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# Temperature and guanidine induced unfolding of dodecameric glutamine synthetase from *E. coli*

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***Abstract***
Glutamine synthetase (GS) of 622000 $M_r$ from *E. coli* is composed of 12 identical subunits which are structurally arranged in two superimposed hexagonal rings with active sites at subunit interfaces. The enzyme undergoes reversible, thermally induced, partial unfolding without dissociation of subunits at pH 7 in the presence of 100 mM KCl and 1.0 mM $MnCl_2$. Cooperative interactions link partial unfolding reactions of all subunits within the Mn•GS dodecamer ($\Delta H_{cal}$ = 750 kJ/mol) and only two, two-state transitions with similar $T_m$ values (324±2 K) are observed. Enthalpies at 310 K for subunit dissociation and subsequent unfolding were estimated to be ~61 and ~55 J/g, respectively, or ~100-fold the value of $\Delta H$ for thermal unfolding.

Differential scanning calorimetry (DSC) and temperature-induced spectral changes of oligomeric proteins give information on the cooperativity of thermally induced unfolding reactions. To apply reversible thermodynamic treatments of DSC data obtained with oligomeric proteins, we should know the following: (1) Unfolding transitions are reversible and the measured parameters ($T_m$ and $\Delta H_{cal}$) are independent of the scan rate used to collect the data. (2) What is the molecular species undergoing unfolding (*i.e.*, can the moles of biopolymer be defined)? (3) Can pre- and post-transitional baselines be precisely drawn and extrapolated to a reasonable $\Delta C_p$ value? (4) How many thermodynamic domains ($\Delta H_{cal}/\Delta H_{vH}$) or cooperative units (two-state transitions)? (5) How do thermodynamic domains compare with molecular structures (if known) and from such a comparison, is there evidence for cooperativity in unfolding? (6) Do ligands uncouple unfolding transitions? Thermodynamic parameters of ligand-protein interactions must be quantitated.

We have been able to apply the criteria of 1-6 in our studies of thermally induced, partial unfolding of glutamine synthetase (GS) from *E. coli* (ref. 1-3). Thermal transitions of this large metalloenzyme ($M_r$ ~622000), which is composed of 12 identical subunits (review ref. 4), are fully reversible and are independent of scan rate (20 - 60°C/h). Heat produces only partial unfolding without dissociation of the dodecamer and recovery of enzymatic activity is >95% after cooling.

An atomic model of Mn•GS at 3.5 Å is available from the X-ray diffraction studies of David Eisenberg and co-workers (ref. 5,6). These studies have confirmed that the 12 subunits of GS are arranged in 2 face-to-face hexagonal rings (ref. 7) 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 ß strands--six ß strands from a large C-terminal domain and two ß strands from a relatively small N-terminal domain of an adjacent subunit. The C-terminal domain of each subunit contains the two active-site metal ions, $n_1$ and $n_2$ (ref. 8), as well as the binding site for *L*-glutamate (*L*-glutamine). ATP spans both domains by binding near Lys-47 (ref. 9) and $Mn^{2+}$ at the $n_2$ site (ref. 8,10). The N-terminal domain with the Trp-57 loop is fairly exposed (ref. 5,6) and contains no tyrosyl residues. The stability of contacts between subunits in opposing rings mainly is provided by the C-terminal 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 ß-sheet interactions have been identified (ref. 6).

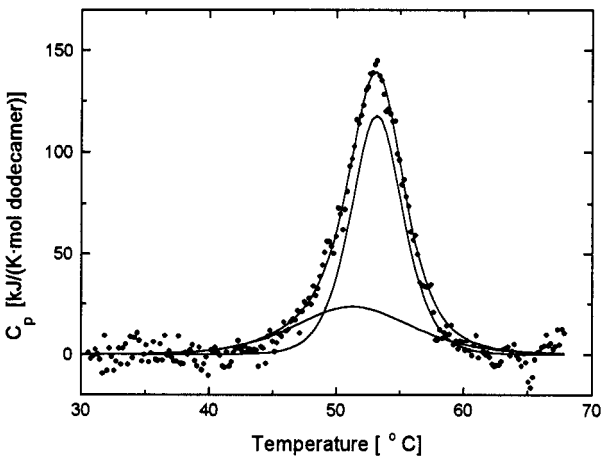


Fig. 1. Deconvolution analysis of DSC data for Mn•GS (11.3 mg) in 50 mM Pipes/KOH, 100 mM KCl, and 1.0 mM $MnCl_2$, pH 7.1 (at $T_m$) obtained with a MicroCal MC-2 instrument: $t_m$ = 53.0°C; $\Delta H_{cal}$ = 862 kJ/mol; and $\Delta H_{vH}$ = 594 kJ/mol. A model for two independent two-state transitions was fitted to the data (solid lines); $t_1$ and $t_2$ = 51.4 and 53.2°C with corresponding $\Delta H$ values of 285 and 594 kJ/mol.

The thermal transition of Mn•GS is non-two-state and involves at least two structural domains: one containing Trp and unfolding at a temperature about 2°C lower than the other containing Tyr residues (ref. 3). The magnitudes of spectral changes correspond to a net exposure of 8 of the 24 Trp and ~24 of the ~144 buried Tyr per GS dodecamer. These changes are smaller than those observed for the formation of the inactive apoenzyme by $Mn^{2+}$ removal and much smaller than those produced by 6 M guanidine•HCl (gdn•HCl) (ref. 3,11). Thus, the thermally induced reactions lead to an intermediate state along the unfolding pathway. The release of metal ions (as well as other active-site ligands) occurs in parallel with dissociation and unfolding of Mn•GS in 6 M gdn•HCl (ref. 12).

DSC profiles of Mn•GS from 15-68°C show a single endothermic peak with a small, positive $\Delta C_p$ of 42 ± 11 kJ/K•mol (ref. 1). After subtracting the buffer baseline, both pre- and post-transitional base lines were linear and these were connected by a progress curve (spanning $\Delta C_p$) which was subtracted from the endothermic peak for data analysis. Fig. 1 shows a deconvolution of such DSC data using the programs of Freire and Biltonen (ref. 13,14) for independent two-state transitions. The data are fitted well by two independent two-state transitions with $T_m$ values < 2 K apart. However, the same data can be fitted equally well with two sequential two-state transitions; (see ref. 1, for example). The $T_m$ values from the deconvolution analysis of Fig. 1 approximate the temperatures of half completion of Trp and Tyr exposures determined spectrally (ref. 1,3). The deconvolution result also is in accord with the cooperative ratio (CR), where CR = $\Delta H_{cal}/\Delta H_{vH}$ = 1.5, which indicates more than one two-state process. From these results we can conclude that the thermally induced, partial unfolding of the Mn•GS dodecamer involves two thermodynamic domains. Since structural domains of each subunit are involved in Trp and Tyr exposures, we can further conclude that partial unfolding reactions in subunit domains are linked so that all subunits within the dodecamer cooperatively undergo partial unfolding.

The specific enthalpy of the thermally induced transition of Mn•GS is small; 1.4 J/g in Fig. 1. We are able to detect this small enthalpy change because there is such a high degree of cooperativity during partial unfolding reactions (ref. 1). We have determined the proton uptake associated with the thermal transitions of Mn•GS to be ~8 $H^+$/mol dodecamer. The proton uptake and the positive $\Delta C_p$ value indicate that there is a net exposure of ionizable groups as well as of hydrophobic groups during partial unfolding reactions. It is possible that partial unfolding transitions within the dodecamer simply involve shifts of ß strands, which lead to a disruption of active sites.

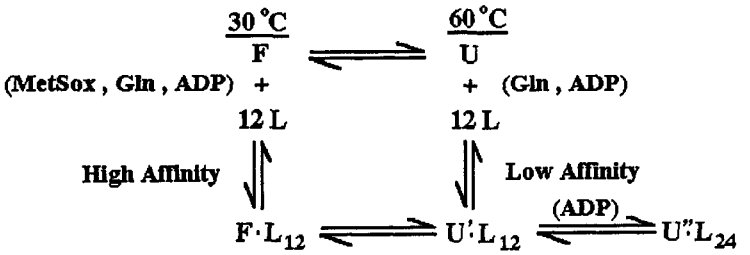


Fig. 2. Folded (F) and unfolded (U) forms of Mn•GS are predominantly populated at 30 and 60 °C, respectively. At 30 °C, log $K_A'$ is 4.0, 2.1 and 5.5 for the binding of MetSox, Gln and ADP, respectively. At 60 °C, log $K_A'$ is 1.3 for Gln; log $K_A'$= 4.3 and 3.6 for binding ADP to 2 sites/subunit in partially unfolded Mn•GS (ref. 2).

The effects of active-site ligands on thermally induced transitions of Mn•GS in DSC also have been investigated (ref. 2). The legend to the scheme in Fig. 2 gives the binding constants (expressed as log $K_A'$ values) which were determined at 30 and 60°C for ligand binding to the folded and unfolded form of Mn•GS, respectively. *L*-Glutamine and the transition state analogue *L*-methionine sulfoximine (MetSox) were found to stabilize Mn•GS and the increases in $T_m$ produced by varying concentrations of these ligands were proportional to the free energies of preferential interactions with the folded form of the enzyme, as discussed by Schellman (ref. 15). High concentrations of ADP were found to destabilize Mn•GS by binding to additional sites which are exposed during partial unfolding (Fig. 2). None of the ligands studied produced an uncoupling of thermodynamic domains; in all cases a single endothermic peak was observed in DSC. The effects of Gln, MetSox, and ADP on $T_m$ and $\Delta H_{cal}$ parameters from DSC were quantitated (ref. 2). Only in the case of ADP (which spans adjacent subunit contacts) did we obtain evidence for an increase in the $\Delta H_{cal}$ value for partial unfolding due presumably to increased interactions between domains.

Guanidine-induced dissociation and unfolding of Mn•GS is being investigated by calorimetry and other techniques. Fig. 3 shows the dissociation of Mn•GS (as monitored by 90° light scattering) and subunit unfolding (as monitored by tyrosyl residue exposure) as a function of increasing concentrations of gdn•HCl. Unfortunately, dilution of Mn•GS into 0.1-2 M gdn•HCl produced turbid solutions which precluded measurements in this gdn•HCl concentration range. Tyr exposure reached a maximum value at ~ 4 M gdn•HCl when the relative light scattering is ~1/12th that for Mn•GS in buffer. It should be noted that the thermally induced, partial unfolding of Mn•GS produces ~20% change in Tyr exposure.

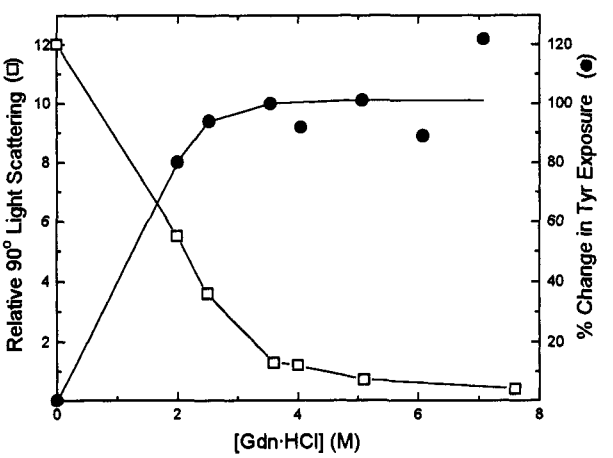


Fig. 3. Light scattering and tyrosyl residue exposure for Mn•GS in gdn•HCl at 37°C. Light scattering ($90^o$) was measured at 360 nm. Tyr exposure was monitored by the second-derivative UV spectroscopy as described in ref. 3. All values were recorded at the end of time dependent changes observed in 2-3 M gdn•HCl solutions. The experiments involved the dilution of 26.1 mg/ml Mn•GS into 40-fold volume of buffer (20 mM Hepes, 100 mM KCl, 0.1 mM $MnCl_2$, pH 7.3) or gdn•HCl solution.

The enthalpy of transfer of Mn•GS into gdn•HCl solutions at 37°C (corrected for dilution of gdn•HCl) has been measured in a LKB 10700 batch calorimeter. In order to minimize errors from the heats of dilution of gdn•HCl, 0.1 ml of buffer or enzyme solution was mixed with 4.0 ml of gdn•HCl solution. This type of measurement has been made previously with less complex proteins (ref. 16,17). For lysozyme, Pfeil and Privalov (ref. 16) found that transfer enthalpies at < 2 M gdn•HCl at 25°C measured the enthalpy of preferential binding of gdn•HCl to the protein and that these values extrapolated to $\Delta H = 0$ at 0 gdn•HCl. Extrapolation of transfer enthalpies obtained at high [gdn•HCl] to 0 gdn•HCl gave approximately the same $\Delta H$ value as obtained by DSC. In the case of Mn•GS, we were unable to obtain transfer $\Delta H$ values from 0 to 2 M gdn•HCl which would be expected to extrapolate to $\Delta H = 0$ at 0 gdn•HCl. Transfer enthalpies for Mn•GS to 2.9-4.7 M gdn•HCl gave linear extrapolations to $\Delta H = 61 \pm 10$ J/g at 0 gdn•HCl. At 4.9 M gdn•HCl, a transition occurred to a line connecting transfer enthalpies at 5.2-7.0 M gdn•HCl which extrapolated to $\Delta H = 115 \pm 18$ J/g at 0 gdn•HCl. These extrapolated transfer enthalpies for Mn•GS appear to represent $\Delta H_{dissociation}$ at $\leq$ 4.7 M gdn•HCl and the sum of dissociation and unfolding enthalpies at $\geq$ 5 M gdn•HCl. Work is in progress to further characterize dissociation and unfolding of subunits of Mn•GS in gdn•HCl.

In summary, our studies demonstrate the importance of intersubunit interactions in an oligomeric protein in maintaining its native structure and their contributions to the thermostability of dodecameric glutamine synthetase.

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