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Acid- and pressure-induced (un)folding of yeast glutathione reductase: Competition between protein oligomerization and aggregation

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Abstract

Glutathione reductase (GR) is a homodimeric flavoenzyme involved in cellular defense against oxidative stress. In the present study, we have used a combination of acidic pH and hydrostatic pressure to investigate the (un)folding transition of yeast GR. Our results indicate that at pH 2 a distinct partially folded state is stabilized, as judged by intrinsic fluorescence, bis ANS binding and circular dichroism (CD) analysis. Further characterization of this partially folded state by size exclusion chromatography revealed that it corresponds to expanded GR monomers. CD analysis at pH 2 showed a significant loss of secondary structure. The partially folded GR monomers stabilized at pH 2 were fully and reversibly unfolded using hydrostatic pressure (up to 3.5 kbar) as a thermodynamic perturbant. By contrast, return to physiological pH after exposure to acidic pH led to a competing reaction between refolding dimerization and aggregation of GR. These results support the notion that a partially folded intermediate state is not only critical for folding of GR but also appears to be a seed for protein aggregation.

Keywords: Glutathione reductase; pH; CD; Fluorescence; Hydrostatic pressure; Aggregation

1. Introduction

Protein folding has evolved from a field of mere academic interest into an area of major biological and medical relevance. There is increasing evidence that a range of disorders, from cystic fibrosis to Alzheimer's disease, originate from the failure of cellular protein folding mechanisms (Temussi, Masino, & Pastore, 2003; Thompson & Barrow, 2002). It appears that the underlying disease-causing event in many such cases is associated with the conversion of normally soluble folded proteins into aggregation-prone conformers which originate from partially folded intermediates (McLaurin, Yang, Yip, & Fraser, 2000). A variety of

Abbreviations: GR, glutathione reductase; CD, circular dichroism; UV, ultraviolet

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cellular proteins have been shown to undergo aggregation in vitro and there is strong evidence that partially folded states formed in solution are precursors of toxic aggregates (e.g., Canet et al., 1999; De Felice et al., 2004). The characterization of protein conformations different from both the native and fully unfolded states may thus provide a platform for deeper understanding of protein misfolding and aggregation phenomena.

In the present study, we have investigated the (un)folding of yeast GR induced by a combination of acidic pH and hydrostatic pressure. GR (a 108-kDa homodimeric enzyme) catalyzes the reduction of glutathione disulfide (GSSG) to GSH using β-nicotinamide-adenine dinucleotide phosphate (NADPH) as a reducing source. The enzymatic properties and (un)folding of GR from a number of species have been extensively characterized (e. g., Louzada, Sebollela, Scaramello, & Ferreira, 2003; Nordhoff et al., 1997; Rendon & Mendoza-Hernandez, 2001; Savvides et al., 2002; Sebollela et al., 2004). The (un)folding of GR was monitored using a combination of intrinsic fluorescence, bis-ANS binding, circular dichroism measurements and size exclusion chromatography (SEC). The results indicate that a monomeric partially folded state of GR is stabilized at pH 2 and exhibits exposed hydrophobic areas and non-native secondary structure. While pressure-induced denaturation of GR at pH 2 was fully reversible, dialysis or pH titration of the acid-denatured protein back to physiological pH brought about a competing reaction between refolding to the native dimeric state of GR and protein aggregation. Collectively, the results presented herein indicate that folding of GR follows a non-cooperative transition including a monomeric intermediate stabilized at acidic pH that appears to be both a productive on-pathway intermediate for folding and a source for aggregation.

2. Materials and methods

2.1. Materials

GR (type IV, from Baker's yeast) was from Sigma Chem. Co. (St. Louis, MO). The enzyme was found to be homogeneous by HPLC analysis using a Superdex 200 HR column (Pharmacia, Uppsala, Sweden) and by SDS-PAGE. Bis-8-anilino-1-naphthalenesulfonate (bis-ANS) was from Molecular Probes (Eugene, OR). The buffer solutions used were sodium phosphate (50 mM) between pH 2 and 3.5, potassium acetate (50 mM) between pH 3.5 and 6, and 1-piperazineethane sulfonic acid, 4-(2-hydroxyethyl)-monosodium salt (HEPES, 50 mM) between pH 6 and 7.6. DTT and mercaptoethanol (140 mM) were from Sigma. All other reagents were of analytical grade. Pre-distilled water was filtered and deionized through a Millipore water purification system.

2.2. Fluorescence measurements

These were carried out on an ISS PC1 (ISS Inc., Champaign, IL) photon-counting spectrofluorometer at 90° in relation to the excitation source. Since yeast GR exhibits a very blue-shifted fluorescence emission spectrum (maximum emission at 317 nm for native GR; Fig. 1C, trace 1) fluorescence was measured with excitation at 270 nm (bandpasses of 8 and 16 nm for excitation and emission, respectively) to minimize contributions from Rayleigh scattering in the emission spectra, which were routinely measured from 295 nm to 420 nm. Fluorescence measurements under pressure were performed using a pressure cell equipped with sapphire optical windows, similar to that originally described by Paladini and Weber (1981). Experiments were carried out at room temperature at the indicated pH values using 0.6 μM protein (dimer concentration). Bis-ANS fluorescence was measured with excitation at 365 nm and emission spectra were recorded from 420 nm to 600 nm. The concentration of bis-ANS was 1.2 μM.

Spectral centers of mass (λ_{av}, average emission wavelength) of the emission spectra were calculated with software provided by ISS Inc., as

$$\lambda_{av} = \frac{\sum \lambda I(\lambda)}{\sum I(\lambda)} \quad (1)$$

where $I(\lambda)$ is the fluorescence intensity at wavelength λ. Shifts in spectral center of mass were converted into extent of dissociation $(\alpha)_p$ at each pressure according to the relationship

$$\alpha_p = \frac{1}{1 + (\lambda_p - \lambda_U)/(\lambda_N - \lambda_p)} \quad (2)$$

where λ_N and λ_U are the spectral centers of mass of native and unfolded protein, respectively,

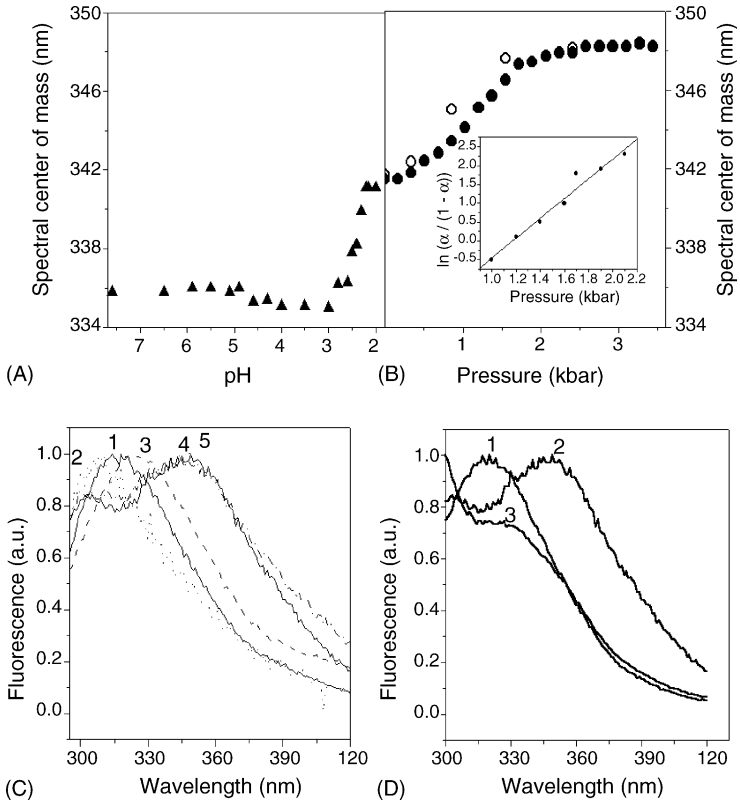


Fig. 1. (Un)folding of GR induced by acid pH (panel A) and hydrostatic pressure (panel B) followed by the spectral center of mass of intrinsic fluorescence emission. Open symbols (in panel B) represent the spectral centers of mass of GR upon decompression. The inset in panel B shows a plot of $\ln[\alpha_p/(1-\alpha_p)]$ as a function of pressure (see Section 2). The errors are smaller than the symbols used. Panel C shows intrinsic fluorescence spectra of native GR (trace 1), acid-denatured GR at pH 4 (trace 2) or pH 2 (trace 3) and fully unfolded GR in the presence of 6 M GdnHCl (trace 4) or at 3.5 kbar and pH 2 (trace 5). Panel D shows intrinsic fluorescence spectra of native GR (trace 1), fully unfolded GR at 3.5 kbar and pH 2 (trace 2) and after dialysis from acidic to physiological pH (trace 3). Spectra are normalized for maximal emission intensities.

and λ_p is the spectral center of mass at pressure p.

2.2.1. Thermodynamic parameters for unfolding of GR at acidic pH

The pressure unfolding data were analyzed using a two-state model for monomer unfolding. The dimensionless equilibrium unfolding constant at atmospheric pressure (K_0) and the molar volume change of folding (ΔV) can be calculated from the following thermodynamic relation

$$K_p = K_0 \exp\left(\frac{p\Delta V}{RT}\right) \tag{3}$$

where K_p is the unfolding constant at pressure p, and R and T have their usual meanings. The equation can be rewritten by introducing the degree of unfolding, α_p, at pressure p,

$$\ln\left[\frac{\alpha_p}{1-\alpha_p}\right] = \ln K_0 + \left(\frac{p\Delta V}{RT}\right) \tag{4}$$

where $\ln[\alpha_p/(1-\alpha_p)]$ equals $\ln K_p$ for the unfolding of a monomer. Thus, a plot of $\ln[\alpha_p/(1-\alpha_p)]$ versus pressure yields the molar volume change of folding (ΔV) from the slope and $\ln K_0$ from the intercept on the ordinate.

2.3. Circular dichroism

Far UV CD spectra of GR were recorded on a Jasco J-715 (Jasco Corporation, Tokyo, Japan) spectropolarimeter (kindly made available by Prof. Jerson L.

Silva, Universidade Federal do Rio de Janeiro) using 0.1-cm path length quartz cells. Spectra were averaged over four scans for each sample containing 2.5 μM GR (dimer concentration) in the corresponding buffer at the indicated pH.

2.4. HPLC analysis

GR samples (15 μM dimers) were incubated for 12 h at pH 7.6 or pH 2 and applied onto a Superdex 200 HR column previously equilibrated at pH 7.6 or pH 2, respectively. The chromatographic analysis was carried out at 23 °C on a Shimadzu (Kyoto, Japan) LC-10AS HPLC system with a sample volume of 50 μl and a flow rate of 0.7 ml/min, with intrinsic fluorescence detection at 330 nm (excitation at 280 nm) as well as absorption at 280 nm. Column calibration was done with a set of 10 proteins of known molecular weights (aprotinin, 6 kDa; cytochrome *C*, 12.4 kDa; lysozyme, 14 kDa; β-lactoglobulin, 36 kDa; ovalbumin, 45 kDa; bovine serum albumin, 66 kDA; phosphorylase B, 97 kDa; β-galactosidase, 116 kDa; apoferritin, 440 kDa and thyroglobulin, 669 kDa). The void volume retention time, t_0, was 11.39 min (measured by the elution of plasmid DNA) and the total volume retention time, t_T, was 41.77 min (measured by the elution of L-tryptophan). From these values, the partition coefficient of a given protein, K_d, was calculated as

$$K_d = \frac{t_e - t_0}{t_T - t_0} \quad (5)$$

where t_e represents the elution time of a given protein. The values for four proteins of known Stokes radii (ovalbumin, 3.05 nm; bovine serum albumin, 3.55 nm; apoferritin, 6.1 nm; thyroglobulin, 8.5 nm; Pharmacia Biotech) were measured to construct the Porath plot according to the empirical relation (Siegel 1966):

$$(K_d)^{1/3} = ar_{Stokes} + b \quad (6)$$

The Stokes radii of native yeast GR and of yeast GR at pH 2 were then estimated from the Porath plot by a linear least-squares fit ($r^2 = 0.99$).

3. Results

Fig. 1 (panels A and B) shows the unfolding of GR induced by acidic pH and by hydrostatic pressure monitored by the shift in the spectral center of mass of the intrinsic fluorescence emission. Between pH 5 and 4, the fluorescence emission of GR initially exhibits a slight blue shift (∼1 nm), which is followed by a significant red-shift (∼6–7 nm) between pH 3 and pH 2 (Fig. 1A). This effect can be rationalized by taking into account the distinct contributions from the 4 tryptophan and 18 tyrosine residues in each GR subunit to the intrinsic fluorescence of the protein. Native GR exhibits a rather blue-shifted fluorescence emission (λ_{max} = 317 nm; Fig. 1C, trace 1), indicating that the 4 tryptophan residues in each subunit are well-buried in the hydrophobic core of the protein. Proteins that contain both tryptophan and tyrosine residues do not usually show tyrosine emission due to extensive energy transfer from tyrosine to the neighboring tryptophan residues (Lakowicz, 1983). Upon protein unfolding, however, the average distance between tyrosine and tryptophan residues may increase, causing a decrease in energy transfer efficiency and allowing for the detection of tyrosine emission. This appears to be the case in the conformational transition of GR that takes place between pH 5 and 4, as evidenced by the shift in the fluorescence maximum to ∼305 nm, which can be attributed to tyrosine fluorescence emission (Fig. 1C, trace 2).

Moreover, tryptophan fluorescence is very sensitive to the polarity of the surrounding environment and exposure of tryptophan residues to the aqueous medium brings about a characteristic fluorescence red-shift (Lakowicz, 1983). A pronounced red-shift of GR fluorescence was observed in the range between pH 3 and pH 2.2, before the transition leveled off at a spectral center of mass of ∼341 nm. Of note is that the fully unfolded state of GR (i.e., in the presence of 6 M GdnHCl) exhibited a considerably more red-shifted fluorescence emission, with a spectral center of mass of 348 nm (Fig. 1C, trace 4). Thus, the plateau observed for the spectral center of mass at pH 2 appears to correspond to a stable partially unfolded state of GR.

A second unfolding transition occurred upon increasing hydrostatic pressure at pH 2 (Fig. 1B) and a final plateau corresponding to fully unfolded GR was reached at ∼2.5 kbar (as indicated by comparison with the fluorescence emission spectrum of GR in the presence of 6 M GdnHCl; Fig. 1C, traces 4 and 5). Upon decompression, the spectral center of mass recovered completely, indicating reversible refolding

of GR to the acid-stabilized state (Fig. 1B, open symbols). However, after dialysis back to physiological pH the fluorescence spectrum of GR showed a peak maximum at about the same wavelength as the native protein but also significant contributions from light scattering (Fig. 1D), likely reflecting partial aggregation of the protein upon return to physiological pH. In essence, both the appearance of characteristic tyrosine emission and the red-shift of the intrinsic fluorescence upon acid-induced and pressure-induced denaturation indicate substantial unfolding of GR, including the presence of a partially folded state stabilized at acidic pH. In addition, the protein exhibited complete reversibility of the pressure denaturation process but, besides refolding to the native state, appeared to partially aggregate upon return to physiological pH. It is interesting to note that the partial reversibility of pH effect shown in Fig. 1D was observed only in the presence of reducing agents (140 mM DTT or β-mercaptoethanol) in the unfolding/refolding buffers. In the absence of reducing agents, the acid-induced unfolding of GR was found to be completely irreversible, suggesting that protein aggregation and lack of reversibility under non-reducing conditions are related to the thiol oxidation and disulfide bond formation in the acid-denatured protein.

We next used the hydrophobic fluorescent probe bis-ANS to further characterize possible partially folded conformations of GR. The fluorescence yield of bis-ANS increases markedly when it binds to organized hydrophobic areas close to positively charged residues (Rosen & Weber, 1969). Partial protein unfolding is usually accompanied by the exposure of hydrophobic side chains, which may serve as binding sites for the dye. Most native proteins and random coil states, however, appear to have either well buried or fully exposed, disorganized hydrophobic side chains, respectively, impeding bis-ANS binding. As expected, native GR did not bind bis-ANS (Fig. 2, trace 1), whereas incubation of the protein at pH 2 led to a substantial increase (∼20-fold) in bis-ANS fluorescence, indicating extensive exposure of hydrophobic domains (Fig. 2, trace 2). Addition of 6 M GdnHCl, (which caused complete unfolding of GR) completely abolished bis-ANS binding (Fig. 2, trace 3). Taken together, both intrinsic and bis-ANS fluorescence results lend support to the existence of a partially folded state of GR at pH 2.

Changes in the secondary structure of GR induced by acidic pH were investigated by CD spectroscopy

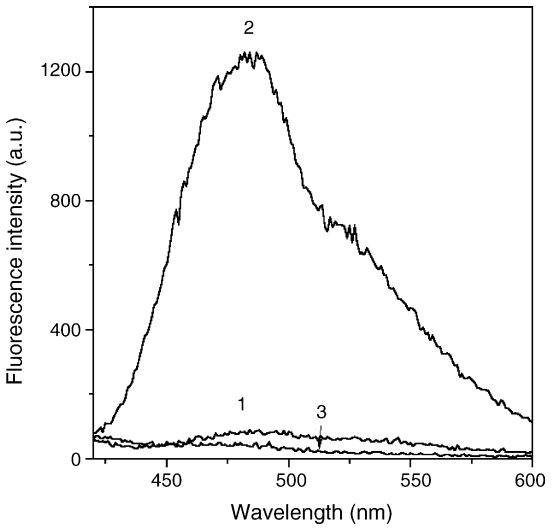


Fig. 2. Bis-ANS fluorescence emission spectra in the presence of native (trace 1) and acid-denatured (trace 2) GR. Trace 3 shows GR in the presence of 6 M GdnHCl.

(Fig. 3). While at pH 4 no major CD changes were observed compared to the spectrum obtained at pH 7.6 (data not shown), a significant drop in ellipticity was observed at pH 2, indicating substantial loss of

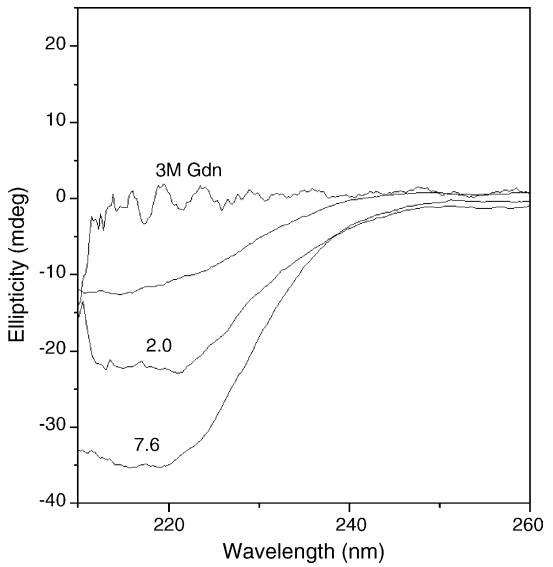


Fig. 3. Circular dichroism spectra of GR. Samples were incubated for 60 min at the indicated pH values or in the presence of 6 M GdnHCl. The second trace from top to bottom shows the CD spectrum of GR after dialysis from pH 2 to physiological pH.

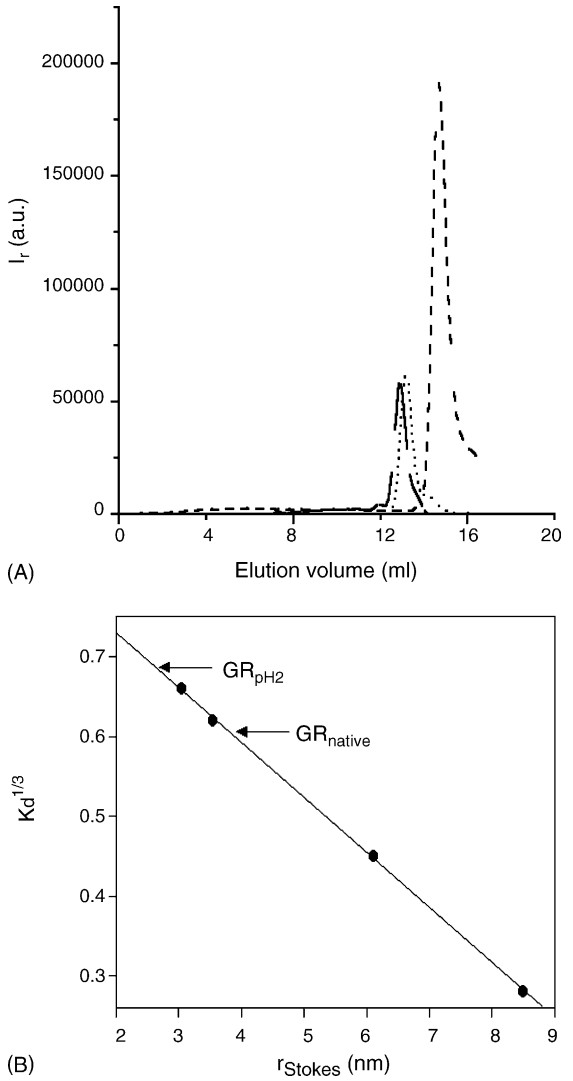


Fig. 4. Size-exclusion HPLC-analysis of GR. (A) Solid line corresponds to chromatographic run carried out at pH 7.6, while dotted and dashed lines correspond to runs carried out at pH 4 or pH 2, respectively. Apparent molecular weights of each peak obtained from the calibrated column are indicated. Detection was carried out using fluorescence emission (excitation 280 nm, emission 330 nm). The differences in fluorescence intensities of the peaks at different pH values are due to differences in fluorescence emission of GR as a function of pH. (B) Porath plot (Siegel 1966) of reference proteins [from left to right, ovalbumin (45 kDa, 3.05 nm), bovine serum albumin (66 kDa, 3.55 nm), apoferritin (443 kDa, 6.1 nm, thryoglobulin (669 kDa, 8.5 nm)]. The cubic root of K_d is plotted against the Stokes radius of each protein. Solid line: linear least-squares fit. Arrows indicate the measured K_d values of GR under the indicated conditions.

secondary structure. It is interesting to note, however, that the CD signal at pH 2 was not completely abolished, in contrast with the spectrum obtained in the presence of 6 M GdnHCl (Fig. 3). Thus, it appears that residual secondary structure of GR persists at pH 2, consistent with the view of a partially folded intermediate state. After dialysis back to physiological pH, the CD spectrum revealed an additional loss in ellipticity when compared to the spectrum measured at pH 2. This result can be understood in conjunction with the intrinsic fluorescence results obtained after dialysis to pH 7. As indicated above, we found evidence for aggregation of GR during refolding from pH 2 to 7.6. Since the amplitude of the CD signal is directly dependent on protein concentration, one would expect a drop in the CD signal intensity upon aggregation as is the case for GR after dialysis to physiological pH. The changes in secondary structure upon acid-induced denaturation of GR further indicate the stabilization of a partially folded state that is prone to aggregate during refolding to neutral conditions.

Changes in the quaternary structure of GR upon acid-induced partial unfolding were investigated by SEC (Fig. 4). As previously reported (Louzada et al., 2003), the elution volume of native GR (at pH 7.6) corresponds to an apparent molecular weight of 85 kDa, in fair agreement with the expected value (108 kDa) for a globular dimer. A very similar elution volume was found when GR was equilibrated at pH 4 (Fig. 4A), indicating that the protein remained dimeric. When the chromatographic run was carried out at pH 2, GR eluted at a volume corresponding to an apparent molecular weight consistent with a monomeric state of the protein (37 kDa) (Fig. 4A). It can, therefore, be concluded that the transition from pH 4 to pH 2 is accompanied by dissociation of the protein. The elution volume of yeast GR under physiological conditions corresponds to a Stokes radius of 38 Å, while a Stokes radius of 28 Å was measured at pH 2, indicating an expanded monomeric conformation of yeast GR at acidic pH (Fig. 4B).

4. Discussion

In this study, we have used a combination of fluorescence and CD spectroscopies and size exclusion chromatography to monitor the conformational changes

of yeast GR during the progress of acid-induced and pressure-induced denaturation. Both intrinsic fluorescence and bis-ANS fluorescence results indicated that the protein unfolds via a non-cooperative transition, including a partially folded state stabilized at pH 2. SEC analysis and CD spectroscopy indicated that the GR dimer is dissociated at pH 2 and that the resulting monomers exhibit non-native secondary structure and expanded hydrodynamic radius. While pressure-unfolded monomers of GR were in equilibrium with the acid-denatured state, we found only partial reversibility in refolding of GR together with protein aggregation upon return to neutral pH.

In general, the driving force of acid denaturation is believed to be the Coulombic repulsion of the excess of positive charges of a protein at low pH (Tanford, 1968). Since hydrophobic interactions, a major determinant of protein stability, are not affected by different charge states, acid denatured proteins usually reveal more structured conformations than those proteins denatured with high concentrations of chemical denaturants. Such partially folded states, however, exhibit a strong propensity for aggregation (Fink, 1995). For instance, staphylococcal nuclease (SNase) forms three different partially folded intermediates at low pH, which form insoluble aggregates especially at higher protein concentrations (Uversky, 1999). Also, glycodelin A, a homodimeric glycoprotein with a molecular mass of 37.5 kDa, exhibits non-native secondary structure at pH 2 and tends to aggregate (Barteri, 2000). Very likely, the exposed organized hydrophobic areas of GR at pH 2, as detected by bis-ANS fluorescence spectroscopy, serve as initial binding sites for aggregation of the protein. It is also of note that the reversibility of acid-denaturation of GR was found to be dependent on the presence of thiol reducing agents in the medium, suggesting that protein aggregation upon refolding may also be related to disulfide cross-linking.

Besides the aggregation characteristics of GR, it is interesting to compare the results from the present study with investigations of folding experiments on GR from yeast and other species. Recent studies have shown that the yeast GR dimer folds via a molten globule monomer that may be stabilized in the presence of 3 M GdnHCl (Louzada et al., 2003). Interestingly, that study also revealed the presence of an expanded predissociated dimer with molten globule characteristics (stabilized at 2.5 M GdnHCl) in the unfolding of GR. In addition, analytical ultra centrifugation experiments on human GR in the presence of 3 M GdnHCl support the existence of monomers that have released the FAD prosthetic group (Nordhoff et al., 1997). Irreversible kinetic unfolding studies on tetrameric GR from the cyanobacterium *Spirulina maxima* found evidence for dissociation to a non-native monomer (Rendon & Mendoza-Hernandez, 2001) and the authors concluded that dimerization is essential to confer the native subunit conformation of this protein. Taken together, the observations from the present study and from studies on GR from other species point to a prominent role of monomeric intermediates in the folding and specific association of the dimer. Indeed, inspection of the structures of human (Karplus & Schulz, 1987) and plasmodial (Sarma et al., 2003) GR shows that the intersubunit interfaces are buried in the hydrophobic core of the proteins, surrounded by the other folding motifs. Partially folded protein conformations are characterized by reduced chain compactness and increased flexibility, which may improve molecular recognition and assembly of the subunits and might ultimately lead to enhanced thermodynamic and proteolytic stability.

The stability of GR appears to depend largely on dimerization, since double or even single amino acid replacements at the interface domain of the *Escherichia coli* protein result in the assembly of enzymatically active dimers with diminished overall stability (Bashir, Perham, Scrutton, & Berry, 1995; Scrutton, Deonarain, Berry, & Perham, 1992). In addition, a single glycine to glutamic acid replacement in the hydrophobic core of human GR is sufficient to inhibit dimerization, and the resulting monomers are unable to bind FAD (Nordhoff, Bucheler, Werner, & Schirmer, 1993). While a variety of partially folded states of proteins have been linked to biological activity (Gee & Katzenellenbogen, 2001; Surendran, Herman, Cheng, Daly, & Ching Lee, 2004) protein folding intermediates stabilized at acidic pH appear to be of particular interest. For example, steroidogenic acute regulatory protein (StAR), which mediates cholesterol transport from the outer to the inner mitochondrial membrane during steroid biosynthesis, has been shown to bind to membranes in a partially unfolded state at pH 3 (Christensen, Bose, Harris, Miller, & Bell, 2001). Similar pH-dependent transitions to molten globule structures have been observed with different proteins that interact with membranes (Banuelos & Muga, 1995; Kim & Kim, 1986). Another

striking example is α-lactalbumin from human milk, which, at pH 2, forms an oligomeric, molten globule state that induces apoptosis in tumor cells, while non-diseased cells remain unaffected (Svensson et al., 1999). Several general aspects related to the potential biological advantages of partially unfolded protein structures have been proposed (Wright & Dyson, 1999). Partially folded proteins may be more malleable, and their increased intrinsic structural plasticity may enhance their responsiveness to different cellular environments.

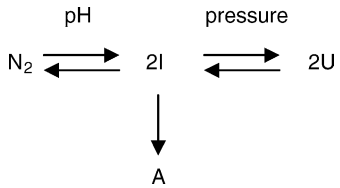
Hydrostatic pressure has been extensively used as a tool to partially denature (Chapeaurouge, Johansson, & Ferreira, 2001, 2002; Lopes, Chapeaurouge, Manderson, Johansson, & Ferreira, 2004; Martins, Chapeaurouge, & Ferreira, 2003; Smeller, Rubens, & Heremans, 1999). or completely unfold proteins (Panick et al., 1998; Rietveld & Ferreira, 1996). The driving force of pressure denaturation is the decrease in specific volume of the protein usually observed upon unfolding. Such a volume decrease may be caused by the hydration of exposed hydrophobic and hydrophilic side chains and by the breaking of internal salt bridges, which become hydrated and cause electrostriction of water. In addition, studies in which cavities were created by site directed mutagenesis in the hydrophobic cores of natural (Frye & Royer, 1998) and de novo designed proteins (Lopes et al., 2004) indicate a significant contribution from internal cavities and solvent-inaccessible void volumes to the changes observed upon pressure unfolding of proteins. Disruption of electrostatic interactions leads to large decreases in volume (Newman, Kauzmann, & Zipp, 1973), while breaking of hydrophobic interactions is accompanied by smaller volume changes. For example, dimeric enolase (80 kDa) (Paladini & Weber, 1981) and hexokinase (96 kDa) (Ruan & Weber, 1988), are reported to undergo specific volume changes of dissociation of 0.6 μl/g and 1.2 μl/g, respectively, reflecting differences in the nature of the protein–protein interactions that are disrupted upon dissociation. Therefore, the relatively large specific volume change of 1.3 μl/g observed in the present study (Table 1) for the folding transition of GR monomers is most likely accompanied by burial of polar side chains. Interestingly, cold denaturation experiments on the acid-denatured state of GR did not result in further denaturation of the protein (data not shown), adding support to the notion that the structure of GR at pH 2 is mainly stabilized by enthalpy-driven, non-hydrophobic interactions.

Table 1
Spectroscopic and thermodynamic parameters for acid-, GdnHCl- and pressure-induced denaturation of yeast GR

SCM[a] of native GR (nm)	336
SCM[a] at pH 2 (nm)	341
SCM[a] at 3.5 kbar (pH 2) (nm)	348
SCM[a] at 6 M GdnHCl (nm)	348
$\Delta G_{pressure}$[b] (kcal/mol)	1.9 ± 00.1
ΔV[b] (ml/mol)	68 ± 8
Specific ΔV[c] (μl/g)	1.3

[a] Spectral center of mass of the intrinsic fluorescence emission (measured from 295 nm to 420 nm).
[b] $\Delta G_{pressure}$ and ΔV correspond to the Gibbs free energy and molar volume changes of the folding transition determined from pressure denaturation data (from Fig. 1B, inset).
[c] From the ratio of ΔV/molecular weight (GR monomers).

In conclusion, we have shown the existence of at least one folding intermediate in the unfolding of GR induced by a combination of acidic pH and pressure. This intermediate appears to be prone to aggregation and the competing folding/dimerization and aggregation pathways of GR may be represented by the following minimal scheme:



The native dimeric state N_2 competes with the aggregated form A of GR upon refolding from the acid-induced intermediate I, which in turn is in equilibrium with the pressure-induced fully unfolded state U obtained at pH 2. The intermediate I exposes much more organized hydrophobic surface to the solvent than N_2 and also shows an expanded hydrodynamic radius (Fig. 3B). This might indicate that dimerization of GR is accompanied by extensive burial of hydrophobic side chains to stabilize a compact overall fold. From the pressure denaturation data, we estimated a free energy of folding of GR monomers of 1.9 kcal/mol (Table 1). Clearly, this value does not represent the overall free energy change of monomer folding since the species stabilized at pH 2 does not exhibit the native fold of GR. Nevertheless, it is sensible to compare our findings to

free energy changes observed upon unfolding of other proteins of similar size. Based on denaturation studies of dimers in the mass range of up to 100 kDa, such as hexokinase (Ruan & Weber, 1988) and tryptophan synthase (Silva, Miles, & Weber, 1986), overall free energies of folding and association of 12–14 kcal/mol have been estimated. We would expect a similar $\Delta G_{overall}$ (folding and dimerization) for yeast GR. Assuming an expected free energy of folding of GR of this magnitude and considering the intimate dimer contacts observed in the crystal structures from human (Karplus & Schulz, 1987) and plasmodial (Sarma et al., 2003) GR, it is tempting to suggest that dimerization is the dominant contributor to the overall stability of GR. It has also been demonstrated that structural elements from both subunits in the dimers of human (Schulz, Schirmer, Sachsenheimer, & Pai, 1978), bacterial (*E. coli*) (Arscott, Drake, & Williams, 1989) and plasmodial (Sarma et al., 2003) GR are important for catalysis and it was concluded that monomers are not enzymatically active. Since yeast GR shares considerable sequence homology with the aforementioned proteins, it is reasonable to expect that dimerization is also critical for its enzymatic activity. Maintenance of tightly bound GR dimers may, thus, be of significant importance for cell survival, especially under oxidative stress conditions.

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