Pressure-Induced Subunit Dissociation and Unfolding of Dimeric β-Lactoglobulin

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ABSTRACT Effects of hydrostatic pressure on dimeric β-lactoglobulin A (β-Lg) were investigated. Application of pressures of up to 3.5 kbar induced a significant red shift (~11 nm) and a 60% increase in intrinsic fluorescence emission of β-Lg. These changes were very similar to those induced by guanidine hydrochloride, which caused subunit dissociation and unfolding of β-Lg. A large hysteresis in the recovery of fluorescence parameters was observed upon decompression of β-Lg. Pressure-induced dissociation and unfolding were not fully reversible, because of the formation of a nonnative intersubunit disulfide bond that hampered correct refolding of the dimer. Comparison between pressure dissociation/unfolding at 3°C and 23°C revealed a marked destabilization of β-Lg at low temperature. The stability of β-Lg toward pressure was significantly enhanced by 1 M NaCl, but not by glycerol (up to 20% v/v). These observations suggest that salt stabilization was not related to a general cosolvent effect, but may reflect charge screening. Interestingly, pressure-induced dissociation/unfolding was completely independent of β-Lg concentration, in apparent violation of the law of mass action. Possible causes for this anomalous behavior are discussed.

INTRODUCTION

In the past 15 years an increasing number of studies have used hydrostatic pressure as a tool for producing reversible subunit dissociation of oligomeric proteins (for reviews, see Silva and Weber, 1993; Gross and Jaenicke, 1994; Mozhaev et al., 1994). These studies have allowed detailed characterization of thermodynamic parameters of subunit association in oligomers ranging from dimers to complex aggregates such as viral particles (Silva and Weber, 1993). Effects of various environmental conditions (such as temperature, pH, solvent composition, or presence of specific ligands) may also be conveniently investigated through pressure perturbation techniques.

β-Lactoglobulin (β-Lg) is the main protein constituent of milk whey from ruminants and several other mammals. β-Lg is a homodimer of 18-kDa subunits, and the dimer forms a central hydrophobic cavity that is thought to be important for binding of hydrophobic molecules such as retinol (Brownlow et al., 1997). The recently available high-resolution crystal structure of β-Lg (Brownlow et al., 1997) makes it an interesting system for correlating pressure effects with structural features of the protein. Previous studies of pressure effects on β-Lg (Dufour et al., 1994; Tanaka and Kunugi, 1996; Tanaka et al., 1996) showed that dimer dissociation/unfolding was partially reversible. In the present work, we have carried out a detailed investigation of pressure effects on β-Lg. Subjecting β-Lg to hydrostatic pressure led to subunit dissociation, unfolding of monomers, and misfolding of monomers and dimers. Effects of various experimental conditions (temperature, solvent composition) on β-Lg stability under pressure, as well as the dependence of pressure dissociation on β-Lg concentration, were investigated.

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MATERIALS AND METHODS

Materials

Bovine milk β-lactoglobulin A (β-Lg), guanidine hydrochloride (GdnHCl), and retinol (all-*trans* isomer) were from Sigma. Protein purity and homogeneity were checked by polyacrylamide gel electrophoresis (PAGE) under both native and denaturing conditions (Laemmli, 1970). All other reagents were of the highest analytical grade available.

Fluorescence measurements

Emission spectra were measured on an ISS PC1 photon counting spectrofluorometer (ISS, Champaign, IL). Excitation was at 283 nm, with 8-nm bandpasses for excitation and emission. Fluorescence measurements under pressure were performed with a pressure cell (ISS) equipped with sapphire optical windows, similar to the cell originally described by Paladini and Weber (1981). Spectra were measured after 10 min of equilibration at each pressure. This waiting period was found to be suficient for the equilibration of pressure effects, as no further spectral changes were observed in samples incubated under pressure for longer times (up to a few hours). Temperature was kept constant at 23 ± 0.5°C by connecting the thermostatted pressure cell to a circulating bath. All experiments (except if otherwise indicated) were carried out in 50 mM Tris-Cl, pH 7.0, with 13.8 μM β-Lg. In guanidine unfolding experiments, β-Lg was incubated with GdnHCl for 24 h at 4°C before measurements.

Fluorescence spectral center of mass (average emission wavelength, λ_{av}) was calculated with software provided by ISS as

$$\lambda_{av} = \Sigma\lambda I(\lambda)/\Sigma I(\lambda), \quad (1)$$

where λ is the emission wavelength and $I(\lambda)$ is the fluorescence intensity at wavelength λ.

Electrophoresis

β-Lg samples (native or pressurized) were analyzed by nondenaturing PAGE and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), with or without β-mercaptoethanol, using the buffer system of Laemmli (1970). Nondenaturing gels contained 12% acrylamide, and SDS-PAGE contained 15% acrylamide. Gels were stained with Coomassie blue.

Size exclusion fast protein liquid chromatography

Size exclusion was performed on a Pharmacia-LKB fast protein liquid chromatography (FPLC) apparatus, using a precalibrated Superdex G-75 HR 10/30 column equilibrated with 50 mM Tris-Cl, 100 mM NaCl, pH 7.0. Where indicated (see Results), the elution buffer also contained 1 M GdnHCl. Elution was monitored by absorption at 280 nm. The flow rate was 0.5 ml/min. Column calibration was done with lysozyme, trypsinogen, pepsin, bovine serum albumin, carbonic anhydrase, β-lactoglobulin, egg albumin, and alcohol dehydrogenase. The presence of up to 1 M GdnHCl in the elution buffer did not affect column calibration.

Retinol binding

This was measured as described by Subramanian et al. (1996), by following the increase in retinol fluorescence at 470 nm (λ_{exc} = 330 nm) upon binding to β-Lg.

RESULTS

Dissociation and unfolding of β-Lg by pressure or GdnHCl

Fig. 1 *A* shows intrinsic fluorescence spectra of native β-Lg (*solid line*), under 3.5 kbar of pressure (*dashed line*), or in the presence of 7.8 M GdnHCl (*dotted line*). Native β-Lg exhibited maximum emission at ~330 nm, and marked red shifts were observed with pressure or GdnHCl. Fluorescence red shifts are indicative of increased solvent exposure of tryptophan residues (Lakowicz, 1983). Thus the spectra in the presence of GdnHCl or at high pressure indicate that similar degrees of solvent exposure of tryptophan residues were obtained with either treatment. In addition to the red shift, GdnHCl or pressure also caused significant increases (~60%; Fig. 1 *A*, *inset*) in fluorescence emission.

Fig. 1 *B* shows fluorescence spectral centers of mass of β-Lg in the presence of increasing concentrations of GdnHCl or at increasing pressures. The spectral red shift reached a plateau at 3 M GdnHCl, corresponding to complete subunit dissociation and unfolding. Size exclusion FPLC analysis of β-Lg in the presence of GdnHCl showed that the protein eluted as a monomeric species (Fig. 1 *B*, *inset*). Application of pressures of up to 3.5 kbar induced a fluorescence red shift similar to that induced by GdnHCl (Fig. 1 *B*), indicating that pressure also promoted subunit dissociation and unfolding of β-Lg.

A compression/decompression cycle on β-Lg is shown in Fig. 2 *A*. After reaching 3.5 kbar, the pressure was released stepwise to 1 atm, revealing a large hysteresis in the recovery of spectral changes. Hysteresis has been reported in pressure dissociation of other proteins and has been related to conformational changes undergone by the dissociated subunits (for reviews, see Weber, 1992; Silva and Weber, 1993). In the case of dimers, hysteresis in pressure effects has been reported for β_2 tryptophan synthase (Silva et al., 1986), hexokinase (Ruan and Weber, 1988), and rubisco (Erijman et al., 1993). The incomplete recovery of spectral center of mass observed upon decompression (Fig. 2 *A*) indicated that dissociated/unfolded β-Lg subunits partially lost their capacity to refold properly into native dimers.

Native β-Lg binds retinol at a hydrophobic cavity at the subunit interface (Brownlow et al., 1997). Retinol binding is independent of the binding of other hydrophobic ligands such as fatty acids (Narayan and Berliner, 1997) and is believed to require specific interactions in the native β-Lg dimer. We have observed that exposure of hydrophobic subunit contact areas of β-Lg upon pressurization caused the release of bound retinol (data not shown), indicating that a specific hydrophobic cavity, and not simply a nonspecific hydrophobic surface, is required for binding. Indeed, retinol binding has been used as a probe of functional structure in a recent investigation of refolding of β-Lg from GdnHCl

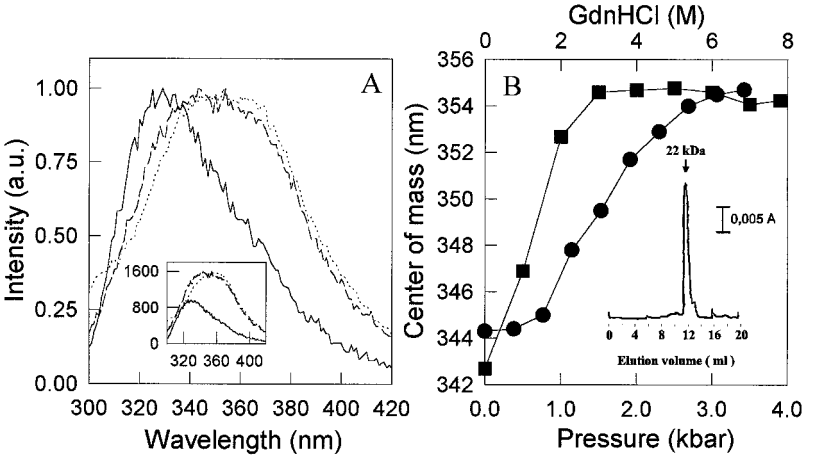

FIGURE 1 Subunit dissociation/unfolding of β-Lg by pressure or GdnHCl (*A*) Fluorescence emission spectra of β-Lg at atmospheric pressure (——), at 3.5 kbar of pressure (– – –), or in the presence of 7.8 M GdnHCl (·····). Spectra are normalized for maximum fluorescence intensity. The inset shows nonnormalized emission spectra. (*B*) Fluorescence spectral centers of mass of β-Lg (calculated as described in Materials and Methods) as a function of pressure (●) or concentration of GdnHCl (■). The inset shows size exclusion chromatography of β-Lg in the presence of 1 M GdnHCl (see Materials and Methods for details). The arrow indicates that under these conditions, β-Lg elutes as a 22-kDa species.

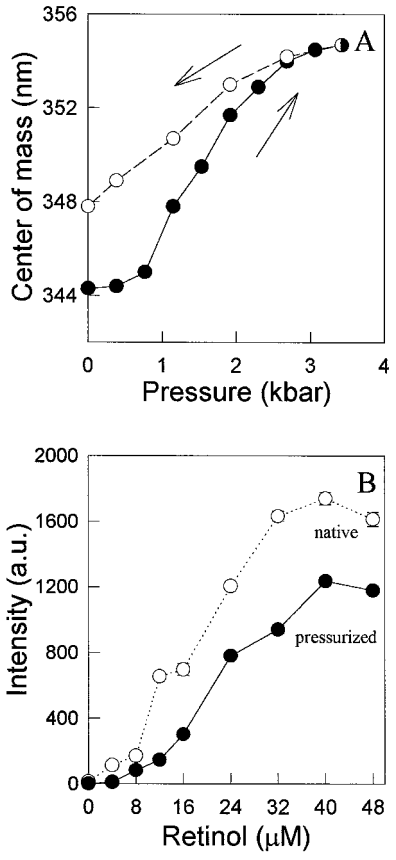

FIGURE 2 Refolding of β-Lg after pressure treatment. (*A*) Spectral centers of mass of β-Lg in a pressure cycle. Closed symbols represent compression and open symbols represent decompression. (*B*) Retinol binding capacity of native (○) or pressurized/decompressed β-Lg (●). The pressurized sample was kept at 3.5 kbar for 10 min and decompressed to 1 atm, and retinol binding was measured immediately after decompression. Total retinol concentrations are indicated in the abscissa. Symbols represent averages ± standard deviations of four or five measurements.

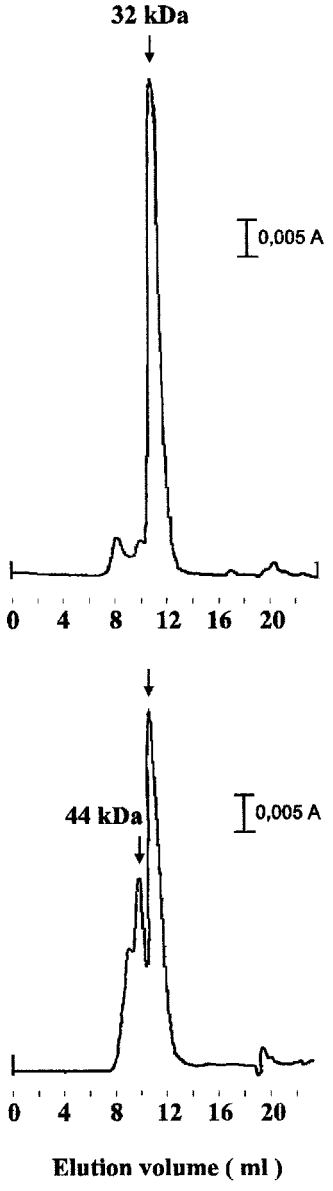

FIGURE 3 Size exclusion chromatography of native or pressurized β-Lg. (*Top*) Native β-Lg. (*Bottom*) A sample of β-Lg was pressurized at 3.5 kbar for 10 min, decompressed, and immediately applied to the column; no difference was observed in the chromatographic profile after incubation of the decompressed sample for up to 24 h at atmospheric pressure. Arrows indicate molecular masses obtained from column calibration.

solutions (Subramanian et al., 1996). Recovery of functional dimers after pressure release was examined by measuring retinol binding to β-Lg after pressure treatment (Fig. 2 *B*). Although native and pressurized β-Lg samples appeared to bind retinol similarly, the extent of binding (as indicated by the maximum fluorescence intensity levels) appeared somewhat lower in pressurized β-Lg than in the control sample (Fig. 2 *B*).

To investigate the origin of incomplete reversibility of pressure effects, native and pressurized β-Lg were analyzed by size exclusion FPLC (Fig. 3). The native sample (*upper panel*) eluted as a single peak of 32 kDa. Pressurized β-Lg (*lower panel*) eluted as two peaks of 32 kDa and 44 kDa. The 44-kDa peak could correspond to β-Lg trimers or to partially folded dimers formed upon compression/decompression. Distinction between these two possibilities was achieved by electrophoretic analysis under both native and denaturing conditions. For control β-Lg, native PAGE showed a single band corresponding to the native dimer, whereas an additional band of decreased electrophoretic mobility appeared in the pressurized sample (data not shown). In SDS-PAGE a single band of ~14 kDa (corresponding to β-Lg monomers) was observed in the native sample, whereas a 30-kDa band (indicative of SDS-resistant dimers) was found in the pressurized sample (not shown). The 30-kDa band disappeared when β-mercaptoethanol was added, suggesting the existence of intersubunit disulfide bonds in pressurized β-Lg. The combined results from FPLC, electrophoretic, and retinol binding analysis indicate that, after pressure release, most of the β-Lg molecules refold into native dimers (Fig. 3). However, a fraction of the molecules (~35%, as judged from data shown in Fig. 3)

form disulfide-linked dimers, which display a larger hydrodynamic radius than native β-Lg.

Effect of temperature on β-Lg stability

Fig. 4 shows pressure-induced dissociation/unfolding of β-Lg at 3°C and 23°C. Decreasing temperature shifted the dissociation/unfolding curve to lower pressures, indicating a decrease in β-Lg stability at low temperature. Because pressure promoted not only subunit dissociation but also unfolding of monomers (Fig. 1), it is not possible to ascertain whether this effect was related to destabilization of dimer, of dissociated monomers, or both.

Stabilization of β-Lg by salt

The addition of 1 M NaCl significantly protected β-Lg from pressure-induced dissociation/unfolding (Fig. 5 *A*). The enhanced stability in the presence of NaCl could be due to charge screening or to a general cosolvent effect involving preferential hydration of the protein (Timasheff and Arakawa, 1989; Timasheff, 1993). The latter possibility was examined by investigating the effect of glycerol, a noncharged cosolvent, on β-Lg stability (Fig. 5 *B*). The addition of glycerol (up to 20% v/v, corresponding to ~2.7 M) promoted a slight blue shift of the fluorescence emission of β-Lg at atmospheric pressure, probably reflecting a decrease in polarity of the medium. The curves obtained in the absence or in the presence of glycerol were approximately parallel, indicating that glycerol did not have any significant stabilizing effect against pressure.

Lack of β-Lg concentration dependence for pressure dissociation/unfolding

From the law of mass action, it follows that the equilibrium between dimers and monomers (whether folded or unfolded) should depend on protein concentration (Silva and Weber, 1993; Rietveld and Ferreira, 1996, 1998). Fig. 6 shows pressure dissociation/unfolding measured at two β-Lg concentrations differing by one order of magnitude (8.3 and 83 μM). The pressure curves are virtually superimposable, showing no dependence on β-Lg concentration. It is interesting to note that a lack of dependence on β-Lg concentration (between 8.3 and 83 μM) was also observed

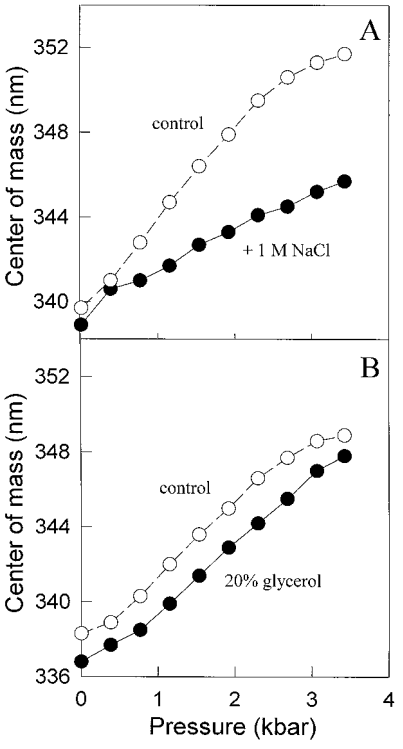

FIGURE 5 Effects of salt or glycerol on the stability of β-Lg. (*A*) Spectral centers of mass as a function of pressure in the absence (○) or in the presence (●) of 1 M NaCl. (*B*) Spectral centers of mass as a function of pressure in the absence (○) or in the presence (●) of 20% (v/v) glycerol.

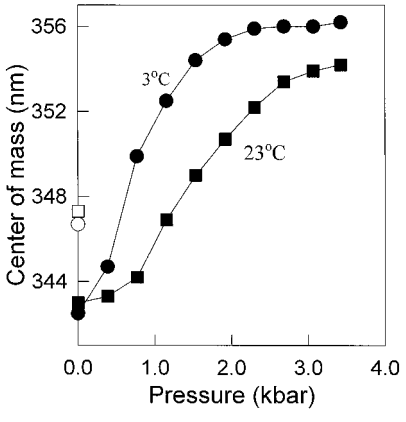

FIGURE 4 Temperature effect on the stability of β-Lg. Spectral centers of mass as a function of pressure for experiments carried out at 3°C (○, ●) or 23°C (□, ■). Open symbols correspond to decompressed samples.

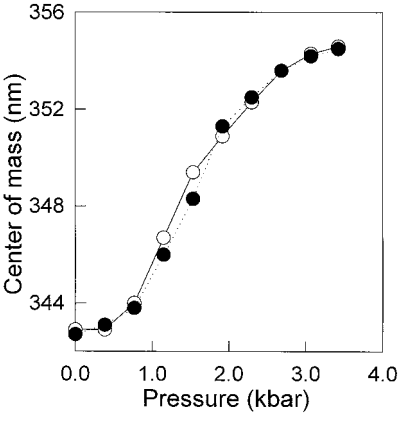

FIGURE 6 Lack of protein concentration dependence of pressure-induced subunit dissociation/unfolding. Spectral centers of mass as a function of pressure for experiments carried out with 8.3 μM (○) or 83 μM (●) β-Lg.

for dissociation/unfolding induced by GdnHCl (data not shown).

DISCUSSION

General features of β-Lg subunit association

The effects of hydrostatic pressure on β-Lg allow a number of general conclusions to be drawn regarding the nature of subunit interactions in this dimer. Application of pressure (up to 3.5 kbar) caused fluorescence changes in β-Lg (spectral red shift and increase in quantum yield) similar to those observed with the addition of denaturing concentrations of GdnHCl, indicating that both perturbing agents caused dissociation and unfolding of β-Lg. This is in line with previous reports of pressure-induced unfolding of β-Lg (Dufour et al., 1994; Tanaka and Kunugi, 1996; Tanaka et al., 1996). Pressure-denatured β-Lg can be partially refolded by decompression (Fig. 2 *A*). However, considerable hysteresis in the recovery of fluorescence spectral properties was observed on decompression, indicating that the dissociated monomers undergo conformational changes that prevent complete refolding of the dimer upon decompression. After a pressure cycle, β-Lg partially recovered its ability to bind retinol (Fig. 2 *B*), indicating that most of the dimers that refolded upon decompression possessed functional structures.

Incomplete recovery of β-Lg spectral properties and retinol binding capacity after pressure treatment appeared to be related to the formation of intermolecular disulfide bonds between pressure-dissociated subunits. This confirms a recent report (Tanaka et al., 1996), which showed the formation of disulfide cross-linked β-Lg dimers after pressurization. We have extended these observations by showing that the disulfide cross-linked dimers formed by pressure displayed significantly larger hydrodynamic radii than native β-Lg dimers (Fig. 3), indicating that they were trapped in incorrectly folded, expanded conformations relative to native dimers.

Investigation of pressure dissociation/unfolding at different temperatures revealed that the stability of β-Lg under pressure was significantly decreased at 3°C relative to 23°C (Fig. 4). This might reflect a decrease in entropic contribution ($T\Delta S$) to the stabilization Gibbs free energy at low temperatures. Alternatively, the loss of stability at low temperatures could be related to a significant heat capacity change, ΔC_p, for denaturation. Large values of ΔC_p for protein unfolding reactions have been reported (for a review, see Privalov, 1992). Previous studies showed that β-Lg undergoes cold denaturation in the presence of high concentrations of urea (Griko and Privalov, 1992; Griko and Kutyshenko, 1994), which was explained on the basis of a large incremental ΔC_p for unfolding. In either case, destabilization by low temperature probably involves the disruption of hydrophobic interactions that are important for protein stability. Such interactions are possibly involved in the formation of the central hydrophobic cavity of the β-Lg dimer (Brownlow et al., 1997). Similar cold denaturation effects have been observed for other proteins containing significant hydrophobic cores, such as myoglobin (Privalov et al., 1986; Griko et al., 1988).

Effects of charged and uncharged cosolvents on β-Lg stability were also examined. NaCl (1 M) caused a pronounced stabilization against pressure-induced subunit dissociation/unfolding (Fig. 5 *A*). However, the addition of up to 20% (~2.7 M) of the noncharged cosolvent glycerol failed to stabilize β-Lg against pressure (Fig. 5 *B*). Therefore, it appears that stabilization by salt cannot be explained on the basis of a general cosolvent effect involving preferential hydration of the protein (Timasheff and Arakawa, 1989; Timasheff, 1993). Rather, salt stabilization is likely due to charge screening and a decrease in unfavorable electrostatic interactions between subunits. Salt stabilization against thermal denaturation of β-Lg has been reported (Kella and Kinsella, 1988). At acid pH, a shift in monomer-dimer equilibrium of β-Lg toward monomers has been reported as a result of decreasing ionic strength (Aymard et al., 1996).

Anomalous concentration dependence for the dissociation of β-Lg dimers

For the stochastic equilibrium between dimers and monomers, a predictable displacement of the pressure dissociation curves to higher pressures should be observed with increasing protein concentration. For two different protein concentrations (C_1 and C_2), the change in pressure (Δp) required to produce a given extent of subunit dissociation can be calculated as (Silva and Weber, 1993)

$$\Delta p = (RT/\Delta V)\ln(C_2/C_1), \qquad (2)$$

where ΔV is the standard volume change upon subunit association, and R and T have their usual meanings.

An interesting and surprising observation in the present work was the lack of dependence of pressure-induced subunit dissociation/unfolding on β-Lg concentration (Fig. 6). Lack of dependence on β-Lg concentration was also found for subunit dissociation/unfolding induced by GdnHCl (data not shown). This type of anomalous behavior has previously been reported for large protein aggregates, such as giant extracellular hemoglobins or viral particles (Silva and Weber, 1993, and references therein), and has been described as deterministic behavior (in contrast with stochastic equilibrium) by analogy to the behavior of macroscopic bodies (Erijman and Weber, 1991). On the other hand, dimers, until recently, have been shown to exhibit stochastic equilibria of subunit association (i.e., subunit association follows the protein concentration dependence predicted by Eq. 2; Silva and Weber, 1993), whereas tetramers show intermediate behavior. It has been proposed that nonstochastic behavior arises from slow or negligible rates of intersubunit exchange in the oligomers, leading to the absence of a true dynamic equilibrium between oligomers and persistent conformational heterogeneity in the ensemble of

particles on the time scale of pressure experiments (Weber, 1992). We have recently shown that trimeric vicilin (Pedrosa and Ferreira, 1994) and dimeric triose phosphate isomerase (TIM) (Rietveld and Ferreira, 1996, 1998) exhibit nonstochastic behavior of subunit association. For TIM, we showed that this behavior resulted from persistent (relative to the experimental time scale, i.e., hours to days) conformational heterogeneity in the ensemble of dimers caused by very slow rates of subunit exchange between dimers (Rietveld and Ferreira, 1998).

For β-Lg, our observations indicate that the rate of subunit exchange under native conditions (atmospheric pressure) is likely to be slow. Under pressure, subunit dissociation is accompanied by the unfolding of monomers and the formation of nonnative intersubunit disulfide bonds. In contrast, β-Lg samples kept at atmospheric pressure for several days do not show appreciable amounts of disulfide cross-linking (data not shown). This indicates that, under native-like conditions, subunit dissociation/unfolding does not occur at a significant rate, which could lead to persistent conformational heterogeneity. This might explain the anomalous lack of protein concentration dependence for dissociation/unfolding.

Although our findings suggest nonstochastic behavior of subunit interactions of β-Lg, interpretation of the results is possibly complicated by the lack of complete reversibility of pressure effects related to disulfide cross-linking. Thus a more thorough analysis in terms of the deterministic behavior described by Weber (1992) will have to await further characterization of this system.

This work was presented at the International Symposium on Protein Condensation in honor of Gregorio Weber.

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