native, low-temperature form. At 150 mM L-glutamine, the difference in binding to the low- and high-temperature forms is ~0.2 equiv/subunit so that the observed value of ΔH_{cal} is only increased by ~8% by the enthalpy of ligand dissociation.

Table IV: Summary of Thermodynamic Parameters for the Thermally Induced Partial Unfolding of GS Dodecamer at pH 7[a]

$[ligand]_{free}$	GS act. (units/mg)	n	t_m[b] (°C)	ΔH_{cal} (kcal/mol)	ΔC_p [kcal/(K·mol)]	ΔH_{vH}[c] (kcal/mol)	CR[c]
1.0 mM Mn^{2+}	122	3	51.5 ± 0.1	183 ± 6	9 ± 6	123 ± 2	1.5 ± 0.1
1.0 mM Mn^{2+}	136	5	51.6 ± 0.1 (50.7, 51.8)	211 ± 4 (73, 151)	10.6 ± 0.9	133 ± 5	1.6 ± 0.1 (1.0, 1.0)
1.0 mM Mn^{2+}, 150 mM Gln	136	2	58.6 ± 0.1 (56.9, 58.7)	227 ± 5 (65, 182)	11.4 ± 0.6	160 ± 2	1.4 ± 0.1 (1.0, 1.0)
3.0 mM Mn^{2+}	122	3	48.4 ± 0.1 (47.2, 48.6)	203 ± 4 (63, 145)	5.7 ± 1.1	128 ± 1	1.6 ± 0.1 (1.0, 1.0)
10.0 mM Mn^{2+}	122	4	43.9 ± 0.2 (43.2, 44.4)	212 ± 2 (76, 141)	3.9 ± 1.7	123 ± 2	1.7 ± 0.1 (1.0, 1.0)

[a] Dialysate buffer at pH 7.0 (50 °C) contained 50 mM Hepes/KOH, 100 mM KCl, and the indicated ligand; enzyme concentrations were 8.8–15.0 mg/mL in DSC experiments. The parameters were obtained from first scans; n was the number of determinations from which the means and standard deviations were calculated. Average parameters from deconvolution analyses are given in parentheses (for first, second two-state transition), assuming a random model. [b] $t_m = T_m - 273.15$ at C_{p_m}, where C_{p_m} is the maximum excess heat capacity over the sigmoidal transitional base line. [c] Calculated from eq 5. The cooperativity ratio (CR), $\Delta H_{cal}/\Delta H_{vH}$, calculated from eq 5 generally was the same as the N value (numbers of moles of cooperative units per mole of dodecamer) obtained from the fit of the data (see text).

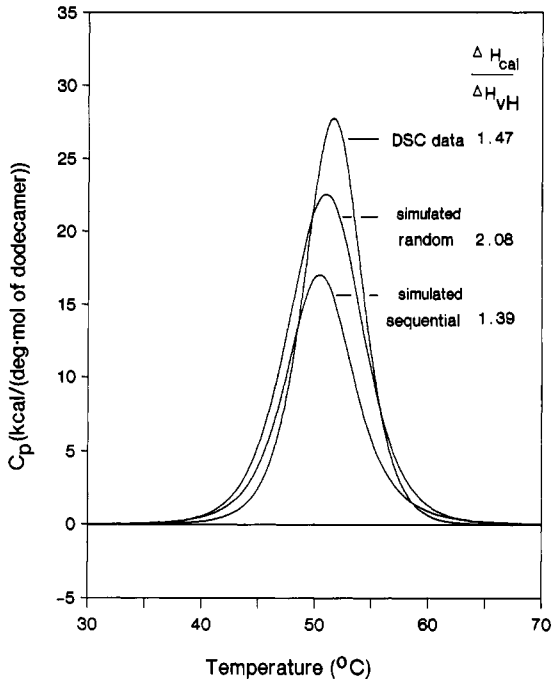

FIGURE 4: Comparison of the DSC endotherm with simulated DSC curves from spectral data for independent (random) and sequential models for the partial unfolding of two domains of GS in the presence of 1 mM free Mn^{2+} at pH 7.0 (50 °C). DSC data were obtained with the same GS preparation used for spectral measurements (in one of the DSC experiments entered in the second row of Table I), and the fitted curve for varying N (see Figure 2B) is shown after subtraction of the sigmoidal transitional base line. Spectral parameters used for generating the simulated DSC curves for random and sequential partial unfolding of two domains are from Shrake et al. (1989). The cooperativity ratio, $\Delta H_{cal}/\Delta H_{vH}$ (eq 5), for each curve is indicated, where ΔH_{cal} values from spectral data are calculated to be 197 and 140 kcal/mol from the sums of ΔH_{vH} values for the random and sequential models, respectively, and ΔH_{cal} from DSC data is the area of the endotherm over the sigmoidal transitional base line, 187 kcal/mol of dodecamer.

GS in DSC experiments conducted at pH 7.0 under different stabilizing/destabilizing conditions. Mean values from first scans of two to five determinations (with 10–18 mg of GS per experiment) and standard deviations are given. In addition to values of t_m (at C_{p_m}) and ΔH_{cal}, the estimated ΔC_p value for partial unfolding of GS and the calculated value for ΔH_{vH} (at T_m) from eq 5 are listed in Table IV for each condition. Each calculated value of CR ($\Delta H_{cal}/\Delta H_{vH}$) from eq 5 agreed within ±0.1 with the N value obtained from fitting the corresponding data file to eq 1 (see Figure 2B). Under all conditions listed in Table IV, the cooperativity ratio (CR) or the number of moles of cooperative units per mole of dodecamer (N) was ~1.6, and the deconvolution analyses indicate that there are two cooperative units per dodecamer with slightly different thermal stabilities.

Interactions between the two intramolecular cooperative units must occur. Deconvolution analyses indicate that t_m values remain less than 2 °C apart over a 14 °C temperature range in t_m (Table IV). Thus, the two intramolecular transitions are not uncoupled by either the stabilizing effect of L-glutamine binding to the folded enzyme form or the destabilizing effects of increased Mn^{2+} binding to the partially unfolded enzyme. As mentioned above, ΔH_{cal} values with a less active enzyme preparation were proportionally lower. Inactive subunits within a dodecamer with impaired Mn^{2+} binding at active sites[4] would not be expected to undergo thermal transitions (Shrake et al., 1989), and apparently these do not interfere with cooperative intramolecular interactions either (Tables II and IV).

In order to compare ΔH_{cal} values obtained under different conditions for thermal unfolding reactions occurring at different temperatures, at least two effects must be considered: *First*, if the transition studied is characterized by $\Delta C_p \neq 0$, then ΔH_{cal} will be temperature-dependent. *Second*, if effectors change the transition temperature, their enthalpy and heat capacity of binding (or dissociation) to the protein will be included in the observed values of ΔH_{cal} and ΔC_p. If the latter ligand effects can be neglected, it is possible to correct ΔH_{cal} values for the temperature change since $\Delta H_{cal}(T_2) = (T_2 - T_1)\Delta C_p + \Delta H_{cal}(T_1)$ (Privalov & Khechinashvili, 1974). However, the measured values of ΔH_{cal} in Table IV appear to reflect both temperature and ligand effects. For example, values of ΔC_p for the partial unfolding of GS in Table IV appear to decrease with increasing extents of Mn^{2+} binding to high-temperature enzyme forms so that values of ΔC_p cannot be used to correct ΔH_{cal} for temperature at varying $[Mn^{2+}]$. Moreover, ΔH_{cal} values in Table IV do not decrease with increasing $[Mn^{2+}]$, which lowers the T_m, even though the binding of Mn^{2+} to GS is almost entirely entropic (Hunt et al., 1972). Further studies on the thermodynamics of ligand binding to the low- and high-temperature forms of GS and their effects on the thermal unfolding reactions now are in progress.[5]

Deconvolution analyses of DSC data obtained for GS in the presence of increasing free Mn^{2+} concentrations for a random model gave the parenthetical parameters in Table IV for two independent two-state transitions. In all cases, an assumption of either a random or a sequential model gave the same standard deviations in fits of the data [~±1 kcal/(K·mol)]. Also, t_m values for the two transitions were similar (±0.5 °C) with either model, and values of ΔH_1 and ΔH_2 were approximately the same as those given in Table II. Since there was no thermal uncoupling of the two transitions under the different conditions, it is not possible to estimate the free energy

of stabilization of either domain by its interaction with the other domain. However, it is possible to speculate on the identity of the domains from previous results obtained for thermally induced exposures of Trp and Tyr residues (Shrake et al., 1989).

Deconvolutions of DSC data (Table IV) give t_1 and t_2 values which approximate $t_{0.5}$ values obtained for Trp and Tyr exposures, respectively (Table III), for the temperature-induced, partial unfolding of GS. Also, ΔH_1 and ΔH_2 values from deconvolution analyses (Tables II and IV) are within 2-fold of ΔH_{vH} values obtained for Trp and Tyr exposures, respectively (Table III). Thermally induced Trp and Tyr exposures appear to reflect partial unfolding of the 12 active-site structures in the GS dodecamer (Shrake et al., 1889). The X-ray crystallographic structure of GS shows that each of 12 active sites is formed by heterologous (noncovalent) contacts between intraring subunits in 2 hexagonal rings (Almassy et al., 1986; Yamashita et al., 1989). Each identical subunit has two domains—a relatively small, flexible N-terminal domain (containing Trp 57 and no tyrosyl residues) and a larger C-terminal domain (containing numerous tyrosyl residues and the two essential Mn^{2+} and the L-glutamine binding sites). Spectral data are consistent with a model in which the Trp-57 domain of all subunits begins to partially unfold before Tyr exposure in C-terminal domains (by either a random or a sequential mechanism). However, the DSC results indicate that there are just two cooperative units in the dodecamer which undergo two-state partial unfolding.

It is of interest to note that if the enzyme subunit had been the cooperative unit in DSC experiments ($N = 12$ with 2^N states), the value of ΔH_{cal} still would be ~17 kcal/mol of subunit or 211 kcal/mol of dodecamer. However, ΔH_{vH} would only amount to 17 kcal/mol (instead of the value 133 kcal/mol; Table IV), and the value of C_{p_m} also would be $^1/_{12}$th the observed value. Furthermore, if each subunit had two cooperative units in roughly 1:2 molecular weight ratios, ΔH_{vH} values would be ~6 and ~12 kcal/mol for domain transitions. These values are totally inconsistent with ΔH_{vH} values obtained from spectral data.

As discussed by others, the number of cooperative units is often less than the number of structural domains identified by X-ray crystallography [see Privalov (1979, 1982) and Ramsay and Freire (1990)]. If there is infinite cooperativity between domains (i.e., strong cooperative interactions that completely couple the unfolding of structural domains), these will behave as a single cooperative unit (CR = 1.0) and unfold in one two-state transition. Obviously, the structure of the GS dodecamer is complex (Yamashita et al., 1989), and thermal transitions involve only partial unfolding of domains without dissociation of subunits. The value of ΔH_{cal} of 211 kcal/mol (0.34 cal/g) for GS dodecamer is <10% of that for the thermal unfolding of lysozyme (~7.5 cal/g; t_m = 63 °C) or of other small globular proteins at 50 °C (Privalov, 1979). Furthermore, proton release and/or uptake during thermal transitions of GS has not as yet been quantitated, although the approximate agreement between ΔH_{cal} values observed in Hepes buffer and the sums of ΔH_{vH} values from spectral studies suggest that proton effects are not large. For these reasons, the relative sizes of the units undergoing partial unfolding can only be estimated. Nevertheless, strong cooperative interactions must exist between structural domains undergoing partial unfolding since a maximum of four states are populated during thermal transitions of GS.

Conclusions

From the above structural and thermodynamic considerations, it appears that strong cooperative interactions link GS subunit domains so that overall partial unfolding of all subunits within the dodecamer occurs. The thermal transitions are dependent upon the enzyme being in an active Mn^{2+}- or Mg^{2+}-stabilized conformation (Shrake et al., 1989), and partial unfolding reactions appear to involve a loosening of active-site structures. On the basis of the GS structure determined by Eisenberg and co-workers (Almassy et al., 1986; Yamashita et al., 1989), thermally labile structures are likely to include Trp-57 in the small N-terminal domain and Tyr in the large C-terminal domain of each subunit, which contribute to active-site structures. The present results indicate that these domains in different subunits cannot unfold independently; if this were the case, the excess heat capacity would be too small to measure. For all subunits to partially unfold in a concerted manner, at least one of the two thermodynamic domains must be composed of both N- and C-domain structures. The cooperative unit with the lower stability apparently involves N-terminal and some C-terminal structures (without Tyr exposure) that affect interactions between neighboring as well as distant subunits [see Yamashita et al. (1989)]. The cooperative unit with the higher stability has structures with buried Tyr, and these apparently communicate with other like domains during partial melting.

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References

Almassy, R. J., Janson, C. A., Hamlin, R., Xuong, N. H., & Eisenberg, D. (1986) *Nature 323*, 304–309.
Bjurulf, C., & Wadsö, I. (1972) *Eur. J. Biochem. 31*, 95–102.
Brandts, J. F., Hu, C. Q., & Lin, L.-N. (1989) *Biochemistry 28*, 8588–8596.
Colombo, G., & Villafranca, J. J. (1986) *J. Biol. Chem. 261*, 10587–10591.
Farber, J. M., & Levine, R. L. (1986) *J. Biol. Chem. 261*, 4574–4578.
Freire, E. M., & Biltonen, R. L. (1978a) *Biopolymers 17*, 463–479.
Freire, E. M., & Biltonen, R. L. (1978b) *Biopolymers 17*, 481–496.
Ginsburg, A. (1972) *Adv. Protein Chem. 26*, 1–79.
Ginsburg, A., Yeh, J., Hennig, S. B., & Denton, M. D. (1970) *Biochemistry 9*, 633–649.
Ginsburg, A., Van Noord, S. C., & Counsil, C. A. (1990) *Biophys. J. 57*, 417a.
Hunt, J. B., Ross, P. D., & Ginsburg, A. (1972) *Biochemistry 11*, 3716–3722.
Hunt, J. B., Smyrniotis, P. Z., Ginsburg, A., & Stadtman, E. R. (1975) *Arch. Biochem. Biophys. 166*, 102–124.
Levine, R. L. (1983) *J. Biol. Chem. 258*, 11823–11827.
Maurizi, M. R., & Ginsburg, A. (1986) *Biochemistry 25*, 131–140.
Pinkofsky, H. B., Ginsburg, A., Reardon, I., & Heinrikson, R. L. (1984) *J. Biol. Chem. 259*, 9616–9622.

Privalov, P. L. (1979) *Adv. Protein Chem. 33*, 167–241.
Privalov, P. L. (1982) *Adv. Protein Chem. 35*, 1–104.
Privalov, P. L., & Khechinashvili, N. N. (1974) *J. Mol. Biol. 86*, 665–684.
Ragone, R., Colonna, G., Balestrieri, C., Servillo, L., & Inace, G. (1984) *Biochemistry 23*, 1871–1875.
Ramsay, G., & Freire, E. M. (1990) *Biochemistry 29*, 8677–8683.
Rhee, S. G., Chock, P. B., & Stadtman, E. R. (1989) *Adv. Enzymol. 62*, 37–92.
Schwarz, F. P. (1989) *Thermochim. Acta 147*, 71–91.
Schwarz, F. P., & Kirchhoff, W. H. (1988) *Thermochim. Acta 128*, 267–295.
Shrake, A., Fisher, M. T., McFarland, P. J., & Ginsburg, A. (1989) *Biochemistry 28*, 6281–6294.
Shriver, J. W., & Kamath, U. (1990) *Biochemistry 29*, 2556–2564.
Stadtman, E. R., & Ginsburg, A. (1974) *Enzymes (3rd Ed.) 10*, 755–807.
Stadtman, E. R., Smyrniotis, P. Z., Davis, J. N., & Wittenberger, M. E. (1979) *Anal. Biochem. 95*, 275–285.
Sturtevant, J. M. (1987) *Annu. Rev. Phys. Chem. 38*, 463–488.
Valentine, R. C., Shapiro, B. M., & Stadtman, E. R. (1968) *Biochemistry 7*, 2143–2152.
Villafranca, J. J., Ash, D. E., & Wedler, F. C. (1976) *Biochemistry 15*, 536–543.
Woolfolk, C. A., Shapiro, B. M., & Stadtman, E. R. (1966) *Arch. Biochem. Biophys. 116*, 177–192.
Yamashita, M. M., Almassy, R. J., Janson, C. A., Cascio, D., & Eisenberg, D. (1989) *J. Biol. Chem. 264*, 17681–17690.

NMR Studies of an Oligoproline-Containing Peptide Analogue That Binds Specifically to the H-2K^d Histocompatibility Molecule†

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ABSTRACT: T lymphocytes expressing variable cell surface antigen receptors recognize "processed" forms of antigen, presented on the surface of other cells by molecules of the major histocompatibility complex (MHC). Naturally processed antigenic peptides can be replaced by synthetic ones. The synthetic peptide AYPPPPPTLA (P5) is an active competitor to the antigenic peptide HLA A24 170–182 (sequence RYLENGKETLQRA) that is recognized by A24 specific T cells in association with the H-2K^d class I MHC molecule. In P5 the five prolines were designed to play the role of a rigid spacer between the residue Y and the T–L unit, so as to mimic the role of Y^{171}, T^{178}, and L^{179} in the HLA A24 antigenic peptide, since these residues have proven to be the most important with respect to the binding of the HLA A24 peptide with the H-2K^d MHC molecule. Nuclear magnetic resonance studies allow us to demonstrate that in aqueous solution P5 adopts at least three long-lived conformations that can be classified with respect to the Y^2–P^3–P^4 amide bonds as trans–trans, cis–trans, and cis–cis. Among these, the trans–trans form is present in 67% of the molecules while the two others share the remaining 33%.

Specific immune responses are mediated by T and B lymphocytes that express clonally variable antigen receptors on cell surfaces. Unlike B lymphocytes that can directly bind antigens in the native conformation, T lymphocytes apparently recognize only "processed" forms of antigens, probably peptides, presented on the surface of other cells by polymorphic molecules encoded by the major histocompability complex (MHC) (Townsend & Bodmer, 1989). Two distinct classes of MHC molecules that differ in structure, biosynthesis, and tissue distribution are known to present antigens to T lymphocytes. In general, antigenic peptides derived from endogenous proteins are presented by class I MHC molecules, whereas those from exogenous proteins are presented by class II (Germain, 1986). Both kinds of antigens can be replaced experimentally by synthetic peptides, provided they are presented by cells expressing the appropriate MHC molecules (Townsend & Bodmer, 1989).

Direct binding studies with antigenic peptides and purified MHC molecules (Buus et al., 1987) support the concept that T cell antigen recognition can be accounted for, at least in part, by the specificity of peptide interaction with MHC molecules. Thus the way in which the peptide binds to the MHC molecule is essential for T cell recognition. In an X-ray crystallographic analysis of MHC class I molecules, a groove containing the most polymorphic residues was identified as the putative antigen-binding site (Bjokman et al., 1987). Class II molecules are presumed to contain a similar site (Brown et al., 1988). The analysis of cocrystals composed of MHC molecules and

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