

In vitro renaturation of bovine β -lactoglobulin A leads to a biologically active but incompletely refolded state

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Abstract

When bovine β -lactoglobulin (β -LG) was refolded after extensive denaturation in 4.8 M guanidine hydrochloride (GuHCl), the functional activity of the protein, retinol binding, as measured by the enhancement of this ligand's fluorescence, was completely recovered. In contrast, the room-temperature tryptophan phosphorescence lifetime of the refolded protein, a *local* measure of the residue environment, was ≈ 10 ms, significantly shorter than the phosphorescence lifetime of the untreated native protein (≈ 20 ms). The lability of the freshly refolded protein, as monitored by following the time course of its unfolding when incubated in 2.5 M GuHCl through the change in fluorescence intensity at 385 nm, was also determined and found to be increased significantly relative to untreated native protein. In contrast to the long term postactivation conformational changes detected previously in *Escherichia coli* alkaline phosphatase (Subramaniam V, Bergenheim NCH, Gafni A, Steel DG, 1995, *Biochemistry* 34:1133–1136), we found no changes in either the lability or phosphorescence decays of β -LG during a period of 24 h. Our results are in agreement with the report by Hattori et al. (1993, *J Biol Chem* 268:22414–22419), using conformation-specific monoclonal antibodies to recognize native-like structure, that long-term changes occur in the protein conformation, compared with the native structure, on refolding.

Keywords: bovine β -lactoglobulin; protein folding; protein lability; protein structure; tryptophan phosphorescence; spectroscopy

During in vitro folding of a protein, the recovery of biological activity traditionally has been assumed to reflect the return of the protein structure to its "native" state, the end product of the in vivo folding process. However, several recent studies using especially sensitive techniques to detect subtle conformational changes in the protein suggest that the structure of a fully reactivated protein may differ from the original native state (Hattori et al., 1993; Subramaniam et al., 1995). Using a combination of biophysical and biochemical methods, a recent report from our laboratory (Subramaniam et al., 1995) demonstrated distinct differences between the structure of enzymatically active refolded *Escherichia coli* alkaline phosphatase (AP) and the native protein. Both the room-temperature tryptophan phosphorescence (RTP) lifetime, a *local* probe of the microenvironment of the emitting Trp, and the protein lability, a global parameter, exhibited a slow recovery to the initial, "native-like" value long after the return of enzymatic activity.

It is interesting to note that there are other reported cases of proteins that possess the functional biological signatures of their native state, yet exhibit significantly different structural and thermodynamic properties. Phosphoglycerate kinase (PGK) has been shown to exist in fully active, slowly interconverting conformations, leading to more stable forms of PGK without any effect on activity (Rothstein, 1985). Hattori et al. (1993) showed that, although renatured β -lactoglobulin (β -LG) exhibited the same retinol binding activity as the native protein, some specific epitopes in refolded β -LG were not recognized by conformation-specific monoclonal antibodies, suggesting that these epitopes did not return to the native conformation from the denatured state. Here we present biochemical and spectroscopic evidence, including RTP data, that extends the initial observation by Hattori et al. (1993) that the renatured protein does not completely refold. Our data indicates that these structural differences affect the environment of Trp 19 in β -LG.

Results

Retinol binding

Figure 1 depicts the results of retinol binding assays of native and renatured β -LG assessed by determining the fluorescence from the

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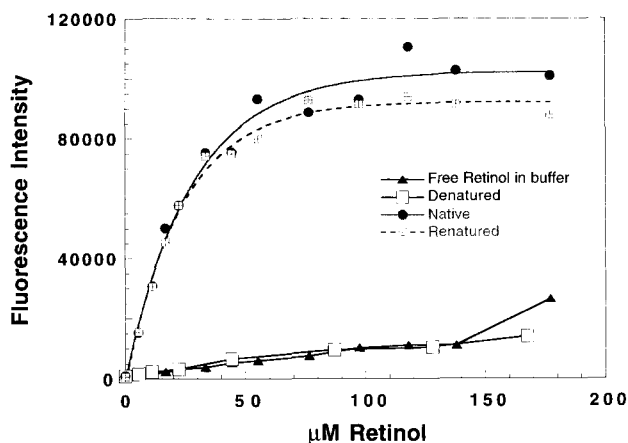


Fig. 1. Fluorescence titration curves of retinol binding to native and refolded β -LG. $\lambda_{ex} = 330$ nm; $\lambda_{em} = 470$ nm. β -LG was denatured in buffer (0.11 M phosphate, 0.04 M NaCl, pH 7.1) containing 4.8 M GuHCl, and renatured by rapid 100-fold dilution into denaturant-free buffer. Renatured protein shows essentially the same ability to bind retinol as the native protein.

β -ionone ring of the retinol molecule following excitation at 330 nm. Although the calculated fits to the data for native and renatured protein (produced by dilution of GuHCl denatured β -LG with excess buffer) indicate a small difference in the saturated value of the fluorescence intensity, possibly representing a small fraction of misfolded protein, the difference is within the experimental error ($\approx 10\%$). We conclude that the binding curves clearly demonstrate that the retinol binding of renatured β -LG is nearly indistinguishable from that of native β -LG. In contrast, β -LG denatured in 6 M GuHCl completely loses its retinol binding activity (Fig. 1). These data indicate that, on refolding, denatured β -LG regains its biological function of retinol binding.

RTP decays of native and refolded β -LG

RTP decay curves of native and refolded β -LG are depicted in Figure 2, showing native β -LG to possess a Trp RTP lifetime of ≈ 20 ms. This phosphorescence is attributed to Trp 19, because inspection of the protein structure indicates that RTP from Trp 61 may be expected to be completely quenched due to its solvent-exposed position and proximity to a disulfide bond, an effective triplet state quencher. The curve marked Native in Figure 2 was obtained for a sample of untreated β -LG at the same concentrations of protein and of GuHCl as those of the renatured samples, i.e., 0.1 mg/mL β -LG and 0.048 M GuHCl. Figure 2 also depicts the RTP decay of renatured β -LG. As is evident from the differences in slopes of the RTP decay curves, renatured β -LG exhibits a markedly shorter RTP lifetime than native β -LG (≈ 10 ms versus ≈ 20 ms). In contrast to the results obtained with alkaline phosphatase (Subramaniam et al., 1995), the RTP of refolded β -LG shows no signs of annealing to its native value, even after 24 h.

Fluorescence-detected protein lability

When β -LG is unfolded in GuHCl, the Trp fluorescence exhibits both a significant red-shift and an increase in yield (Fig. 3A), reflecting the change in emitting Trp environment from hydrophobic to solvent exposed. In order to assess the significance of the

subtle structural changes in the refolded protein by means other than phosphorescence, we evaluated the lability of the native and freshly refolded protein by following the increase in tryptophan fluorescence intensity at 385 nm (Fig. 3A) as a function of incubation time in intermediate concentrations of GuHCl (Fig. 3B). We note the following salient features of the lability data for native and refolded proteins:

1. The baseline fluorescence intensity (i.e., the fluorescence intensity in the absence of GuHCl) has a higher value for refolded β -LG than for native β -LG. We believe this increase reflects a slightly more open conformation for the refolded protein, which increases the exposure of Trp residues to solvent relative to the native conformation. Alternatively, this increase may reflect the presence of some misfolded β -LG; however, inspection of the equilibrium fluorescence intensity data for native and unfolded protein suggest that $\approx 25\%$ of the protein needs to be fully unfolded to account for the difference in baseline intensities. This is clearly inconsistent with the extent of retinol binding exhibited by the refolded protein.
2. The fluorescence intensity observed immediately following addition of denaturant is higher for refolded β -LG than for native β -LG, a result that indicates a larger relative amplitude for fast inactivation processes (within the dead time of manual mixing) for refolded β -LG. This difference in fluorescence intensity persists even when the initial fluorescence baselines are subtracted.
3. The kinetics of unfolding (reflected in the fluorescence increase) are faster for refolded β -LG than for the native protein (Fig. 3B, inset), showing that the renatured protein is more labile than native β -LG; the respective rate constants are 0.0286 ± 0.0002 s $^{-1}$ and $0.0225 \pm 9.4 \times 10^{-5}$ s $^{-1}$, with linear correlation coefficients, R , of 0.99931 and 0.99972, respectively.
4. The decrease in fluorescence intensity is attributed to photobleaching. Under these intermediate unfolding conditions, the fluorescence intensity of the partially denatured refolded protein decreases faster than that of partially denatured native pro-

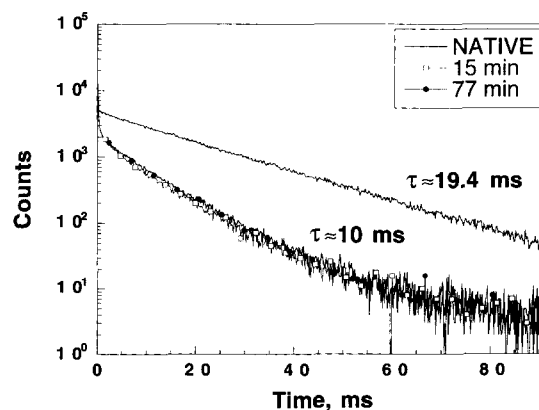


Fig. 2. Representative room-temperature phosphorescence decays of native and renatured β -LG. β -LG was denatured in buffer containing 4.8 M GuHCl and renatured by rapid 100-fold dilution and mixing. RTP lifetime of the refolded protein does not recover to that of native protein. Native control sample contained the same concentration of GuHCl as the final concentration of GuHCl in the renatured sample.

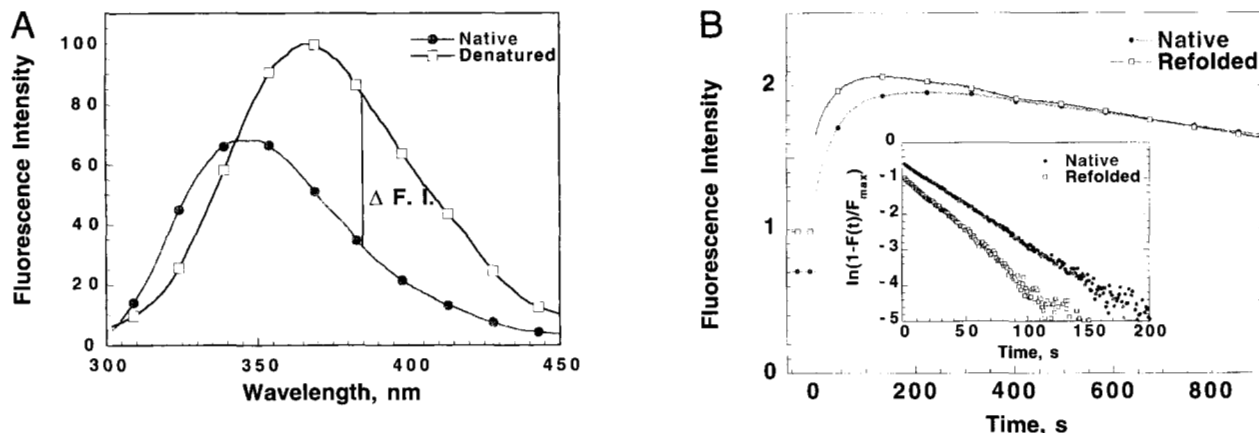


Fig. 3. Lability of native and refolded β -LG as followed by the increase in fluorescence intensity at 385 nm that accompanies unfolding of β -LG. Aliquots of native and refolded β -LG were mixed with buffer containing 6 M GuHCl to a final GuHCl concentration of 2.5 M and the change in fluorescence intensity was monitored as a function of time in the denaturant. **A:** Fluorescence spectra for native and denatured β -LG, indicating the wavelength (385 nm) at which the unfolding of the protein is monitored. **B:** Raw data for unfolding of native and refolded β -LG. Note the higher fluorescence baseline exhibited by refolded protein. Inset: Initial unfolding kinetics of native and refolded protein. $F(t)$ is the fluorescence intensity at time t , and F_{max} is the maximum value of the fluorescence intensity. Refolded β -LG clearly unfolds faster than native β -LG: respective rate constants are $0.0286 \pm 0.0002 \text{ s}^{-1}$ and $0.0225 \pm 9.4 \times 10^{-5} \text{ s}^{-1}$.

tein during long continuous exposure to ultraviolet light (280 nm). A possible explanation is that the denaturation proceeds by formation of an unfolding intermediate, and that the interim state formed by partial denaturation of the refolded protein is more photolabile than the intermediate formed by partial denaturation of the native protein. This result also confirms that the refolded protein is different from the native protein.

The lability of the refolded protein does not approach native-like levels as a function of time after refolding (even after 24 h, data not shown), thus indicating that the structural modification in the refolded protein is likely to be permanent, although it does not affect the biological function of the protein.

In addition, when aliquots of native and refolded protein are extensively unfolded in 5 M GuHCl, the fluorescence intensity at 385 nm of both samples is equal (within experimental error, data not shown), indicating that no protein is lost on refolding.

Discussion

β -LG is a prevalent whey protein found in the milk of mammals. It is a predominantly β -sheet protein, with a calyx-like structure composed of nine antiparallel beta strands and one alpha helix (Papiz et al., 1986), and has received significant attention recently as a model for the folding of beta sheet proteins and for the implications for theories about nonhierarchical folding of proteins (Kuwajima et al., 1987; Hattori et al., 1993; Shiraki et al., 1995). The function of the protein has not yet been unequivocally determined, but its structural homology to retinol-binding protein suggests the binding and transportation of retinol in suckling animals. At neutral pH, β -LG is a homodimer, with each subunit being composed of 162 amino acid protein residues with molecular weight of 18,400, and contains two tryptophan residues, Trp 19 and Trp 61. Examination of the structure of β -LG determined by X-ray crystallography shows that Trp 61 is in a substantially solvent-exposed position and is also in close proximity to a disulfide bond formed between Cys 66 and Cys 160. As explained in further detail

below, several factors affect the RTP lifetime of Trp residues in proteins; however, solvent-exposed tryptophans, as a rule, do not show long-lived RTP. Moreover, triplet states are known to be quenched very effectively by disulfide bonds (Bent & Hayon, 1975; Gonnelli & Strambini, 1995), and the RTP from Trp 61 is thus expected to be extensively quenched. Trp 19, on the other hand, is buried in the calyx and, from its relative inaccessibility to the solvent, is expected to be the sole phosphorescent Trp.

In extensively deoxygenated solution, the RTP lifetimes of tryptophan residues in proteins may become exceedingly long (milliseconds to seconds) (Vanderkooi et al., 1987; Vanderkooi, 1992) and are known to be remarkably sensitive to the local environment of the emitting Trp (Strambini & Gonnelli, 1985; Vanderkooi et al., 1987) or to quenching interactions (Gonnelli & Strambini, 1995). Whereas X-ray temperature factors typically vary by a factor of ≈ 3 –5 between deeply buried and solvent exposed residues, the corresponding RTP lifetimes typically range over 3–4 orders of magnitude. The enhanced sensitivity of RTP, coupled with the ability to time resolve the emission, allows RTP to be used as a near-real-time probe of subtle conformational changes around the Trp moiety as the protein refolds. Although most changes in RTP lifetimes on ligand binding, unfolding, or refolding have been interpreted as reflecting differences in the rigidity or microviscosity of the Trp environment due to conformational changes (Strambini & Gonnelli, 1985), specific short-range interactions with some amino acid side chains can also affect the RTP decay (Gonnelli & Strambini, 1995).

The RTP and lability data (Figs. 2, 3B) clearly indicate a significant conformational change in the refolded protein. The decrease in the RTP lifetime of the refolded protein is consistent with the increased protein lability and may reflect a decrease in the rigidity of the environment surrounding Trp 19, leading to enhanced out-of-plane vibrational modes of the indole ring, which have been postulated as the primary mechanism for indole triplet state quenching (Lower & El-Sayed, 1966). However, the conformational changes reflected in the increased lability may also increase the frequency of collisional quenching of the Trp by nearby

side chains, or may allow for a more favorable orientation between Trp 19 and nearby groups that exhibit a high propensity for intrinsic quenching of the Trp phosphorescence, such as Tyr, Cys, or His (Gonnelli & Strambini, 1995). Two possible candidates are the residues Tyr 102 and Tyr 20, which are within 5 Å of Trp 19 in β -LG. Irrespective of the specific mechanism of quenching, fundamentally the origin of the shorter RTP lifetime observed for the renatured form of β -LG lies in the local environmental changes in the vicinity of the emitting tryptophan (assumed to be Trp 19).

The results obtained in the present study confirm earlier work on β -LG by Hattori et al. (1993), who reported the detection of local structural differences between native and renatured β -LG molecules using a panel of four monoclonal antibodies. Of these four, only two mAbs showed the same affinity toward both renatured and native β -LG, as measured by competitive enzyme linked immunosorbent assay. The other two mAbs exhibited different binding affinities to native and renatured β -LG. The binding epitopes for these mAbs have been identified and are depicted in Figure 4. It is particularly interesting to note that the two mAbs that detected a structural modification in refolded β -LG both recognize regions around Trp 19, the putative phosphorescent Trp. Thus, our interpretation of the short RTP lifetime of renatured β -LG as reflective of conformational changes is consistent with incomplete binding of monoclonal antibodies recognizing epitopes in the vicinity of Trp 19.

Interestingly, Trp 19 in β -LG is the only invariant residue throughout the lipocalin superfamily (Katakura et al., 1994), a class of proteins possessing significant sequence homology and performing similar biological functions, primarily the binding and transport of small hydrophobic molecules (North, 1989; Flower et al., 1993). Experiments on retinol-binding affinity and stability of a W19Y mutant of β -LG reveal that this strictly conserved residue is important for stabilizing the bound retinol and in maintaining the structural environment of the retinol-binding pocket (Katakura et al., 1994). This is of particular relevance to the expected func-

tion of β -LG—binding, delivery, and release of retinol. The W19Y mutant exhibits a reduction in stability of 6.9 kcal/mol compared with wild-type β -LG. One would thus expect that any changes in the environment of Trp 19 would be manifested in the lability of the protein; our results indicate that this is indeed the case, and the reduced RTP lifetime further corroborates a change in the vicinity of the residue.

Our recent work on *E. coli* alkaline phosphatase (Subramaniam et al., 1995) has shown that freshly refolded and biologically active proteins may exhibit differences in various biophysical parameters relative to the native forms of these proteins (defined as the *in vivo* produced species). The results suggested that what is commonly defined as the native state may represent a fully active, long-lived intermediate state on the pathway to folding to a more rigid conformation of the protein, which may be nearer the global thermodynamic energy minimum. This observation indicates that, contrary to the established ideas, during *in vitro* folding of a protein, the recovery of biological activity may not reflect the return to the final native state.

In the framework of the rugged energy model (Frauenfelder et al., 1991; Bryngelson et al., 1995; Wolynes et al., 1995), the folding process is pictured to be driven by a reduction in the free energy of the system toward the global thermodynamic minimum. The potential energy function is not smooth, but exhibits considerable local structure corresponding to activation barriers and intermediate states in the folding pathway. The depth of these local minima determines the time spent in a particular intermediate state. In the case of AP, these barriers are clearly larger than kT , as reflected in the long interconversion times between states. In the case of β -LG, we find no changes in the lability or RTP decays over a period of 24 h, in agreement with the observation of Hattori et al. (1993) that complete refolding, as determined by recognition of native epitopes by conformation-specific monoclonal antibodies, is never accomplished. It seems likely that the protein is irrevocably trapped in a near-native minimum, exhibiting full bio-

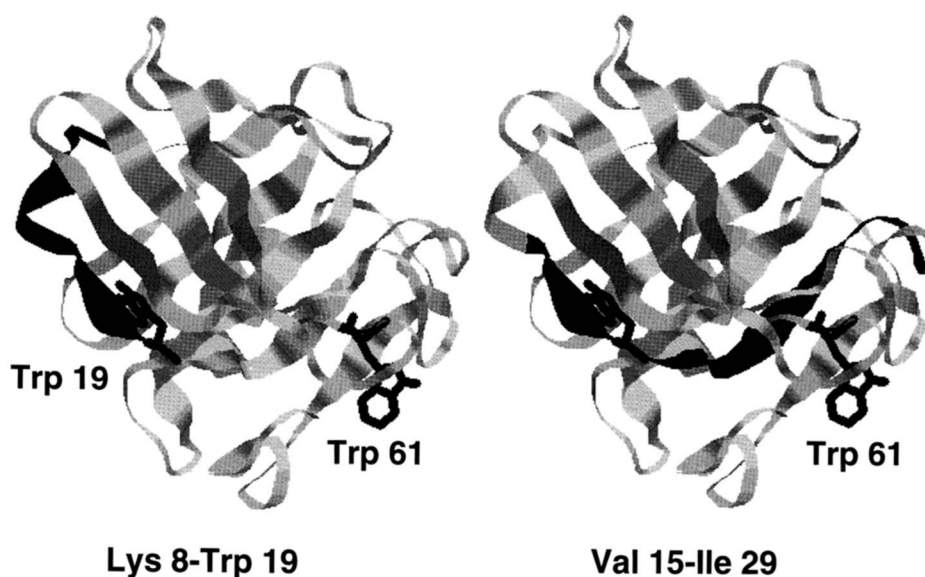


Fig. 4. Binding epitopes for monoclonal antibodies used by Hattori et al. (1993). Both epitopes include Trp 19, assumed to be the phosphorescent residue. X-ray structure coordinates of β -LG were kindly provided by Dr. Lindsay Sawyer, University of Edinburgh (S. Brownlow, J.J. Morais Cabral, R. Cooper, D.R. Flower, S.J. Yewdall, I. Polikarpov, A.C.T. North, & L. Sawyer, in prep.).

logical activity, but with very high activation energy barriers that prevent relaxation to the native state. Such high energy barriers have been demonstrated previously in the folding of α -lytic protease (Baker et al., 1992) and of bacterial luciferase (Clark et al., 1993).

The results raise the intriguing possibility that a fully folded structure (i.e., the global-energy-minimum species) is not required for biological function of a protein. It is not clear whether the stable conformation reached in our *in vitro* experiments is a folding intermediate trapped in a deep energy well, or the final result of folding along pathways that never lead to the "native," *in vivo*, conformation. Potential reasons for the inability of the protein to refold completely include: (1) differences between conditions in the cell and those *in vitro*; (2) shifts in the β -LG monomer-dimer equilibrium on refolding; (3) the requirement of a pro-sequence for correct folding, as for folding of α -lytic protease; and (4) artifacts introduced into the structure during the preparation of purified β -LG. Although β -LG is a secreted protein with a leader recognition sequence, there is no evidence that this protein requires a pro-sequence for folding. Furthermore, an examination of the preparation and purification procedure for β -LG (Aschaffenburg & Drewry, 1957) indicates no temperatures higher than 40 °C in the process, making it unlikely that a heating step is responsible for the achievement of what we define as the native conformation. In addition, we subjected the renatured protein to acidification to pH 2.0 followed by salting out with ammonium sulfate, both being steps in the protein preparation. Neither procedure had a discernible "annealing" effect on the RTP from refolded β -LG.

At neutral pH, β -LG exists as a dimeric species, dissociating into monomers below pH 3.0. It has been reported that the monomer binds retinol with essentially the same binding constant as the dimer (Fugate & Song, 1980). It is conceivable that, on refolding, the monomer-dimer equilibrium shifts toward the monomer, causing changes in spectroscopic signatures. However, we believe this to be unlikely; light scattering experiments with the native and renatured protein indicate no discernible differences in the state of association of the protein (data not shown). Because we find no evidence of slow changes in RTP or lability, we do not expect slow processes such as proline isomerization (Brandts et al., 1975) to play a role in the refolding. Based on these observations, we are led to suggest that the inability of the protein to refold completely may lie in the differences between conditions in the cell and *in vitro*. This indicates that β -LG may well require the presence of molecular chaperones or other components present in the cellular milieu to achieve the "native" conformation that characterizes the mature, *in vivo* processed protein.

The implications of these observations are potentially very significant. There now exist a number of distinct examples of proteins, including AP, β -LG, and PGK, whose refolding produces biologically active states that are clearly not the traditionally defined "native" structure, i.e., the state of lowest free energy. Similarly, plasminogen activator inhibitor Type 1 (PAI-1) (Sancho et al., 1995) undergoes a spontaneous transformation from the originally folded, active, state to a latent, but more stable conformation. Rietveld and Ferreira (1996) have demonstrated persistent heterogeneity in pressure sensitivity of a population of refolded triose phosphate isomerase (TIM) dimers that was not detected by traditional methods. Their data suggested that not all dimers are energetically identical in solution, and the energetic heterogeneity was attributed to conformational heterogeneity in the refolded species. These studies reveal that the *in vivo* "native" structure may

not be universally accessible *in vitro*. The subtle differences in conformations between refolded and native proteins may have important consequences, such as an increased propensity for aggregation, or enhanced lability. These observations may also assume physiologic relevance because the inability of a protein to form its native structure under physiologic conditions may be the basis of a large number of human diseases (Thomas et al., 1995) and has been implicated, for example, in the development of cystic fibrosis and in the formation of amyloid plaques in Alzheimer's disease. Finally, if the *in vitro* folded molecule is indeed different from the native protein structure, it might be necessary to modify the understanding of the accepted dogma that the native conformation of a protein is unique and determined only by its amino acid sequence; the influence of external conditions on which folded state is produced may have to be considered.

Materials and methods

Preparation of protein samples

Electrophoretically pure β -lactoglobulin A was purchased from Sigma (St. Louis, Missouri) and used without further purification. The protein was dissolved in 0.1 M phosphate buffer, pH 7.1, containing 40 mM NaCl.

Denaturation and renaturation of β -LG

β -LG (10 mg/mL) was denatured extensively by incubating overnight in buffer containing 4.8 M GuHCl. Renaturation was initiated by a 100-fold dilution of the denatured protein to a final protein concentration of 0.1 mg/mL and final GuHCl concentration of 0.048 M. Refolded protein was incubated at room temperature for 30–60 min before measuring retinol binding or lability. Fluorescence and far-UV CD signals recover to native-like values within about 1 min on initiation of renaturation.

Measurement of retinol binding

Retinol-binding curves of native and renatured β -LG were derived from fluorescence titration (Futterman & Heller, 1972). Small aliquots (5 μ L) of retinol dissolved in ethanol at 3 mM concentration were added to a cuvette containing 1 mL of 1 mg/mL protein. The mixture was thoroughly mixed and allowed to equilibrate for a few minutes before the fluorescence intensity was recorded. Fluorescence measurements were performed on a Spex (Edison, NJ) Fluorolog fluorimeter with excitation at 330 nm and emission at 470 nm.

Fluorescence detected protein lability

The labilities of the native and freshly refolded protein were assessed by comparing the rates of unfolding by denaturant as measured by following the change in Trp fluorescence at 385 nm as a function of incubation time in 2.5 M guanidine hydrochloride. All measurements were performed at room temperature on a Spex Fluorolog spectrofluorimeter, using excitation wavelength set at 280 nm, with 3.6 nm excitation and emission band widths. The data presented are an average of four separate measurements. Rate constants for the initial kinetics were extracted by least-squares fitting of the data to a first-order reaction using Kaleidagraph software (Synergy Software, Reading, PA); the quoted errors were

determined from the least-squares fit. Photolability of the putative unfolding intermediate from native and refolded protein is inferred from the decrease in Trp fluorescence intensity with continuous exposure to UV excitation light (280 nm).

RTP

RTP measurements were performed using a laser-based system of our own design (Mersol et al., 1993; Subramaniam et al., 1995). Briefly, the experimental configuration consists of a Spectra-Physics model DCR-11 Nd:YAG laser emitting pulses at a wavelength of 532 nm to pump a Spectra-Physics model PDL-3 dye laser operating at 560 nm. The latter light is frequency-doubled externally in a nonlinear crystal (Inrad KDP) to produce 5-ns pulses of 280-nm light. Phosphorescence was detected at 440 nm using an Instruments SA HR 320 single monochromator; alternatively, broad-band detection was accomplished by directly close-coupling to the photomultiplier tube through a 0.5 M potassium nitrite solution serving to cut off fluorescence light. Data was collected using an EG&G Ortec ACEMCS multi-channel scaler card, with a minimum dwell time of 2 μ s per channel, installed in an IBM personal computer. Phosphorescence decays were analyzed using Photon Technology International fluorescence decay analysis software, which fits the decay curve to a discrete sum of exponential decays; the quality of the fit is determined by a Marquardt χ^2 parameter. Deconvolution of the instrument response is not necessary because the laser pulse is contained entirely in the first channel of the data.

Because molecular oxygen is an extremely effective quencher of the room-temperature phosphorescence, great care was taken to deoxygenate the sample extensively by passing a stream of ultra pure argon gas over the sample for several hours, after which the sample was kept sealed at room temperature without further deoxygenation for the remainder of the experiment. The phosphorescence lifetime obtained in deoxygenated solution prior to denaturation was used to define the lifetime of the native state. All decays were collected for the same number of laser shots. Laser intensity variations were monitored by measuring the number of counts from a native β -LG standard immediately following data acquisition.

Acknowledgments

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