Urea-induced dissociation and unfolding of dodecameric glutamine synthetase from *Escherichia coli*: Calorimetric and spectral studies

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Abstract

Urea-induced dissociation and unfolding of manganese·glutamine synthetase (Mn·GS) have been studied at 37 °C (pH 7) by spectroscopic and calorimetric methods. In 0 to ~2 M urea, Mn·GS retains its dodecameric structure and full catalytic activity. Mn·GS is dissociated into subunits in 6 M urea, as evidenced by a 12-fold decrease in 90° light scattering and a monomer molecular weight of 51,800 in sedimentation equilibrium studies. The light scattering decrease in 4 M urea parallels the time course of Trp exposure but occurs more rapidly than changes in secondary structure and Tyr exposure. Early and late kinetic steps appear to involve predominantly disruption of intra-ring and inter-ring subunit contacts, respectively, in the layered hexagonal structure of Mn·GS. The enthalpies for transferring Mn·GS into urea solutions have been measured by titration calorimetry. After correcting for the enthalpy of binding urea to the protein, the enthalpy of dissociation and unfolding of Mn·GS is 14 ± 4 cal/g. A net proton uptake of ~50 H⁺/dodecamer accompanies unfolding reactions. The calorimetric data are consistent with urea binding to multiple, independent sites in Mn·GS and the number of binding sites increasing ~9-fold during the protein unfolding.

Keywords: calorimetry; glutamine synthetase; protein unfolding; subunit dissociation; urea denaturation

The important regulatory role of multisubunit proteins and multiprotein complexes in biological systems is widely recognized. Understanding the folding and self-assembly processes of such macromolecules remains a major problem in protein chemistry. Although inter- and intrasubunit interactions are of the same physical nature, the denaturation/renaturation reactions in oligomeric proteins are more complex than those of smaller, monomeric proteins and involve many local and global processes that occur by sequential and/or concerted mechanisms (Jaenicke, 1987; Seckler & Jaenicke, 1992). In order to characterize these reactions fully it is necessary to use various experimental methods, each of which can monitor a different aspect of the folding/unfolding process.

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Abbreviations: GS, unadenylylated *Escherichia coli* glutamine synthetase dodecamer containing 0.8 equivalent of covalently bound AMP; Mn·GS, manganese ion complex with GS (12 Mn²⁺/native GS with 0.1 mM free Mn²⁺ at pH 7); DSC, differential scanning calorimetry; LS, light scattering; PMPS, *p*-(hydroxy-mercuri)benzenesulfonate.

Guanidine-HCl and urea are most often used in the studies of chemically induced protein denaturation. The mechanism of reactions involving these denaturants has been a subject of many experimental and theoretical studies (Alonso & Dill, 1991; Timasheff, 1992). Recently, Makhatadze and Privalov (1992) have shown that the calorimetric titration isotherms for urea- and GdnHCl-protein interactions are consistent with the binding of these denaturants to multiple, independent sites in proteins.

Glutamine synthetase from *Escherichia coli* is a large oligomeric enzyme ($M_r \sim 622,000$) consisting of 12 identical subunits arranged in two stacked hexagonal rings (Stadtman & Ginsburg, 1974). The 12 active sites of GS are located at the interfaces between adjacent subunits within each ring of 6 (Almasy et al., 1986; Yamashita et al., 1989). Each subunit has two essential metal ion binding sites, which can be occupied by Mn²⁺ or Mg²⁺ (Hunt et al., 1975; Villafranca et al., 1976; Hunt & Ginsburg, 1980).

Earlier studies of the urea- and GdnHCl-induced denaturation of Mn·GS have shown that there are many intermediate states with different levels of protein oligomerization (Woolfolk & Stadtman, 1967; Maurizi & Ginsburg, 1982). Active-site ligands

stabilize intersubunit contacts in dodecameric Mn·GS and inhibit dissociation and unfolding (Ciardi et al., 1973; Maurizi & Ginsburg, 1982, 1985). Because mild dissociation conditions yield only even-numbered, active oligomers (4-, 6-, 8-, and 10-mers), inter-ring subunit contacts appear to be stronger than heterologous intra-ring contacts (Maurizi & Ginsburg, 1982, 1985).

A transition from the native Mn·GS to a partially unfolded, nondissociated state can be induced thermally at $\sim 51^\circ\text{C}$ (Shrake et al., 1989). This unfolding step is reversible, highly cooperative, and affects the ligand binding properties of the enzyme (Ginsburg & Zolkiewski, 1991; Zolkiewski & Ginsburg, 1992). The transition enthalpy, as measured by differential scanning calorimetry, is low: ~ 0.3 cal/g (Ginsburg & Zolkiewski, 1991).

For large oligomeric proteins, it has been impossible to measure thermodynamic parameters for complete unfolding by using DSC. This is due to thermostable regions in proteins and to a tendency of unfolding intermediates to aggregate at high temperature. In this study, we have obtained the enthalpy of complete unfolding and dissociation of Mn·GS by measuring the heat of transfer of the protein into denaturant solutions. These data add to only a small number of enthalpy values obtained in this direct way and reported thus far only for monomeric proteins (Pfeil & Privalov, 1976; Lapanje et al., 1977; Makhatadze

& Privalov, 1992). Calorimetric transfer experiments have been paralleled by analysis of protein structural changes occurring at different denaturant concentrations. Changes in the size of Mn·GS and intermediate oligomers have been monitored by light scattering, nondenaturing gel electrophoresis, and ultracentrifugation, whereas losses in secondary protein structure have been estimated by CD measurements. Decreases in enzymatic activity and the exposures of Trp, Tyr, and Cys residues accompanying the denaturation of Mn·GS also have been investigated.

Results

It is known that the chemical denaturation of proteins is a multi-step process and some physical parameters have been found to change for more than 100 days after initialization of the reaction (Tanford, 1966). It is often difficult, therefore, to demonstrate that the protein–denaturant system has reached thermodynamic equilibrium. What is usually observed instead, is a steady-state level of one or more measured parameters.

Chemical denaturation of glutamine synthetase is indeed a complex reaction. For example, a partially unfolded state has been observed after 1–2 days of incubation in ~ 3 M urea (Fisher & Stadtman, 1992). This intermediate tends to aggregate due in part to the formation of intersubunit disulfide bonds (M. Zolkiewski, unpubl. results). In the present study, we show that detectable heat effects occur within < 10 min after transfer of Mn·GS into urea solutions (see below). Therefore, our goal has been to identify protein structural changes that correspond to those stages of the denaturation process that contribute to the measured enthalpy change. Denaturation experiments have been performed at 37°C , where the changes in many properties of Mn·GS on exposure to a denaturant occur rapidly (Maurizi & Ginsburg, 1982).

Urea-induced denaturation of Mn·GS

Figure 1 shows the relative changes in various parameters measured after 10 min incubation of Mn·GS in buffer solutions containing different amounts of urea. This incubation time is sufficient for the measured parameters to reach steady-state levels in < 2.4 M and > 3.8 M urea. In intermediate urea concentrations, a slow drift of the measured values has been observed for periods longer than 10 min (see below).

No changes in the weight-average molecular weight, α -helical content, Mn·GS activity, Tyr, and -SH group exposure have been found up to ~ 2 M urea (Fig. 1). In contrast, the changes in Trp exposure occur at lower urea concentrations. In addition, sedimentation velocity measurements for Mn·GS (1 mg/mL) in 1.5 M urea at 20.0°C and 35,000 rpm show a single, symmetrical sedimenting boundary with $s_{20,w} = 19.6 \pm 0.1\text{S}$. This sedimentation coefficient corresponds to 97% of that of native Mn·GS and is the same value as that previously measured for the apo- or “relaxed” form of the dodecameric enzyme (Shapiro & Ginsburg, 1968).

As the urea concentration increases, dissociation of the dodecamer occurs and this is accompanied by changes in other elements of protein structure (Fig. 1). In 6 M and higher urea concentrations, the LS decreases to 1/12th the value of native

Fig. 1. Progress of urea-induced denaturation of Mn·GS at 37°C . Mn·GS (final concentration 0.22 mg/mL) was transferred into 20 mM Hepes/KOH, 100 mM KCl, 0.1 mM MnCl_2 , pH 7.3, containing different amounts of urea. After 10 min incubation, the following parameters were measured (see Materials and methods for experimental details). **A:** $\circ$, ellipticity at 222 nm; $\bullet$, enzymatic activity (10 min assay); $\blacktriangle$, Trp and $\square$, Tyr exposures. **B:** $\bullet$, LS; $\circ$, -SH exposure. Solid lines are for illustration only.

dodecameric Mn·GS (Fig. 1B). The secondary structure is disrupted because Mn·GS in 5–7 M urea has a random coil-type CD spectrum (not shown), and no further changes in molar ellipticity at 222 nm are observed in this urea concentration range (Fig. 1A). All four -SH groups/subunit, which are nonreactive in native Mn·GS, become exposed to the solvent and maximum changes in Trp and Tyr environments occur in >5 M urea. Also, the enzymatic activity is lost in 4–7 M urea (Fig. 1A).

Sedimentation equilibrium experiments have demonstrated that the protein is 100% monomeric in 6 M urea ($M_r = 51,800$) and ~90% monomeric (with ~10% oligomers of average 6-mer size also present) in 5 M urea. For these experiments, Mn·GS had been exposed to urea solutions for 3–5 days at 20 °C, and the buffer contained 2 mM 2-mercaptoethanol to prevent possible crosslinking of GS subunits by disulfide bond formation.

Figure 2 shows the time courses for the change of various parameters following the transfer of Mn·GS from the native buffer into urea solutions. The rate of the LS decreases occurring in >2 M urea becomes greater as the urea concentration increases (Fig. 2A). All the parameters studied in 4 M urea (Fig. 2B) reach a steady-state after ~10 min of incubation at 37 °C; however, the time course for the change in each parameter is different. The LS decrease parallels the change in Trp exposure with an approximate half-time of ~100 s. The changes in the α -helical content and Tyr exposure are slower than that of the decrease in the average size of Mn·GS.

Fig. 2. Time course of the urea denaturation of Mn·GS at 37 °C. **A:** LS changes were monitored for Mn·GS (final concentration 0.22 mg/mL) transferred into ○, buffer, or ▲, 2 M; ◆, 2.8 M; ×, 3.2 M; +, 3.6 M; ●, 4.0 M; ■, 5.9 M urea. **B:** Changes of ○, ellipticity at 222 nm; ●, LS; ▲, Trp and □, Tyr exposure during incubation of Mn·GS in 4 M urea.

Full regain of enzymatic activity has been observed during 1-min assays of Mn·GS incubated in 4 M urea up to 2 min and ~75% regain after 6 min incubation (data not shown). This indicates that dissociation reactions can be reversed under assay conditions.

Nondenaturing gradient-pore gel electrophoresis has been used to identify possible dissociation reactions corresponding to the LS decreases for Mn·GS in urea solutions (Fig. 3). The Pharmacia Phast system allows a rapid electrophoretic separation of oligomeric species formed during denaturation/renaturation of Mn·GS (Fisher, 1994). No subunit dissociation occurs in 1 M urea (lane 3). Sharp bands that appear below the major band of dodecameric Mn·GS (see reference lane 2) correspond to even-numbered oligomers, as identified earlier by electron microscopy (Haschemeyer et al., 1982; Maurizi & Ginsburg, 1982). The amounts of decamers, octamers, hexamers, and tetramers increase after 10 min incubation in 2–3 M urea and after 1 min in 4 M urea (lanes 4–6). All of these bands stain positively for GS-catalyzed γ -glutamyl transfer activity, as has been previously reported for stabilized oligomers (Maurizi & Ginsburg, 1985).

Monomeric GS is found in 3–7 M urea, but it cannot be observed after 1 min incubation in 4 M urea. Multiple weaker, diffuse bands can be seen in >4 M urea and their position does not coincide with those of sharp bands found in lower urea concentrations (lane 7). These diffuse bands are inactive in catalyzing γ -glutamyl transfer. The location and intensity of these broad bands does not change after overnight incubation of Mn·GS in urea or when 2 mM dithiothreitol has been present in incubation mixtures (gels not shown). Thus, the diffuse bands are not due to incompletely dissociated or crosslinked oligomers. Rather, the broad bands appear to correspond to inactive intermediates formed during renaturation of Mn·GS from the monomers during the gel electrophoretic separation in the absence of urea. Similar bands have been observed during chaperonin-assisted renaturation of Mn·GS (Fisher, 1994). When gel electrophoresis is performed in the presence of 7 M urea, only a monomer subunit band is observed (not shown).

The gel electrophoresis results shown in Figure 3 indicate that the early phases of the LS decreases in >2 M urea (Figs. 1, 2) may be due to dissociation of the dodecameric Mn·GS into even-numbered oligomers. Disruption of these oligomeric species and formation of unfolded monomers occurs during the later dissociation phase.

Enthalpy of dissociation and unfolding of Mn·GS

A major difficulty in the determination of the heat of transfer of a protein into denaturant solutions is the large, endothermic heat effect produced by dilution of the denaturant. The heat of dilution of urea constitutes a major portion of the total measured heat effect, even for 5- μ L injections of a buffered protein solution into a calorimetric cell of 1.4 mL volume filled with the buffer containing urea (Fig. 4). Nevertheless, when Mn·GS is present in injected solutions, the endotherms are smaller than those observed without the protein present (Figs. 4, 6 below). This indicates that the binding of urea to the protein is exothermic. Detectable heat effects occur within ~2 min after the injection of either buffer or the Mn·GS solution into 1.2 M urea (Fig. 4A). However, the injection of Mn·GS into more concentrated urea solutions results in additional endothermic reactions that last for ~10 min (Fig. 4B, inset). The slower endothermic

Fig. 3. Distribution of Mn-GS oligomers after incubation in urea solutions analyzed using 8–25% nondenaturing gradient gel. Lanes 1 and 11: molecular weight standards (from the top): thyroglobulin (M_r 669,000); ferritin (M_r 440,000); catalase (M_r 232,000); aldolase (M_r 158,000); bovine serum albumin (M_r 67,000); and ovalbumin (M_r 43,000). Lane 2: native Mn-GS. Lanes 3–5 and 7–10: Mn-GS incubated for 10 min at 37 °C in 1, 2, 3, 4, 5, 6, and 7 M urea. Lane 6: Mn-GS incubated for 1 min in 4 M urea. Bands corresponding to GS monomer and even-numbered oligomers are identified with subunit numbers.

processes correspond to urea-induced dissociation and unfolding of the Mn-enzyme as identified in Figures 1 and 2.

Integrated heats for the transfer of Mn-GS into urea solutions (after correction for the heat of dilution of urea) are shown in Figure 5. The enthalpy of transfer is negative, which reflects the exothermic binding of urea to Mn-GS. Assuming a mechanism of multiple, independent binding sites for urea (Makhatadze &

Privalov, 1992), the enthalpy of transfer (per g of a protein) can be expressed as:

$$\Delta H_{\text{trans}}(a) = (n/M_r)\Delta H_{\text{bind}}K_A a / (1 + K_A a) + \Delta H_d(a), \quad (1)$$

where n is the number of urea binding sites (per mol of Mn-GS), $M_r = 622,000$ is the molecular weight of Mn-GS, K_A is the as-

Fig. 4. Representative calorimetric data of the heat effects of transfer Mn-GS into urea solutions at 37 °C. **A:** 5-μL injection of 20 mM Hepes/KOH, 100 mM KCl, 0.1 mM MnCl₂, pH 7.3 (solid line) or Mn-GS in the same buffer (117 μg/injection) (dashed line) into 1.2 M urea. **B:** 5-μL injection of 1.2 M urea (solid line) or Mn-GS (86 μg/injection) in 1.2 M urea (dashed line) into 4 M urea. Insets: Expanded scale for the data in the 150–660-s range.

Fig. 5. Enthalpy of transfer Mn·GS from ●, 20 mM Hepes/KOH; ○, 20 mM Tris/HCl; or △, 20 mM Pipes/KOH, with 100 mM KCl, 0.1 mM MnCl₂, pH 7.3, into urea at 37 °C. Data points correspond to the averages from 6 to 10 injections into solutions of specific urea concentrations after integration of the heat output curves and correction for the heat of dilution of urea (see Figs. 4, 6). The data point for 4 M urea in Hepes shows the mean value and standard deviation from 15 independent determinations of the heat of transfer Mn·GS either directly or stepwise from 0 to 4 M urea solutions. The data points for >4 M urea were obtained using the ΔH_{trans} value for 4 M urea and the measured enthalpy values for the transfer from 4 M to higher urea concentrations. Solid lines show the least-squares fits of the data obtained in Hepes in 0–1 M and 6–8.6 M urea concentration regions using Equations 2 and 3, respectively. Dashed line shows the linear least-squares fit for the data in 6–8.6 M urea.

sociation constant for urea binding, ΔH_{bind} is the single-site enthalpy for urea binding, a is the activity of urea in solution, and ΔH_d is the heat of protein denaturation (including the enthalpy of subunit dissociation and unfolding). The activity of urea was calculated from the molarity of urea solutions using the activity coefficient data at 40 °C of Stokes (1967).

The binding of urea to proteins is weak ($K_A = 0.055 \text{ M}^{-1}$ at 40 °C for three unfolded proteins at pH 2.5; Makhatadze & Privalov, 1992). Thus, for 0–1 M urea, we can assume that $K_A a \ll 1$. From the data in Figures 1 and 3, it is clear that no significant changes in the structure of Mn·GS occur below 1 M urea ($\Delta H_d = 0$). This leads to:

$$\Delta H_{\text{trans}}(a) = (n_f/M_r)\Delta H_{\text{bind}}K_A a \quad \text{for } 0 < [\text{urea}] < 1 \text{ M}, \quad (2)$$

where n_f is the number of urea binding sites in native, folded Mn·GS.

From the data presented in Figures 1 and 3 and from sedimentation–equilibrium studies conducted in the ultracentrifuge, we can conclude that Mn·GS is completely dissociated and unfolded in $\geq 6 \text{ M}$ urea and $\Delta H_d(a) = \text{constant} = \Delta H_d$ for these urea concentrations:

$$\Delta H_{\text{trans}}(a) = (n_u/M_r)\Delta H_{\text{bind}}K_A a/(1 + K_A a) + \Delta H_d \quad \text{for } [\text{urea}] > 6 \text{ M}, \quad (3)$$

where n_u is the number of urea binding sites in the dissociated and unfolded protein.

Equations 2 and 3 have been used to fit the data for the enthalpy of transfer in the low- and high-urea concentration ranges, respectively (Fig. 5). The data for intermediate urea concentrations show a continuous transition between the two limiting behaviors, consistent with Equations 2 and 3. The fitting parameters are: $(n_f\Delta H_{\text{bind}})$ for Equation 2 and $(n_u\Delta H_{\text{bind}})$ and ΔH_d for Equation 3. The value of K_A is assumed not to change

with pH and is taken from Makhatadze and Privalov (1992). This leads to the following values for the fitting parameters: $n_f\Delta H_{\text{bind}} = -9.3 \pm 0.4 \text{ cal/g}$, $n_u\Delta H_{\text{bind}} = -83 \pm 14 \text{ cal/g}$, and $\Delta H_d = 13.6 \pm 3.7 \text{ cal/g}$ (in the absence of urea).

Calorimetric measurements in three buffers with different protonation enthalpies¹ give very similar results (Fig. 5), which indicates that the heat of proton uptake/release during the Mn·GS denaturation is small and is certainly within the error of the ΔH_d determination. The observed ΔH_{trans} values obtained for the transfer of Mn·GS into 4 M urea in Tris, Hepes, and Pipes at pH 7 have been plotted against the heats of protonation of these buffers. From the linear least-squares fit of these data, the slope gives a net uptake of $\sim 50 \text{ H}^+/\text{Mn}\cdot\text{GS}$ dodecamer, which is coupled to unfolding reactions of Mn·GS. Thus, the observed ΔH_d in Hepes buffer, for example, will contain a contribution of $\sim 0.4 \text{ cal/g}$ from proton uptake. An uptake of 24 protons is anticipated from the release of the 12 Mn^{2+} bound to structural sites of Mn·GS (Hunt et al., 1972) upon urea-induced unfolding (Maurizi & Ginsburg, 1982).

It should be noted that the K_A value reported by Makhatadze and Privalov (1992) is higher than values obtained by others for urea binding to peptide bond-containing model compounds in nonbuffered aqueous solutions (Schoenert & Stroth, 1981; Sijpkens et al., 1993). For such weaker urea binding $K_A a \ll 1$ for 6–8.6 M urea and a linear fit of the ΔH_{trans} data (dashed line in Fig. 5) gives $\Delta H_d = 8.2 \pm 3.5 \text{ cal/g}$.

The above fitting parameters give a ratio for $n_u/n_f = 8.9$, which indicates that there is a significant increase in the number of urea binding sites during the dissociation and unfolding of Mn·GS. By assuming $\Delta H_{\text{bind}} = -2.15 \text{ kcal/mol}$ (Makhatadze & Privalov, 1992), we also can estimate that $n_f \approx 2,700$ and $n_u \approx 24,000$.

¹ The enthalpies of buffer protonation at 37 °C were calculated from those measured at 30 and 50 °C (Beres & Sturtevant, 1971; Chen & Wadso, 1982; Zolkiewski & Ginsburg, 1992) and are $-3.2 \text{ kcal/mol H}^+$ for Pipes, $-5.4 \text{ kcal/mol H}^+$ for Hepes, and $-11.7 \text{ kcal/mol H}^+$ for Tris.

Whereas Mn·GS is soluble in all concentrations of urea, solutions of the Mn·enzyme in 0.1 to ~2 M GdnHCl are turbid. In 3–7 M GdnHCl, however, the LS signal reaches ~1/12 of that of native Mn·GS, and the maximum changes in Trp and Tyr exposures are observed (Ginsburg & Zolkiewski, 1994). Measurements of the enthalpy for transfer of Mn·GS into GdnHCl solutions have been more difficult and less accurate than those with urea, due to the very high heats of dilution of GdnHCl. Data points for the transfer of Mn·GS into 2.9–7.0 M GdnHCl (not shown) are scattered (± 3 cal/g) around the fitted (solid) curve constructed for the GS–urea interaction isotherm (Fig. 5).

Discussion

The urea-induced changes in different structural elements of Mn·GS show that the denaturation of this protein is a multistep process. This is obviously different from the two-state unfolding behavior observed for many small, single-domain proteins (Jaenicke, 1987).

Dissociation of the dodecameric Mn·GS appears to be among the first steps in the denaturation process (Figs. 1, 2). Previously, it has been found that the interactions between opposite, neighboring GS subunits from two hexagonal rings (homologous contacts) of Mn·GS are stronger than those between adjacent subunits within the same ring (heterologous contacts) (Haschemeyer et al., 1982; Maurizi & Ginsburg, 1982, 1985). This conclusion is based on the observation that Mn·GS forms only even-numbered oligomers (with the homologous interactions intact) under mild denaturing conditions in the presence of saturating amounts of strongly bound active-site ligands. The atomic structure of Mn·GS, which has been determined from elegant X-ray crystallography studies in David Eisenberg's laboratory (Almassy et al., 1986; Yamashita et al., 1989), shows the existence of strong, stabilizing inter-ring subunit contacts: an apolar carboxy-terminal "helical thong" inserts into a hydrophobic pocket formed by two neighboring subunits on the opposite ring and a hydrogen bonded β -sheet interaction, which also exists between subunits within a ring. It has been concluded that hydrophobic interactions account for most of the intersubunit stability.

The gel electrophoresis results of Figure 3 suggest that early in the dissociation phase breakage of subunit contacts occurs primarily between heterologous interfaces in the Mn·GS dodecamer, which leads to the formation of even-numbered, enzymatically active oligomers. At later times, dissociation of homologous contacts and formation of unfolded monomers are evident. Thus, the rates of tyrosyl residue exposure and secondary structure decay are slower than that of the overall dissociation (Fig. 2B). This interpretation is entirely consistent with the structural information on Mn·GS (Yamashita et al., 1989).

Tryptophanyl residue exposure closely parallels the decrease in the size of Mn·GS (Fig. 2B). There are two Trp residues in each GS subunit and they are located near intersubunit contact regions (Almassy et al., 1986; Yamashita et al., 1989). Trp-57 is in the active-site region at each heterologous interface and Trp-158 is located in structures that maintain homologous contacts. Thus, tryptophanyl residues are effective spectral probes for Mn·GS dissociation. However, Trp exposure also occurs during thermally induced, partial unfolding of the dodecameric enzyme (Shrake et al., 1989) and during the conformational change produced by removal of Mn^{2+} or Mg^{2+} (Shapiro & Ginsburg,

1968; Hunt & Ginsburg, 1972). The removal of Mn^{2+} makes the enzyme susceptible to dissociation (Shapiro & Ginsburg, 1968), and it is quite likely that Mn^{2+} is released during urea treatment, as has been observed for GdnHCl-induced unfolding (Maurizi & Ginsburg, 1982).

The 17 Tyr residues are located both in surface and interior regions of the GS subunit structure. The four Cys residues of each subunit are buried in Mn·GS and only become reactive with sulfhydryl reagents upon removal of Mn^{2+} (Shapiro & Ginsburg, 1968). Therefore, observed changes in Tyr and -SH exposures during urea-induced denaturation reflect a progressive disruption of the protein tertiary structure (Figs. 1, 2B).

Observations on the relative stabilities of different elements of Mn·GS structure possibly are relevant to understanding the mechanism of folding and assembly of this enzyme. Newly synthesized GS subunits may form homologous contacts simultaneously with early folding events. Dimers of GS subunits have a transient stability, but once tetramers are formed in the presence of Mg^{2+} or Mn^{2+} , association of the heterologous contacts occurs with the formation of hexamers, octamers, decamers, and, finally, the most stable dodecameric species.

The enthalpy of protein denaturation is determined by compensating effects of the enthalpy of disruption of contacts between buried amino acids and their enthalpy of hydration (including hydrophobic and hydrophilic hydration) (Makhatadze & Privalov, 1993). The specific enthalpy of dissociation and unfolding Mn·GS ($\sim +14$ cal/g) estimated from the transfer experiments (Fig. 5) is higher than the values found for a number of smaller proteins (2–8 cal/g at 37 °C) (Makhatadze & Privalov, 1994). It should be noted that the method used in this work to obtain ΔH_d has been shown to give the same enthalpy values as DSC for small proteins, where thermal reactions lead to complete unfolding (Pfeil & Privalov, 1976).

A large enzyme of the size of Mn·GS has a lower fraction of amino acid residues exposed on its surface than a smaller protein. (Assuming that proteins are spherical and have similar packing density, it can be estimated that the volume of Mn·GS is 62 times greater than that of a 10,000 M_r protein, whereas its surface area is only 15 times greater.) It can be anticipated therefore that the enthalpy of unfolding (per g protein) is higher for Mn·GS than for unfolding small proteins because the fraction of amino acid residues transferred from the protein interior into the solvent is proportionally higher. The same effect makes the urea binding site ratio $n_u/n_f \approx 9$ higher for Mn·GS than the value of $n_u/n_f \approx 2$ –3, which has been obtained for three proteins with $M_r \sim 12,000$ –14,000 (Makhatadze & Privalov, 1992).

The enthalpy of the temperature-induced, reversible transition of Mn·GS occurring at ~ 50 °C (Ginsburg & Zolkiewski, 1991) is only $\sim 2\%$ of the enthalpy for complete denaturation reported here. Thus, as concluded earlier (Ginsburg & Zolkiewski, 1991; Zolkiewski & Ginsburg, 1992), this transition represents a highly cooperative structural rearrangement of the dodecameric Mn·GS without a significant exposure of the hydrophobic core of the protein.

From their studies on the urea and GdnHCl interactions with lysozyme, ribonuclease, and cytochrome c, Makhatadze and Privalov (1992) concluded that the number of denaturant binding sites correlated well with the number of hydrogen bonding groups exposed in a folded or unfolded protein. Assuming that all amino acid residues are exposed to the solvent in unfolded

GS, it follows from the GS amino acid sequence (Colombo & Villafranca, 1986) that the number of hydrogen bond donor and acceptor groups is 2,590 per GS subunit. When compared with the estimated n_u , this leads to a value of 1.3 for the ratio of hydrogen bonding groups to urea binding sites. Although the value of n_u is only approximate, it supports the conclusion of Makhatadze and Privalov (1992) that hydrogen bonding is the main mechanism of urea-protein interactions.

Materials and methods

Chemicals and enzyme solutions

Hepes and Pipes were obtained from Sigma; Tris, ultrapure urea, and GdnHCl were from Schwarz/Mann Biotech. Chemicals for enzyme preparation and activity measurements were as described previously (Ginsburg & Zolkiewski, 1991). All aqueous solutions were prepared using deionized and filtered water from Millipore Milli-Q Plus system.

Unadenylylated glutamine synthetase was isolated from an overproducing *Escherichia coli* strain (Maurizi & Ginsburg, 1986) using the purification procedure of Woolfolk et al. (1966), with minor modifications (Ginsburg & Zolkiewski, 1991). The enzyme was stored at 4 °C as a suspension in 52% $(\text{NH}_4)_2\text{SO}_4$. GS samples were collected by centrifugation and then dialyzed extensively at 4 °C against a buffer containing 20 mM Hepes/KOH (or 20 mM Tris/HCl or 20 mM Pipes/KOH), 100 mM KCl, 0.1 mM MnCl_2 , pH 7.30 ± 0.01 , at 30 °C. Protein concentrations were determined from the published absorption coefficients (Ginsburg et al., 1970).

Concentrated urea and GdnHCl solutions in water were prepared by weight using the density data of Kawahara and Tanford (1966) and then adjusted to desired concentrations with water and 10× concentrated buffer. GS samples were diluted into urea or GdnHCl solutions of known concentrations, except for the ultracentrifugation experiments described below. Enzyme activity was measured in the saturating pH 7.57 γ -glutamyl transfer assay as described by Stadtman et al. (1979).

LS

Denaturant-induced changes in 90° LS were measured using a Perkin-Elmer 650-40 fluorometer with a jacketed cell holder connected to an external thermostated water bath. The excitation and emission wavelengths were 360 nm, which is much greater than the size of Mn·GS. Under such conditions, the Rayleigh LS intensity is proportional to weight-average molecular weight and concentration of the solute and also depends on the refractive properties of the solvent (Tanford, 1966). The effects of urea on the LS of aqueous solutions of proteins were estimated by measuring the LS of bovine serum albumin in the buffer with different urea concentrations. Assuming the refractive properties of the buffer = 1, the corrected LS = $\text{LS}_{\text{obs}}C$, where the correction factor $C = 1/(1 - 0.117x + 0.0093x^2)$ (at 37 °C) and x is the molarity of urea solution.

CD

CD spectra were measured with a Jasco J-710 spectrometer using a water-jacketed, cylindrical cell with 1-mm optical pathlength (Jasco). Cell temperature was controlled by an external pro-

grammable water bath. The spectra were corrected for the solvent CD signal.

Changes in Trp and Tyr exposure

UV spectra were measured with a Hewlett Packard 8450A rapid-scan, diode-array spectrophotometer with temperature-controlled cell holders (HP 89101A). The changes in tryptophanyl and tyrosyl residue exposure were calculated from the second-derivative absorption spectra as described before (Ragone et al., 1984; Shrake et al., 1989; Zolkiewski & Ginsburg, 1992).

-SH group reactivity

The exposed -SH groups were quantitated using the reaction with PMPS at pH 7 to produce absorption increases at 250 nm (Boyer, 1959). Glutathione solutions of known concentrations were used as standards. PMPS (final concentration 28 μM) was added to Mn·GS (0.27 mg/mL, corresponding to a maximum 20 μM -SH) after incubation of the protein in urea solutions, and the change in $A_{250\text{nm}}$ was measured at 20 °C for 5 min and extrapolated back to the reaction initialization time.

Analytical ultracentrifugation

The Beckman Optima model XL-A analytical ultracentrifuge equipped with a four-place An-Ti (titanium) rotor was used at 20.0 °C. Densities of buffers were determined at 20.00 ± 0.01 °C using an Anton Paar model DMA 58 densitometer and viscosities were determined as described previously (Shapiro & Ginsburg, 1968). For determining the sedimentation velocity coefficient of Mn·GS in 1.5 M urea, the protein was equilibrated in standard buffer containing 1.5 M urea by gel filtration through a P-10 column at room temperature and loaded into the right side of a 4° double-sector 12-mm Kel-F centerpiece (0.30 mL of 1.08 mg/mL Mn·GS) with 0.31 mL buffer placed in the left sector. The absorption spectrum of this protein solution was identical to that of native Mn·GS. After equilibration at 20.0 °C at 3,000 rpm, the rotor was accelerated to 35,000 rpm for radial scans at 280 nm (with a 0.003-cm step size) at 4-min intervals. Sedimentation coefficients were calculated using the software program "XLAVEL" (Beckman, version 2.0, 1992). Native Mn·GS was run also under the same conditions. For sedimentation-equilibrium runs of GS (0.3 mg/mL) in 5 and 6 M urea, samples were dialyzed at room temperature for 27 h against standard buffer containing 2 mM 2-mercaptoethanol in addition to either 5 or 6 M urea. Double-sector cells loaded with 110 μL of GS and 120 μL dialysate and decelerated to 14,000 (49 h) and 12,000 (44 h) rpm (after overspeeding) were used. Base lines were determined at the end of the run after centrifugation at 48,000 rpm for 5 h followed by deceleration to 14,000 and 12,000 rpm. Radial scans at 276 nm (10 averages) with a 0.001-cm step size were collected. Data analysis was performed using software kindly provided by Allen P. Minton with $V_{\text{bar}} = 0.715$ mL/g (Shapiro & Ginsburg, 1968). For GS in 5 M urea, it was necessary to do a global fit of the data for a nonequilibrium mixture of monomer and higher molecular weight species. For GS in 6 M urea, a global fit of the data showed a single component of $M_r = 51,800 \pm 1,000$.

Fig. 6. Cumulative heat effects in the series of calorimetric transfer experiments illustrated in Figure 4. Data sets correspond to sequences of 5- μ L injections of $\bullet$, the buffer or $\circ$, Mn·GS into 1.2 M urea, and $\blacktriangle$, 1.2 M urea or $\triangle$, Mn·GS in 1.2 M urea into 4 M urea. Data points were obtained by integration of the calorimetric output curves (Fig. 4). The difference in linear slopes for each pair of data sets ($\pm$ Mn·GS) was used to calculate specific enthalpies of transfer shown in Figure 5.

Nondenaturing gel electrophoresis

Mn·GS oligomers formed after incubation in urea solutions were rapidly separated (30–40 min) using Pharmacia Phast electrophoretic system with a discontinuous buffer system. The protein samples (~ 0.3 mg/mL) were loaded on a precast 8–25% polyacrylamide gradient gel (Phastgel). After separation, the gels were stained with Coomassie blue or for GS activity (Maurizi & Ginsburg, 1985).

Isothermal reaction calorimetry

Enthalpy of Mn·GS transfer into urea solutions was measured with a MicroCal Omega titration calorimeter (MicroCal Inc., Northampton, Massachusetts). The signal response of this calorimeter is fast with a primary time constant < 10 s (Wiseman et al., 1989). The sample cell (1.406 mL) was filled with urea solutions of known concentration. Mn·GS or buffer was added to the cell in a series of injections. The heat effects of the additions were calculated (using data analysis software from MicroCal) and plotted as illustrated in Figure 6. The slopes of the data obtained for buffer and protein additions were determined by linear least-squares regression, and the difference in slopes was used to calculate the transfer enthalpy in each case. Effects of protein volume (0.71 mL/g) on the final urea concentrations are negligible. To obtain better accuracy of the heat of Mn·GS transfer into 4–8 M urea, the protein samples were first transferred into 4 M urea and then loaded in the calorimeter syringe and injected into a higher urea concentration.

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