Thermal unfolding of dodecameric glutamine synthetase: Inhibition of aggregation by urea

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Abstract

Thermal unfolding of dodecameric manganese glutamine synthetase (622,000 M_r) at pH 7 and ~ 0.02 ionic strength occurs in two observable steps: a small reversible transition ($T_m \sim 42^\circ\text{C}$; $\Delta H \cong 0.9$ J/g) followed by a large irreversible transition ($T_m \sim 81^\circ\text{C}$; $\Delta H \cong 23.4$ J/g) in which secondary structure is lost and soluble aggregates form. Secondary structure, hydrophobicity, and oligomeric structure of the equilibrium intermediate are the same as for the native protein, whereas some aromatic residues are more exposed. Urea (3 M) destabilizes the dodecamer (with a tertiary structure similar to that without urea at 55°C) and inhibits aggregation accompanying unfolding at ≤ 0.2 mg protein/mL. With increasing temperature (30 – 70°C) or incubation times at 25°C (5 – 35 h) in 3 M urea, only dodecamer and unfolded monomer are detected. In addition, the loss in enzyme secondary structure is pseudo-first-order ($t_{1/2} = 1,030$ s at 20.0°C in 4.5 M urea). Differential scanning calorimetry of the enzyme in 3 M urea shows one endotherm ($T_{max} \sim 64^\circ\text{C}$; $\Delta H = 17 \pm 2$ J/g). The enthalpy change for dissociation and unfolding agrees with that determined by urea titrations by isothermal calorimetry ($\Delta H = 57 \pm 15$ J/g; Zolkiewski M, Nosworthy NJ, Ginsburg A, 1995, *Protein Sci* 4:1544–1552), after correcting for the binding of urea to protein sites exposed during unfolding (-42 J/g). Refolding and assembly to active enzyme occurs upon dilution of urea after thermal unfolding.

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

Intermediates detected in the refolding pathways of small proteins often are off-pathway, kinetically trapped, misfolded forms (Sosnick et al., 1994; Baldwin, 1995; Dobson, 1995; Privalov, 1996). In order to rejoin fast, sequential refolding pathways, some kinetically trapped intermediates require rescuing by an “unfoldase” activity of molecular chaperones. Alternatively, misfolded intermediates can be degraded in vivo by energy-dependent protease complexes. Refolding and assembly of oligomeric, multisubunit proteins is even more complex and involves local and global processes that occur by sequential or concerted mechanisms for which chaperones often maximize yields (Jaenicke, 1991).

Glutamine synthetase from *Escherichia coli* is a large, highly regulated, dodecameric metalloenzyme ($\sim 622,000$ M_r) with 12 identical subunits arranged in two face-to-face hexagonal rings (Ginsburg, 1972; Stadtman & Ginsburg, 1974). The X-ray crystallographic structure of Mn·GS revealed that each of the 12 active

sites are formed by intraring heterologous contacts and that the conformational stability of the dodecamer arises primarily from interranging subunit contacts (Almasy et al., 1986; Yamashita et al., 1989). Indeed, the observed even-numbered oligomers (tetramer, hexamer, octamer, and decamer) in the dissociation studies of Maurizi and Ginsburg (1982, 1985) and Haschemeyer et al. (1982) resulted from a preferential destabilization of intraring subunit contacts in the dodecamer.

Previous studies on reversible, partial unfolding transitions in dodecameric Mn·GS ($T_m \cong 51.6^\circ\text{C}$) have shown that cooperative interactions link unfolding reactions of all subunits and that the stability of N- and C-terminal domains of each subunit differ by $\sim 2^\circ\text{C}$ (Shrake et al., 1989; Ginsburg & Zolkiewski, 1991). The partially unfolded, high-temperature form of Mn·GS is a fully populated unfolding intermediate that is in equilibrium with the folded, native dodecamer. The effects of active-site ligands on partial unfolding transitions have shown that the high-temperature form of the enzyme at 60°C can bind Mn^{2+} , L-glutamine, and ADP (Ginsburg & Zolkiewski, 1991; Zolkiewski & Ginsburg, 1992). The larger C-terminal domain of each subunit contains the two essential divalent metal ion-binding sites (n_1 and n_2) for Mn^{2+} or Mg^{2+} (Hunt et al., 1975) as well as sites for L-glutamate and ammonia. ATP spans both C- and N-terminal domains by binding near Lys-47 (Pinkofsky et al., 1984) and Mn^{2+} at n_2 sites (Hunt et al., 1975; Villafranca et al., 1976).

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Abbreviations: GS, *Escherichia coli* unadenylylated glutamine synthetase dodecamer containing 0.8 equivalent of covalently bound AMP; Mn·GS, manganese ion complex of GS (20 Mn^{2+} /GS with 0.1 mM free Mn^{2+} at pH 7); HEPES, [4-(2-hydroxyethyl)-1-piperazineethane sulfonic acid] neutralized with KOH; 2-ME, 2-mercaptoethanol; DSC, differential scanning calorimetry; LS, 90° light scattering.

Thermodynamic measurements of unfolding/refolding reactions for oligomeric, multisubunit proteins is made difficult by the propensity of such biopolymers to aggregate during thermal denaturation. Urea has been used to reversibly denature proteins (Tanford, 1968) and inhibit aggregation. This report is on the thermal unfolding of dodecameric Mn·GS at pH 7 in the absence and presence of 3 M urea. Under the latter conditions, no stable intermediates between dodecamer and unfolded monomers were detected, whereas in the absence of urea, the partially unfolded dodecameric intermediate aggregated at 80 °C upon further unfolding. The addition of 3 M urea to Mn·GS at 20 °C produced a marginally stable dodecamer with a conformation similar to those of the partially unfolded high-temperature form and the inactive apoenzyme. The enthalpy of the thermally induced dissociation and unfolding of Mn·GS in 3 M urea has been determined by DSC.

Results and discussion

Heating dodecameric GS at pH 7 in the presence of 1 mM free MnCl_2 and 100 mM KCl produces a small reversible transition ($T_m = 51.6^\circ\text{C}$; $\Delta H = 1.42 \text{ J/g}$) and precipitation at $\sim 67^\circ\text{C}$ (Ginsburg & Zolkiewski, 1991). The reversible transition is characterized by exposure of ~ 0.7 of the 2 Trp and ~ 2 of the 17 Tyr residues/subunit (Shrake et al., 1989). Deconvolution of DSC data for Mn·GS shows two two-state transitions, with T_m values of 50.4 and 51.7°C (Ginsburg & Zolkiewski, 1991). Saturation of Mn^{2+} binding sites necessary for activity expression (Hunt et al., 1975) stabilizes the dodecamer against partial unfolding (Shrake et al., 1989). The substrates L-glutamine and L-methionine sulfoximine also stabilize the Mn·GS dodecamer through the free energies of ligand binding (Zolkiewski & Ginsburg, 1992).

The use of low ionic strength buffers with 0.1 mM free Mn^{2+} at pH 7 in the present report allowed study of the thermal denaturation of Mn·GS without precipitation. Preliminary experiments indicated that heating Mn·GS (1 mg/mL) from 20 to 90°C in 20 mM HEPES buffer at pH 7 containing 100–500 mM KCl increasingly lowered the solubility of the protein, as measured by 280-nm absorbance in supernatants after centrifugation. DSC profiles of Mn·GS at pH 7 showed that removal of KCl decreased the T_m of the small reversible transition ($T_m \sim 42^\circ\text{C}$; $\Delta H \sim 0.9 \text{ J/g}$), and resulted in the appearance of a much larger endotherm ($T_m \sim 81^\circ\text{C}$; $\Delta H \sim 23.4 \text{ J/g}$), which was irreversible, scan rate-dependent, and accompanied by aggregation (Fig. 1A). Although the irreversible transition was concentration-independent (0.25–2.0 mg/mL), it was scan rate-dependent, with a shift in $T_{max} \approx 3^\circ\text{C}$ for scan rates of 30– 90°C/h . The absence of an endotherm on a rescan, the lack of a posttransitional baseline, and the fact that the unfolded products form high molecular weight species precluded deconvolution of the high-temperature endotherm using the equations for reversible thermodynamics (Freire et al., 1990). Although no dissociation of the dodecamer took place up to 80°C as indicated by 90° light scatter, aggregation occurred during the irreversible transition (Fig. 1A). After heating Mn·GS to 85°C , protein eluted in the void volume of a Superose 6 column.

DSC scans of the inactive apoenzyme (3–10 mg) in the presence of 1 mM EDTA at pH 7 showed no low-temperature transition (or further Trp exposure; see also Shrake et al., 1989) and only a large endotherm (20.2 J/g) at $\sim 70^\circ\text{C}$ that was accompanied by aggregation (data not shown). Thus, the dodecamer already was partially unfolded in the absence of Mn^{2+} binding and the major unfolding

Fig. 1. Thermal unfolding of Mn·GS in 20 mM HEPES and 0.1 mM MnCl_2 , pH 7. **A:** DSC profiles [$\text{kJ (mol dodecamer)}^{-1} \cdot \text{K}^{-1}$] at scan rates of 30 (-----), 60 (----), and 90 (—) $^\circ\text{C/h}$ (2.0 mg GS/mL) are plotted with 90° light scattering data (— · — · —) obtained at 60°C/h (1.0 mg GS/mL). LS is expressed as a ratio of 1.0 for the dodecamer. **B:** Progress curves are shown for changes at 50°C/h in the mean residue molar ellipticity in the near UV (290 nm) with 2.0 mg GS/mL (○) and in the far UV (222 nm) with 1.0 mg GS/mL (+). Corresponding measurements in the presence of 6 M urea show the temperature dependence of CD changes at 290 (○) and 222 (+) nm for dissociated and unfolded GS.

reactions were destabilized $\sim 10^\circ\text{C}$ in comparison to observations made in the presence of Mn^{2+} .

With high concentrations of Mn·GS (8.9 mg/mL) in the absence of KCl, the low-temperature endotherm could be deconvoluted as described previously (Ginsburg & Zolkiewski, 1991) into two two-state transitions with $T_1 = 41.5$ and $T_2 = 42.1^\circ\text{C}$. The latter values are $\sim 10^\circ\text{C}$ lower than those obtained previously in the presence of 100 mM KCl. Elution profiles of Mn·GS were identical at 20, 30, 40, and 56°C in HPLC gel filtration experiments, which confirmed that the quaternary structure was unchanged by the partial unfolding reactions. Mn·GS also expressed full enzymatic activity upon incubation for 5 min at increasing temperatures to 60°C (tested by adding the assay solution at the same temperature), and no activity at 90°C .

Progress curves for thermally induced changes in ellipticity of Mn·GS in the near- and far-UV are shown in Figure 1B. For reference values, corresponding measurements are shown for the completely unfolded protein in 6 M urea. The transition at 290 nm

in the absence of urea occurred at the same T_m value as did the small endotherm in Figure 1A, and represents changes in the environment of aromatic residues. Changes in the secondary structure of GS (as monitored by CD at 222 nm) occurred only at the temperatures of the major unfolding transition shown in Figure 1A. In addition, the CD spectrum of GS from 190–240 nm was identical at 20 and 65°C, which confirmed that secondary structure remained the same after the small reversible transition at 42°C (data not shown). The CD change at 222 nm for GS at 90°C was 50% of that expected based on the CD value in 6 M urea (Fig. 1B). The CD spectrum from 208 to 240 nm at 90°C gave no structural information on unfolded species because soluble aggregates were present. In measurements with Nile Red (not shown), fluorescence increases were not observed for GS heated to 60°C (just after the reversible transition), whereas fluorescence increased markedly due to increased hydrophobicity at 90°C after irreversible reactions had occurred at the high-temperature endotherm of Figure 1A.

In order to inhibit aggregation during the major unfolding reactions of Mn·GS, thermal studies were conducted in the presence of increasing concentrations of urea. It was found that the dodecamer was marginally stable for ~2 h in 3 M urea at 20°C and that this concentration of urea was sufficient to inhibit aggregation at low protein concentrations.

Near-UV CD spectra are shown in Figure 2 for Mn·GS at 20 and 55°C and in 3 M urea at 20°C. The similarity of the latter two spectra indicate that the tertiary structure of GS in 3 M urea approximates that of the dodecamer at 55°C. Moreover, the low-temperature, reversible transition was absent in DSC scans of Mn·GS in 3 M urea. The near-UV CD spectrum of apo-GS (which is enzymatically inactive with tryptophanyl and tyrosyl residues partially exposed; Ginsburg, 1972) also was similar to those for GS at 55°C and GS with 3 M urea at 20°C (Fig. 2).

Sedimentation velocity measurements at 20°C of Mn·GS (~1 mg/mL) gave sedimentation coefficients ($s_{20,w}$) of 20.3 and

Fig. 2. Near-UV CD spectra of Mn·GS (1.8 mg/mL) in 20 mM HEPES, 0.1 mM MnCl₂, pH 7, buffer at 20°C (—) and 55°C (---), and immediately after adding the enzyme to the same buffer containing 3 M urea at 20°C (Δ). For comparison, the dotted line shows the CD spectrum of the apo-enzyme (1.9 mg/mL) in 20 mM HEPES and 1 mM K-EDTA, pH 7, at 20°C.

Fig. 3. Progress curves for 90° LS changes for Mn·GS in 20 mM HEPES, 0.1 mM MnCl₂, and 3 M urea, pH 7, as a function of increasing temperature at a rate of 60°C/h. Concentrations of protein (mg/mL) were 0.50 (■), 0.25 (+), 0.19 (○), 0.10 (Δ), and 0.050 (—).

17.7 S in 20 mM HEPES, pH 7.3, 0.1 mM MnCl₂, and 2 mM 2-ME in the absence and presence of 3 M urea (<2 h), respectively. The $s_{20,w}$ value in the absence of urea was the same as that measured previously at higher ionic strength, whereas the value of 17.7 S in the presence of 3 M urea was ~90% of the value measured for the apo-dodecamer (Shapiro & Ginsburg, 1968); in both cases, a single, monodisperse sedimenting boundary of solute was observed.

The 90° light scattering was monitored as the temperature was increased at a rate of 60°C/h for 0.5 to 0.05 mg/mL concentrations of Mn·GS in 3 M urea (Fig. 3). A decrease of the 90° LS to ~1/12th (for monomer formation) during heating from 20 to 70°C was only observed at <0.19 mg/mL of Mn·GS in 3 M urea, at which protein concentrations aggregation of unfolded species was completely inhibited (Fig. 3). Similar results were obtained when Mn·GS was diluted into 3 M urea at 65°C. At 0.05 or 0.10 mg/mL final concentrations of protein, the LS value rapidly decreased to ~1/12th and remained constant, whereas, at 0.24 mg/mL, the LS decrease was more gradual and then began to increase with time as aggregates formed.

Because the dissociation and unfolding reactions of Mn·GS in 3 M urea at elevated temperatures were not reversed by cooling to 20°C, the experiments shown in Figure 4 were possible. Samples of Mn·GS (0.05 mg/mL in 3 M urea) were heated to the indicated temperatures and then were subjected immediately to HPLC gel filtration at 20°C in the presence of 3 M urea. Only two states, dodecamer and unfolded monomer, were observed for Mn·GS heated to 50–60°C with 3 M urea at pH 7. At <40°C, only the dodecamer with a slightly increased Stokes radius was eluted; at 65°C, only unfolded monomer (with a Stokes radius greater than that of bovine serum albumin) was observed (Fig. 4). Similar gel filtration profiles to those shown in Figure 4 were obtained by incubating Mn·GS in 3 M urea at 25°C for 0, 2, 5, 6, 10, and 35 h. Only at ≥5 h was there a significant amount of monomer formed and only at 35 h was the dodecamer fully dissociated to monomer (data not shown). These results were confirmed by gel electrophoresis of GS samples after treatment with glutaraldehyde, which showed only two protein-stained bands (dodecamer and monomer)

Fig. 4. HPLC gel filtration at 20°C of Mn-GS in 3 M urea, 20 mM HEPES, and 0.1 mM MnCl₂, pH 7, after heating each protein sample (0.05 mg/mL) at 60°C/h in the same buffer to the indicated temperature and immediately loading onto a Superose 6 column (equilibrated at 20°C). Elution was monitored at 280 nm for Mn-GS heated to 25°C (----), 39°C (.....), 50°C (-.-.-), 55°C (—), 60°C (Δ), and 65°C (○). Heating to 95°C gave the same elution profile as observed for the 65°C sample. Arrows indicate the elution positions of GS dodecamer in the absence of urea and bovine serum albumin (BSA) in the presence of 3 M urea.

at the different temperatures or time at 25°C in 3 M urea.¹ In addition, sedimentation equilibrium experiments conducted at 20°C with samples of GS (0.05–0.07 mg/mL) that had been heated to 65°C with 3 M urea or incubated for 72 h at 25°C gave the monomer molecular weight of 52,800. These preparations of GS monomer had a far-UV CD spectrum typical for a random coil configuration (not shown) with $[\theta]_{222\text{ nm}} \approx -3,200 \text{ deg}\cdot\text{cm}^2/\text{dmol}$ at 20°C.

The enzymatic activity of Mn-GS in 3 M urea also decayed with time and was approximately proportional to the amount of dodecamer remaining. Recovery of GS activity produced by a 10-fold dilution of GS in 3 M urea (0.05 mg/mL) that had been heated to 65°C was slow and better at 25°C than at 4°C (Table 1). Approximately 75% of GS activity was recovered overnight by incubation at 25°C (correcting for protein loss during heating and/or incubation at 25°C). The majority of unfolded monomers therefore were competent to refold and assemble upon dilution because activity expression required an oligomeric structure (Maurizi & Ginsburg, 1982, 1985; Almasy et al., 1986). Gel electrophoresis of diluted samples confirmed that dodecamer was formed. The fact that GS γ -glutamyltransferase activity was recoverable from 3 M urea after heating indicates that protein modification by cyanate formation from urea hydrolysis was not a serious problem in these studies.

¹Electrophoresis of GS in 3 M urea on nondenaturing gels at pH 8.8 showed sharp bands corresponding to dodecamer and apparently partially folded dissociation intermediates containing 10, 8, 6, and 4 subunits in addition to monomer. Such intermediates were not observed in gel filtration experiments (Fig. 4) or in gel electrophoresis when GS samples were pretreated with glutaraldehyde to form intramolecular crosslinks or when the running buffer was at pH 7. It is concluded that dissociation intermediates are stabilized by the gel matrix at pH 8.8, and possibly also by glycine or L-alanine present in running buffers (see Woolfolk & Stadtman, 1967; Fisher, 1994; Zolkiewski et al., 1995).

Table 1. Recovery of γ -glutamyltransferase activity after heating GS (0.05 mg/mL) at 60°C/h to 65°C in 3 M urea (20 mM HEPES and 0.1 mM MnCl₂, pH 7), cooling, and diluting 10-fold into 20 mM imidazole, pH 7.3, 100 mM KCl, 1 mM MnCl₂, and 10 mM 2-ME

Time (min)	% Activity recovered ^a	
	4°C	25°C
0	0	0
15	4	8
30	5	16
60	11	26
120	16	32
180	22	37
1,000	26 ± 3	50 ± 3 ^b

^aPercent activity was measured relative to native GS (without urea) incubated under the same conditions.

^bBio-Rad microprotein assays gave 66% of the protein in control GS, which indicated that the refolded, soluble GS had regained 75 ± 5% activity at 25°C.

The HPLC gel filtration experiments demonstrated that there was an absence of detectable intermediates in the dissociation and unfolding reactions of GS in 3 M urea because only dodecamer and unfolded monomers were observed after heating or incubating up to 35 h in 3 M urea. Dissociation and unfolding reactions of GS in guanidine/HCl and urea have been observed to occur in parallel (Maurizi & Ginsburg, 1982; Zolkiewski et al., 1995). Transient intermediates during incubation of GS in 3 M urea might well be undetected in gel filtration experiments. However, intermediates also were not visible after gel electrophoresis if proteins were treated with glutaraldehyde to produce intramolecular crosslinks. In the absence of crosslinking, even-numbered subunit intermediates have been observed for GS in 3 M urea using rapid electrophoretic separation with a discontinuous buffer system and running pH ≈ 8.8 (Zolkiewski et al., 1995).¹

Stable, folded (enzymatically active) oligomeric intermediates (with 4, 6, 8, 10 subunits) have been produced by partial saturation of the dodecamer with a transition state complex to lock Mn²⁺ into active sites (Hunt & Ginsburg, 1980) followed by treatments with EDTA and dithiobisnitrobenzoic acid at pH 8 (Maurizi & Ginsburg, 1982, 1985; Haschemeyer et al., 1982). Intraring subunit contacts were disrupted by sulfhydryl modification to produce partially active oligomers containing interrupting subunit contacts. Rapid electrophoretic separation of Mn-GS in 3 M urea at high pH apparently stabilized similar oligomers.¹

For estimating the calorimetric enthalpy of dissociation and unfolding of Mn-GS, DSC experiments were performed with low concentrations of protein in the presence of 3 M urea (Fig. 5A). Similar DSC profiles were obtained at scan rates of 50 and 60°C/h. Because dissociation was involved, the asymmetry in the endotherm was expected, as was a higher T_{max} value than T_m (Freire, 1989). Asymmetry in the DSC profile also could be partially due to the temperature dependence of the unidirectional unfolding rate. Nevertheless, the average area under the endotherms shown in Figure 5A gave $\Delta H \approx 16.8 \pm 2.1 \text{ J/g}$ (or $\sim 4.0 \pm 0.5 \text{ cal/g}$). This value must be corrected for the heat contributed by urea binding to newly exposed groups on unfolded monomer chains, which could

Fig. 5. Thermal unfolding of Mn·GS in 3 M urea, 20 mM HEPES, and 0.1 mM MnCl₂, pH 7. **A:** DSC profiles for GS at 0.25 (—), 0.50 (---), and 1.00 (-----) mg/mL at a scan rate of 50 °C/h. Results were normalized for protein concentration and scan rate after subtraction of the buffer baseline, and expressed as kJ (mol dodecamer)⁻¹·K⁻¹. **B:** Loss in secondary structure as monitored by CD changes at 222 nm at a heating rate of 50 °C/h for 0.15 mg GS/mL. Data for Mn·GS in 3 and 6 M urea are shown by the solid and dotted lines, respectively. The experiment was begun immediately after adding the protein to 3 M urea, whereas GS was incubated in 6 M urea for ~20 min to allow complete unfolding to occur before CD measurements.

be estimated to be -41.8 ± 2.1 J/g (or approximately -10 cal/g) from the values given in Zolkiewski et al. (1995). Thus, the corrected enthalpy change in Figure 5A is $\sim 58 \pm 4$ J/g, which is in good agreement with the value of 57 ± 15 J/g measured previously for dissociation and unfolding of Mn·GS by urea titrations (Zolkiewski et al., 1995).

In comparing the above values, it should be noted that the ΔH value for dissociation and unfolding of GS was measured by DSC at ~ 60 °C (Fig. 5A), whereas that reported from isothermal titrations with urea was obtained at 37 °C. A correction for the temperature dependence of ΔH (9.6 J/g) estimated from ΔC_p data for protein denaturation (Privalov, 1979) is within the error estimates of the unfolding enthalpy for Mn·GS (Zolkiewski et al., 1995). The initial states of Mn·GS also differ in the two types of experiments. Previously, the urea titration data gave the enthalpy change for dissociation and unfolding of the fully folded, native Mn·GS, whereas GS in 3 M urea already has the conformation of a high-temperature form that has undergone a small partial unfolding with changed tertiary but not secondary structures. Because ΔH for the partial unfolding reaction of Mn·GS is only ~ 1 J/g (or $\sim 2\%$ of that for complete unfolding), this correction also is insignificant. However, the heat of binding urea to polypeptide chains ($-9.0 \pm$

2 kJ/mol at 25 °C; Makhatadze & Privalov, 1992) during dissociation and unfolding is a large correction that may be in error if temperature-dependent.

Far-UV CD measurements confirmed that secondary structure was completely disrupted during the thermal unfolding of Mn·GS in 3 M urea (Fig. 5B; compare with Fig. 1B). The midpoint of ellipticity change at 222 nm occurred at ~ 61 °C, which is ~ 3 °C lower than the maximum of the endotherm shown in Figure 5A for the lowest concentration of Mn·GS.

In order to measure the kinetics of the loss in secondary structure of Mn·GS, it was necessary to increase the concentration of urea. With 4.5 M urea, the time course of ellipticity changes at 222 nm could be followed conveniently at 20.0 °C (Fig. 6A). Surprisingly, the data could be fitted quite well to a first-order decay of secondary structure with $k = 6.8 \pm 0.4 \times 10^{-4}$ s⁻¹. A first-order rate plot of the CD data from Figure 6A is shown in Figure 6B with the corresponding half-time indicated. Thus, the data of Figure 6 suggest that, in addition to the absence of stable intermediates formed between dodecamer and monomer during thermal unfolding in the presence of urea (Fig. 4), cooperative unfolding occurs at a single pseudo-first-order decay rate.

Similar kinetic results have been obtained for urea-induced inactivation and denaturation of the clostridial glutamate dehydrogenase hexamer, for which no stable dimeric or trimeric intermediates were detected (Aghajanian et al., 1995).

Fig. 6. Kinetics of unfolding of Mn·GS (0.05 mg/mL) in 4.5 M urea, 20 mM HEPES, and 0.1 mM MnCl₂, pH 7.3, at 20.0 °C. The reaction was initiated by addition of 2.5 μ L of GS (20 mg/mL) to 1.0 mL of buffer with 4.5 M urea at 20 °C, mixing, and placing in a CD cell (2-mm pathlength) that was equilibrated at 20 °C by an external bath (~ 20 –40 s dead-time). **A:** Time course of CD changes at 222 nm (representing mainly disruption of α -helices); position of the arrow indicates the approximate start of the unfolding reaction. These data were fitted by means of a nonlinear least-squares program to the equation $CD_t = (CD_\infty - CD_0)e^{-kt} + A_\infty$, where CD_t , CD_∞ , and CD_0 are the instantaneous time, infinite time, and zero-time ellipticities, respectively, k is the first-order rate constant, and t is the time in seconds. The computer program, which treats CD_∞ , CD_0 , and k as adjustable parameters, gave $CD_\infty = -4,300$ and $k = 6.8 \times 10^{-4}$ s⁻¹. **B:** First-order rate plot of the data from A after multiplying by -1 and subtracting the value of CD_∞ together with the fit (solid line). Half-time for the conversion of GS to unfolded monomer is shown.

Conclusion

The studies reported here show that urea can be used to study the thermal unfolding of a multisubunit protein. A concentration of urea was chosen that was sufficiently low to not dissociate dodecameric Mn·GS, but high enough to inhibit aggregation during thermal dissociation and unfolding. The selected 3 M urea concentration gave a conformation similar to that of the fully populated, partially unfolded dodecameric intermediate of Mn·GS that is in equilibrium with the folded dodecamer on the unfolding pathway (Ginsburg & Zolkiewski, 1991), and allowed study of the dissociation and subunit unfolding. Refolding and assembly of the enzymatically active dodecamer occurred only upon dilution of the protein and urea in the presence of Mn^{2+} and not upon temperature decreases. Thus, the binding of urea to unfolded polypeptide chains inhibited refolding and assembly reactions and thereby made the thermal unfolding reactions apparently irreversible. In fact, it is the redistribution of urea to unfolded monomer sites that promotes the concerted dissociation of dodecameric GS to unfolded monomers without detectable intermediates. Nevertheless, a characterization of the initial and final states of Mn·GS in 3 M urea and an estimate of the enthalpy for dissociation and unfolding of the dodecamer (corrected for the heat of urea binding) that agrees with previous isothermal titration results (Zolkiewski et al., 1995) suggests that a similar approach could be useful for studying unfolding of other oligomeric proteins.

Materials and methods

Enzyme solutions and chemicals

Unadenylylated GS was purified by the method of Woolfolk et al. (1966) from an overproducing *E. coli* strain (Maurizi & Ginsburg, 1986) as described previously (Ginsburg & Zolkiewski, 1991). Protein concentrations were determined from published absorption coefficients (Ginsburg et al., 1970); $A_{280\text{nm}, 1\text{cm}} = 0.738 \text{ mL/mg}$ after applying fourth-power light scattering corrections from the absorbance at 340 nm. Enzyme activity was measured using the saturating pH 7.57 γ -glutamyl transferase assay (Stadtman et al., 1979). Concentrated urea in water solutions were prepared by weight using the density data of Kawahara and Tanford (1966) just prior to use and adjusted to 3 M urea with water and concentrated buffer. Nile Red was obtained from Fisher Scientific and glutaraldehyde was from Electron Microscopy Sciences (Ft. Washington, Pennsylvania). All other materials were of the highest grade commercially available and all solutions were prepared using deionized and filtered water from a Millipore Milli-Q Plus system. The main buffer used in thermal unfolding studies was 20 mM HEPES and 0.1 mM $\text{MnCl}_2 \pm$ urea, adjusted to pH 7.3 at 30 °C (buffer A). For long incubations, sedimentation velocity runs, and refolding and assembly studies, 2 or 10 mM 2-ME was included to prevent disulfide formation.

DSC

DSC experiments were performed with a model MC-2 calorimeter with a sample cell volume of 1.1954 mL (MicroCal Inc., Northampton, Massachusetts) as described previously (Ginsburg & Zolkiewski, 1991). DSC profiles are shown after subtraction of the buffer baseline. At the time that protein in urea was loaded into the sample cell, it was necessary to replace the reference cell with fresh buffer

when urea was present because even a small amount of urea hydrolysis changed baselines. DSC scans of urea solutions were initiated at 15 °C immediately after loading samples without the usual pre-equilibration period. By $\sim 30^\circ\text{C}$, the calorimeter was balanced.

CD spectrophotometry

CD measurements were made with a Jasco J-710 spectrometer equipped with water-jacketed cells having 1- or 10-mm pathlength for far- or near-UV measurements, respectively. Thermal progress curves were obtained at 50 °C/h. Temperature was controlled with an external programmable water bath (Neslab RTE-110). Protein spectra were the average of four scans recorded at 10 nm/min and were corrected for the solvent CD signal. A mean residue molecular weight of 110.8 calculated from the amino acid sequence (Colombo & Villafranca, 1986) was used for conversion to mean residue ellipticity.

Light scatter measurements

Thermally induced changes in 90° light scatter were measured using a Perkin Elmer 650-40 fluorometer with a jacketed cell holder that was equipped with a programmable water bath (Neslab RTE-111). Temperature was measured with an Omega thermocouple placed into an identical cell adjacent to the cell being measured. Excitation and emission wavelengths were 360 nm and slit widths were set at 3 nm. Under these conditions, the Rayleigh light scattering intensity is proportional to the weight average molecular weight and concentration of the solute, after correcting for solvent refractive index changes (Tanford, 1966).

Gel filtration

Gel chromatography was performed using a Pharmacia FPLC HR high-resolution Superose 6 column (1.0 × 30 cm; 24 mL bed volume) attached to a Hewlett Packard HPLC (HP 1090) system at a flow rate of 0.25 mL/min with 0.20 mL of sample injected/run. The column was immersed in a water bath for temperature control.

Gel electrophoresis

Electrophoresis was performed using the Pharmacia Phast system and the manufacturers instructions. Precast 4–15 and 8–25% gradient, nondenaturing Phastgels, and a discontinuous running buffer at pH 8.8 were used. Also, electrophoresis was performed after soaking buffer strips in buffer A with 3 M urea when anomalous results were obtained at pH 8.8. In order to resolve the discrepancy between results obtained at pH 7 and 8.8, GS samples were treated with glutaraldehyde to crosslink any associated protein species.¹ For this purpose, glutaraldehyde was added to a final concentration of 0.005% in buffer A $\pm$ 3 M urea and allowed to react for 1 h with GS samples (0.050 mg/mL) before and after heating in the presence of urea. One hour was found to be sufficient time for inter-subunit linkage, but short enough for no crosslinkage between dodecamers. GS in buffer A without urea migrated as a single species at either pH 7 or 8.8 with or without crosslinking with glutaraldehyde. After separation, the gels were stained with Coomassie blue.

Estimation of hydrophobicity

Relative hydrophobicity was estimated by adding Nile Red to GS (Sackett & Wolff, 1987). Nile Red (0.25 mM in dimethylsulfoxide)

was added to GS (0.20 mg/mL in buffer A without urea) to a final concentration of 10^{-6} M Nile Red. After 10 min at 10, 60, or 90°C, fluorescence emission spectra were measured at 20°C with excitation at 550-nm and 5-nm slits.

Analytical ultracentrifugation

The Beckman Optima model XL-A analytical centrifuge equipped with a four-place An-60 Ti rotor was used at 20°C. Buffer densities were determined using an Anton Paar model DMA 58 densitometer. Buffer viscosities were measured as described previously and V_{bar} was estimated to be 0.715 mL/g (Shapiro & Ginsburg 1968). Sedimentation velocity experiments were conducted using double sector 12-mm Kel-F centerpieces. In the absence of urea, GS (1 mg/mL) was dialyzed at 4°C overnight against buffer A with 2 mM 2-ME. In the presence of 3 M urea, GS in buffer A and 2 mM 2-ME was equilibrated rapidly with the same buffer by gel filtration through a PD-10 Sephadex G-25M column (Pharmacia), and immediately run in the ultracentrifuge. Radial scans at 40,000 rpm were made at 280 nm with a 0.003-cm step size and 3-min intervals. The time between addition of urea to GS and the completion of the centrifuge run was 1.5 h. Sedimentation equilibrium experiments were conducted at 20°C and 13,000 rpm with GS after (a) heating 0.05 mg GS/mL to 65°C in buffer A with 3 M urea and after (b) dialyzing 0.05 mg GS/mL for 48 h against buffer A with 3 M urea at 25°C (without heating). A carbon-filled, six-channel centerpiece in a 12-mm cell was used to load 0.12 mL of 0.025 and 0.050 mg/mL in separate channels of either (a) or (b) GS sample (with 0.13 mL of the same buffer in reference channels). Radial scans at 230 nm with 30 averages and 0.001-cm step size were made from 16 to 24 h. A monomer molecular weight of 52,800 was calculated by global fitting procedures for both (a) GS heated to 65°C in 3 M urea and (b) GS incubated for 3 days in 3 M urea. Analysis of ultracentrifuge data were performed with software provided by Allen P. Minton (NIDDK, NIH) which can be downloaded from the following World Wide Web site: <http://bbri-www.eri.harvard.edu/RASMB/rasmb.html>.

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