Effect of Sodium Dodecyl Sulfate and Palmitic Acid on the Equilibrium Unfolding of Bovine β-Lactoglobulin†

Lawrence K. Creamer*

New Zealand Dairy Research Institute, Palmerston North, New Zealand

Received October 24, 1994; Revised Manuscript Received March 27, 1995⊗

ABSTRACT: The unfolding of bovine β-lactoglobulin, a small globular protein that unfolds reversibly at low pH in the presence of urea or guanidine hydrochloride, has been studied at pH 6.72 in phosphate buffer at 21 °C. The midpoint urea concentration for the loss of CD intensity at 220 nm, loss of CD intensity at 293 nm, quenching of intrinsic fluorescence, shift in the wavelength of the maximum of the intrinsic fluorescent emission, and loss of fluorescence intensity from 1-anilino-8-naphthalenesulfonate (ANS) (and probably the hydrophobic binding site) was close to 4.4 M. Addition of sodium dodecyl sulfate (SDS) at concentrations less than 100 μM to the β-lactoglobulin solutions increased the midpoint urea concentration for the CD and intrinsic fluorescence parameters to about 5.8 M. Palmitic acid had a similar effect to that shown by SDS in altering the CD intensity at 293 nm, and both SDS and palmitic acid attained a maximum effect in altering the CD at 293 nm at a 1:1 molar ratio to β-lactoglobulin. It seems likely that the β-sheet structure of β-lactoglobulin breaks down simultaneously with the loss of the hydrophobic binding site and exposure of tryptophan-19 to the external environment, supporting the view that the major hydrophobic binding site of β-lactoglobulin is closely involved with the β-sheet core of the protein. The increased stability of the protein toward unfolding in the presence of SDS or palmitate suggests that each of these ligands occupies the cavity of the β-barrel of β-lactoglobulin and stabilizes the protein against urea unfolding via strengthened hydrophobic interaction and a greater exclusion of water from the cavity. This conclusion supports the notion that bovine β-lactoglobulin binds hydrophobic ligands in an analogous fashion to human serum retinol-binding protein (RBP).

Bovine β-lactoglobulin is a globular protein with a monomer molecular weight of about 18 300 (Hambling et al., 1992), and it is usually associated into dimers. It has the ability to bind a range of amphiphilic and hydrophobic ligands (Hambling et al., 1992), including retinol (Futterman & Heller, 1972), long-chain fatty acids (Spector & Fletcher, 1970), and sodium dodecyl sulfate (SDS)[1] (McMeekin et al., 1949; Ray & Chatterjee, 1967; Seibles, 1969; Jones & Wilkinson, 1976; Lamiot et al., 1994). Although there is no obvious biological function for β-lactoglobulin, it may be involved in lipid digestion as a fatty acid- and retinol-transporting protein within the gut (Pérez et al., 1992). It is now recognized that bovine β-lactoglobulin is one of a group of proteins that are capable of binding a wide variety of amphiphilic or hydrophobic ligands and have eight-stranded β-barrels as the major structural motif (Flower et al., 1993; Banaszak et al., 1994). These have been called *e*-LBPs by Banaszak et al. (1994) while a functionally similar group of proteins with a 10-stranded β-barrel have been called *i*-LBPs.

Papiz et al. (1986) reported a preliminary high-resolution X-ray crystal structure of the orthorhombic form. It has a slightly distorted eight-stranded β-barrel structure with a three-turn α-helix that runs parallel to β-sheet strands G, H, and A and covers the free thiol, cysteine-121. Pervaiz and Brew (1985) and Papiz et al. (1986) noted sequence and structural similarities to serum retinol-binding protein (RBP). Papiz et al. (1986) proposed that β-lactoglobulin was capable of binding retinol, and with a minimum of side-chain movement, they were able to define a possible site within the β-barrel of the protein. In 1987, Monaco et al. reported the preliminary X-ray crystal structure of the trigonal form of β-lactoglobulin. They also noted that retinol appeared to be bound to a surface site, essentially in a cleft alongside the helix and the exterior of the β-barrel. Cho et al. (1994) used site-directed mutagenesis to show that replacement of lysine-70, which is in the entryway of the binding site suggested by Papiz et al. (1986), by alanine decreased the binding of retinoic acid considerably, but that changing lysine-141, which is in the external cleft that Monaco et al. (1987) assigned as the likely binding site, to alanine made little difference to retinoic acid binding. This suggested that retinoic acid, and possibly retinol, was bound within the β-lactoglobulin calyx as suggested by Papiz et al. (1986).

Some years ago the stability of β-lactoglobulin was studied at pH 2.5−3.5 using urea and guanidine hydrochloride as unfolding agents, and it appeared to display classic two-state behavior (Tanford & De, 1961; Pace & Tanford, 1968). Similar studies, using optical techniques, at neutral pH revealed that the unfolding reaction was not entirely revers-

† This work was supported by research grants from the Foundation for Research, Science and Technology.

* Address correspondence to this author at the New Zealand Dairy Research Institute, Private bag 11029, Palmerston North, New Zealand. Phone: 64-6-350-4711. Fax: 64-6-356-1476.

⊗ Abstract published in *Advance ACS Abstracts,* May 15, 1995.

[1] Abbreviations: ANS, 1-anilino-8-naphthalenesulfonate; CD, circular dichroism; CRABP, cellular retinoic acid-binding protein; CRBP, cellular retinol-binding protein; *e*-LBP, *extra*-cellular lipid-binding protein; *F*, fluorescent intensity; FABP, fatty acid-binding protein; *i*-LBP, *intra*-cellular lipid-binding protein; laurodan, 6-dodecanoyl-2-(dimethylamino)naphthalene; NATA, *N*-acetyltryptophanamide; RBP, serum retinol-binding protein; RET, resonance (or radiationless) energy transfer; retinol, *all-trans*-retinol; SDS, sodium dodecyl sulfate; UV, ultraviolet; λ_{max}, apparent wavelength of maximum fluorescent emission.

0006-2960/95/0434-7170$09.00/0

ible (McKenzie et al., 1972; Ralston, 1973) and that the single free thiol was involved, probably by catalyzing inter- and intramolecular disulfide interchange reactions. More recently Griffin et al. (1989) noted that β-lactoglobulin unfolded in guanidine hydrochloride solution at pH 6.92 with the concomitant loss of the ability to bind retinol. This finding was substantiated by Hattori et al. (1993), who were also able, under some conditions, to refold the protein to a native, retinol-binding conformation. A similar study (Müller & Skerra, 1993) showed that porcine RBP unfolded in guanidine hydrochloride but when retinoic acid was present the unfolding took place at a higher guanidine hydrochloride concentration.

The present study was undertaken to expand our understanding of the equilibrium unfolding of bovine β-lactoglobulin and then extend this to examine the effect of an amphiphilic ligand, SDS, known to bind strongly to a single site on β-lactoglobulin (e.g., Lamiot et al., 1994), on this equilibrium. It was anticipated that by working quickly at relatively low pH (6.7) with freshly prepared solutions that the complicating effects of the disulfide interchange reactions on the unfolding (McKenzie et al., 1972; Ralston, 1973) could be minimized.

EXPERIMENTAL PROCEDURES

Materials. Bovine β-lactoglobulin (3× crystallized, L 0130), *all-trans*-retinol, butylated hydroxytoluene (BHT) and *N*-acetyl-L-tryptophanamide (NATA) were obtained from Sigma Chemical Co. (St. Louis, MO). "High purity" 1-anilinonaphthalene-8-sulfonate (ANS) and laurodan [6-dodecanoyl-2-(dimethylamino)naphthalene] were obtained from Molecular Probes Inc. (Eugene, OR). Acrylamide was obtained from BioRad (Richmond, CA). Urea, SDS, and hexadecanoic (palmitic) acids were obtained from BDH Chemicals Ltd., New Zealand, as the specially purified grades and all other chemicals were analytical reagent grade from BDH. Artesian bore water was purified by reverse osmosis treatment followed by carbon treatment and de-ionization using a Milli-Q apparatus (Millipore Corp, Waltham, MA).

Protocol. Solutions were made as follows: (a) phosphate buffer (0.025 M NaH_2PO_4, 0.068 M NaCl adjusted to pH 6.72); (b) a series of urea solutions made from weighed quantities of urea in phosphate buffer; (c) β-lactoglobulin made to about 23 or 45 mg/mL by weight in phosphate buffer; (d) SDS made to 3.33 mg/mL in phosphate buffer; (e) palmitic acid made to 1 mg/mL in deoxygenated ethanol. β-Lactoglobulin concentration was determined from the 278 nm absorbance using an extinction coefficient of 9.6 (Bell & McKenzie, 1967).

For the near-UV CD measurements, a 3 mL aliquot of a buffered urea solution, no more than 30-h old (Pace, 1986), had 200 μL of 46 mg/mL (by weight) β-lactoglobulin added. For the far-UV spectra, 50 μL of 23 mg/mL β-lactoglobulin solution was added to a 2 mL aliquot of buffered urea solution. The spectrum of each solution was run about 10 min after protein addition, and then 15 or 20 μL of SDS (or palmitic acid) solution was added to the mixture. After about 2 h, the spectrum of each mixture was redetermined.

For the fluorescence measurements, 2 mL aliquots of the various urea solutions were placed directly into the fluorometer cell, aliquots of the other reagents were added and mixed, and the spectra were measured.

CD Spectroscopy. CD spectra were obtained using a Jasco Model 720 spectropolarimeter (Jasco, Ishikawa-cho, Hachioji City, Tokyo, Japan). The wavelength calibration was checked with benzene vapor (266.7 nm) and a neodymium filter (585.9 nm) using the absorbance mode, and the sensitivity and rotation at 290.5 nm were checked using pure ammonium *d*-camphor-10-sulfonic acid from Katayama Chemical, Japan and supplied by Jasco. The spectra of the protein solutions were measured in 10 mm (for near-UV) and 0.1 and 0.5 mm (for far-UV) cells. The solutions were scanned at 50 nm/min using a 2 s time constant, a 1 nm bandwidth, and a sensitivity of 10 or 20 mdeg. The average of 9 scans was recorded. For some preliminary measurements, the 0.1 mm cell was used so that the same solutions could be used for both near- and far-UV measurements. In this narrow cell, the effect of urea absorbance on the signal-to-noise ratio was minimized. Filling and emptying the narrow cell was difficult, and all the results reported here were determined using a 0.5 mm cell.

Fluorescence Measurements. These were made using 10 mm square quartz cells in a Perkin-Elmer MPF 2A spectrofluorometer fitted with a water-jacketed cell holder held at 21 °C with a thermostatically controlled water bath. Intrinsic protein fluorescence was determined using excitation wavelengths in the range 275−305 nm with excitation and emission band-passes of 8 and 9 nm respectively, while ANS was excited at 370 nm with excitation and emission band-passes of 8 and 14 nm, respectively. Protein and fluorophore concentrations were usually such that the absorbances of the solutions were less than 0.35 (0.17 for 5 mm solution thickness, i.e., to the cell center) at the excitation wavelength. Because the measurements were of a comparative nature, corrections for inner filter effects were not made. Emission spectra were scanned across the peak, and λ_{max} and the peak height at λ_{max} determined.

Quenching. Solutions of β-lactoglobulin (or *N*-acetyl-tryptophanamide, NATA) in buffer were placed in fluorometer cells, and the emission spectrum was measured and the peak height at λ_{max} (F_0) determined. Aliquots (20 μL) of 5 M acrylamide solution, made up in phosphate buffer, were mixed into the cell contents, and the emission spectrum was redetermined. The peak height at λ_{max} (F) was divided into F_0, and the resultant ratio was plotted versus acrylamide concentration (a Stern−Volmer plot; Eftink & Ghiron, 1981). Correction was made for the dilution of the fluorophore and the urea but not for light absorption by the acrylamide. In the determination of the effect of urea and SDS on the effectiveness of the quenching by acrylamide, the results from a single acrylamide concentration are reported.

RESULTS

Intrinsic Fluorescence. Spectra typical of those found in the present study of bovine β-lactoglobulin in which both the emission intensity (F) and λ_{max} increased in the presence of high concentrations of urea are shown in Figure 1. In the absence of urea, the apparent wavelength of maximum emission (λ_{max}) was about 332 nm regardless of excitation wavelength (λ_{ex}) in the 275−305 nm range (Figure 1, curves a, c), which is typical of tryptophan-containing proteins (Demchenko, 1992). In the presence of 9.48 M urea, excitation at 275 nm gave a λ_{max} of 350 nm but with a pronounced shoulder near 305 nm (Figure 1, curve b) due

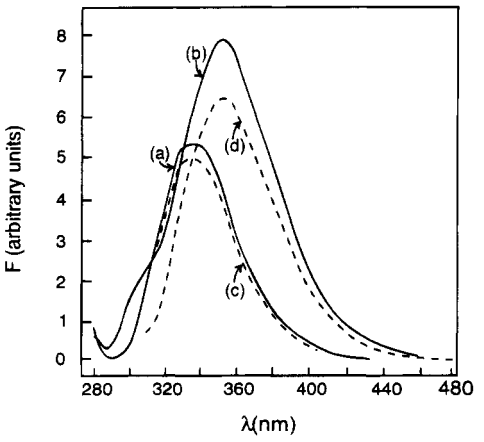

FIGURE 1: Fluorescence emission spectra of β-lactoglobulin AB in the absence and presence of urea. β-Lactoglobulin concentration was 16.5 μM in a 0.025 M phosphate, 0.068 M NaCl buffer at pH 6.72 and at 21 °C. The measurements were made using a 10 mm square cell and with excitation and emission spectral bandwidths of 8 and 9 nm, respectively. (a) Buffer only, λ_{ex} 275 nm; (b) 9.48 M urea solution, λ_{ex} 275 nm; (c) buffer only, λ_{ex} 297 nm; (d) 9.48 M urea solution, λ_{ex} 297 nm.

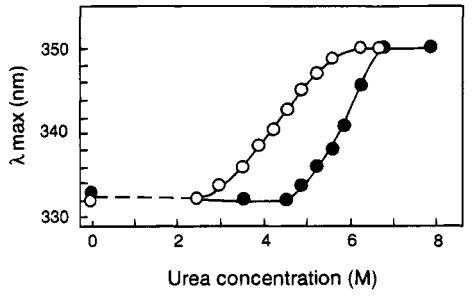

FIGURE 2: Effect of urea concentration on the λ_{max} of β-lactoglobulin intrinsic fluorescent emission in the absence (○) and presence (●) of SDS. The protein concentration was 25.8 μM in phosphate buffer at pH 6.72, while the SDS concentration was 83 μM and λ_{ex} was 294 nm.

to tyrosine emission (Wu & Brand, 1994), while excitation at 297 nm gave rise to the 350 nm peak alone (Figure 1, curve d). β-Lactoglobulin contains two tryptophan and four tyrosine residues (Hambling et al., 1992), and this behavior is consistent with radiationless energy transfer (RET) between the tyrosine in the structured protein to one or both of the tryptophan residues in the protein. In the presence of urea, the tertiary structure is lost, and, consequently, the conditions for successful RET are no longer present (Wu & Brand, 1994). Consequently, for this study, a λ_{ex} above 287 nm was selected so that tyrosine was not being excited and thus RET did not occur. In addition, the choice of a longer λ_{ex} allowed the use of higher protein concentrations without the primary inner filter effect becoming a constraint.

Although both λ_{max} and F changed with urea concentration, only λ_{max} changed in a simple sigmoidal fashion (Figure 2) with a midpoint transition at a urea concentration of about 4.3 M. In the presence of 83 μM SDS, this transition midpoint occurred at a urea concentration of about 5.8 M.

Fluorescence Quenching. The addition of acrylamide, a neutral quencher (Eftink & Ghiron, 1981), to NATA in phosphate buffer diminished the fluorescent emission markedly. A plot of F_0/F versus acrylamide concentration (Figure 3) shows a slight upward curvature. Similar results were obtained for NATA in the presence of 9.84 M urea and added

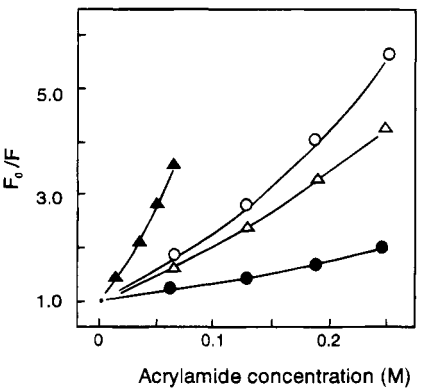

FIGURE 3: Effect of acrylamide concentration on the ratio of F_0 (the intrinsic fluorescence of β-lactoglobulin, or NATA, in the absence of acrylamide) to F (the intrinsic fluorescence of β-lactoglobulin, or NATA, in the presence of acrylamide). The protein and NATA concentrations before acrylamide addition were 16.5 and 10.8 μM, respectively. (●) protein in buffer; (△) protein in 4.92 M urea solution; (○) protein in 9.84 M urea solution; (▲) NATA in buffer.

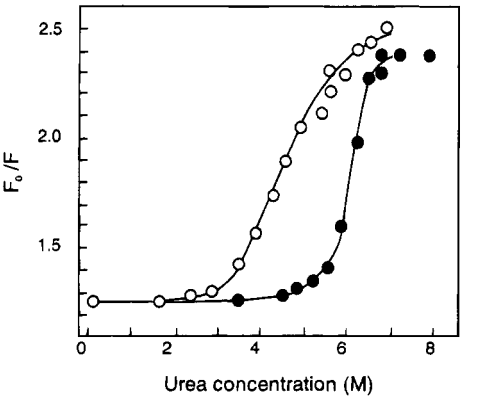

FIGURE 4: Effect of urea concentration on F_0/F in the absence (○) and presence (●) of SDS at pH 6.72 in phosphate buffer and an acrylamide concentration of 0.119 M. The protein and SDS concentrations before acrylamide addition were 25.8 and 83 μM, respectively.

dithiothreitol had a negligible effect on the F_0/F values, indicating that NATA fluorescence was quenched by acrylamide but not by dithiothreitol. The effect of the quencher on β-lactoglobulin unfolded in urea (with or without added dithiothreitol) was substantially less than for NATA (Figure 3), indicating that the two tryptophan residues of β-lactoglobulin were not fully accessible to the acrylamide, and probably to the aqueous solvent, under normal unfolding conditions. The intrinsic fluorescence of the native β-lactoglobulin was not quenched to any extent by the acrylamide (Figure 3), suggesting that the major fluorophore(s) was (were) well protected from the environment.

The change in acrylamide quenching as a function of urea concentration is shown in Figure 4. It can be seen that the apparent midpoint is at a urea concentration of about 4.4 M. With SDS in the mixture, the midpoint of the transition was close to a urea concentration of 6 M (Figure 4).

Circular Dichroism. (i) Near-UV. The near-UV CD spectrum of β-lactoglobulin in phosphate buffer (Figure 5) is very similar to those obtained earlier (e.g., Timasheff et al., 1967). The two deep troughs at 286 and 293 nm are believed to be a consequence of one or both of the β-lactoglobulin tryptophan residues being in a chiral environment (Kahn, 1979). In 7.3 M urea solution, the spectrum

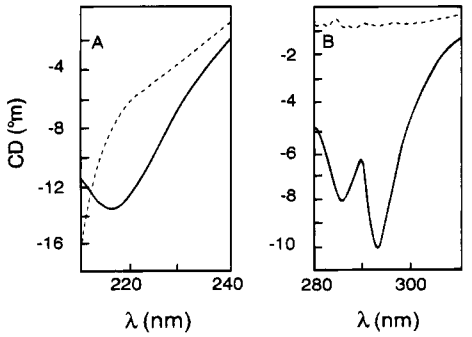

FIGURE 5: Selected portions of the near- and far-UV CD spectra of β-lactoglobulin AB in pH 6.72 phosphate buffer in the absence (—) and presence (- - -) of 7.24 M urea. (A) Far-UV spectra using a 0.5 mm cell with a β-lactoglobulin concentration of 26.2 μM. (B) Near-UV spectra using a 10 mm cell with a β-lactoglobulin concentration of 67.3 μM. The instrumental settings were as follows: 50 nm/min scan rate; 2 nm band width; 20 mdeg sensitivity; 9 accumulations; 2 s time constant.

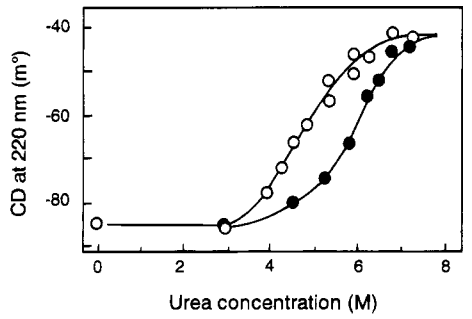

FIGURE 6: Effect of urea concentration on the intensity of the 293 nm CD trough of β-lactoglobulin in the absence (○) and presence (●) of 74 μM SDS. Experimental details are described in the Figure 5 caption.

collapsed toward the base line, which suggests that the environment of the tryptophan and tyrosine residues became less chiral.

Preliminary experiments showed that if the β-lactoglobulin stock solution was added to 5 mL of 10 M urea stock solution and this was diluted to a final concentration with 5 mL of buffer, the CD spectrum was less intense than if the β-lactoglobulin stock solution was diluted directly into 10 mL of 5 M urea solution. This confirmed an earlier report (Franklin & Leslie, 1968) which suggested that 10 M urea solution denatures β-lactoglobulin irreversibly at pH 7.5. Addition of SDS to a solution of β-lactoglobulin in 5 M urea increased the 293 nm CD signal. If the SDS was added to the 5 M urea solution before β-lactoglobulin addition, the CD signal was the same. Thus, the order of addition of SDS and β-lactoglobulin to the buffered urea solutions did not alter the CD at 293 nm. As a consequence of all these preliminary experiments, the stock β-lactoglobulin was always added to buffer solutions that were close to the final urea concentration, a spectrum was determined, SDS (or palmitic acid) was added, and the spectrum was redetermined.

Increasing the urea concentration decreased β-lactoglobulin CD at 293 nm (Figure 6) with a midpoint urea concentration of about 4.3 M, confirming earlier reports that the spectral properties of β-lactoglobulin were altered with urea concentration (e.g., Ralston, 1973). The presence of about 74 μM SDS (or palmitic acid) in the solution shifted the transition

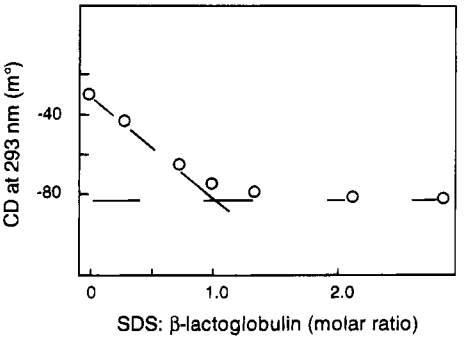

FIGURE 7: Effect of added SDS on the intensity of the 293 nm CD peak at pH 6.72. The initial protein and urea concentrations were 25 μM and 4.8 M, respectively. Other experimental details are as described in the Figure 5 caption.

to a higher urea concentration with the midpoint close to a urea concentration of 5.6 M (Figure 6).

When β-lactoglobulin was in a buffered 4.8 M urea solution and small quantities of SDS or palmitic acid were added, the CD signal became greater until a molar ratio of ligand to β-lactoglobulin of approximately 1 was attained (Figure 7), indicating that a 1:1 complex was possibly involved in the 293 nm CD changes. In the case of palmitic acid, the solutions were distinctly turbid, indicating that the solubility of the ligand had been exceeded. A similar experiment with laurodan as the additive gave no change that could be distinguished from noise. As with palmitic acid, the solutions were turbid. A third supplementary experiment using ANS showed that ANS did not affect the 293 nm signal, indicating that this probe did not affect the midpoint urea concentration of the unfolding.

(ii) Far-UV. The far-UV CD spectrum of β-lactoglobulin in phosphate buffer (Figure 5) was very similar to those obtained earlier (e.g., Timasheff et al., 1967) and consistent with the secondary structure content of 50% β-sheet and 8% α-helix found more recently by Papiz et al. (1986) using X-ray crystallography. When β-lactoglobulin was in 7.3 M urea, the trough between 205 and 235 nm diminished markedly while a deep trough developed near 200 nm. The shape of this new trough could not be determined accurately because of the increased noise as a consequence of the high absorbance of the solution. This was caused by the high urea concentration, and using a cell with a path length as short as 0.1 mm did not diminish the noise sufficiently to give usable results below about 205 nm. Because of the practical difficulties of using this narrow cell, the remainder of the measurements were made using a 0.5 mm cell and concentrating on the variations that occurred at 220 nm. The CD signal at 220 nm increased slightly up to a urea concentration of about 3.3 M and then diminished with further increases in urea concentration and gave a midpoint urea concentration of 4.6 M (Figure 8). Inclusion of 83 μM SDS did not appear to alter the shape of this curve greatly although the midpoint of the transition was at a urea concentration of nearly 5.8 M (Figure 8).

Extrinsic Fluorescence of Laurodan and ANS. Addition of β-lactoglobulin to suspensions of laurodan in buffer increased the fluorescent emission at 470 nm, presumably as a consequence of the laurodan binding to the protein at a hydrophobic site. When the β-lactoglobulin was in urea solutions, the fluorescence of the laurodan was less when the urea concentration was above 4 M. Further preliminary

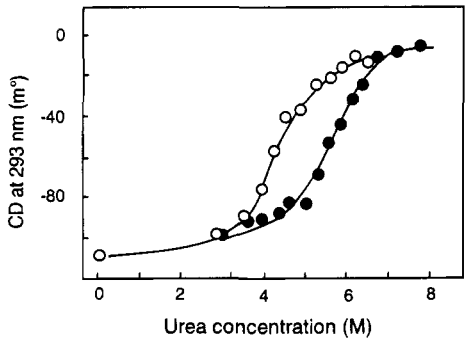

FIGURE 8: Effect of urea concentration on the intensity of the 220 nm CD of β-lactoglobulin in the absence (○) and presence (●) of 83 μM SDS. Experimental details are described in the Figure 5 caption.

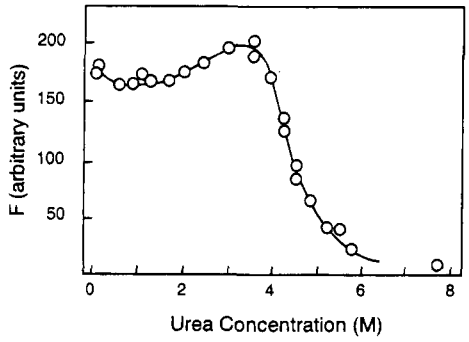

FIGURE 9: Effect of urea concentration on the extrinsic fluorescence emission intensity of ANS at 21 °C. λ_{ex} was 380 nm, and λ_{em} was 490 nm. Protein concentration was 50.8 μM while ANS concentration was 3.23 μM. The excitation and emission spectral bandwidths were 8 and 14 nm, respectively.

experiments showed that after addition of laurodan to the β-lactoglobulin solution, laurodan fluorescence increased with time but usually attained a stable value after about 10 min. In the presence of urea, a stable reading was attained much more quickly. However, addition of concentrated urea solution (10 M) to a mixture of β-lactoglobulin and laurodan in buffer in 2 M urea to give a final urea concentration of 4 M reduced the fluorescent emission by about 20%, not the 50% expected on the basis of the experiment in which the urea and protein solutions were admixed prior to laurodan addition. Nevertheless, consistent results were obtained on the addition of laurodan dissolved in alcohol to β-lactoglobulin in urea-containing buffer solutions. The midpoint in the decrease in extrinsic fluorescent intensity was at a urea concentration of 4.1 M.

Repeating these experiments with ANS, the well-established hydrophobic probe (Slavík, 1982), dissolved in phosphate buffer in place of laurodan gave results in which the time dependence was slight and only occurred at low urea concentrations. Although urea concentrations greater than 6 M were not examined, there were no difficulties with reversibility; that is, the fluorescent emission was related to the final urea concentration and was not dependent on the order of urea and buffer additions. The resultant curve for ANS is shown in Figure 9. There is a slight increase in fluorescent emission with increasing urea concentration to a maximum at about 3.4 M urea followed by a decrease with a midpoint at a urea concentration of about 4.3 M. At a urea concentration of 8.1 M, the ANS emission spectrum was essentially the same irrespective of the presence of β-lactoglobulin. The small increase in fluorescence at low urea concentrations was not reflected in changes in other parameters at similar urea concentrations. One possibility is that the normally dimeric protein is dissociating to monomers at these concentrations. The substantial decrease with a midpoint near 4.3 M seems to follow similar patterns shown by the other measured parameters (Figures 2, 4, 6, and 8) and is probably a consequence of the same structural changes in β-lactoglobulin with urea. Inclusion of SDS in the mixture was impractical as it is a strong competitor for the ANS-, retinol-, and laurodan-binding sites (Creamer, unpublished results).

DISCUSSION

The present results show that there are significant changes in the spectral parameters of β-lactoglobulin that relate to tryptophan residues as a function of urea concentration. These include λ_{max} (Figure 2), accessibility of the neutral quencher, acrylamide, to the tryptophans (Figure 4), and CD intensity at 293 nm (Figure 6). Bovine β-lactoglobulin has two tryptophan residues, and from the Papiz et al. (1986) crystal structure, tryptophan-19 is within the central hydrophobic β-barrel or calyx of the protein while tryptophan-61 is part of an external loop and close to the cystine-160−66 disulfide bond. Cho et al. (1994) genetically modified tryptophan-19 to alanine-19 and showed that the intrinsic fluorescence was substantially diminished, demonstrating that tryptophan-61 was a minor contributor to the overall fluorescent emission. This result supports the view of Mills (1976) that tryptophan-61 fluorescence was quenched in the native protein; possibly by the nearby (cystine-160−66) disulfide bond. The marked increase of F in the presence of high concentrations of urea (Figure 1) or guanidine hydrochloride (Hattori et al., 1993) may be partly as a result of tryptophan-61 moving out of a quenching environment. It seems likely that the intensity shifts of the 293 nm CD trough are related to the chiral environment of one or both tryptophan residues. It is probable that a site within the binding pocket is more likely to be chiral than one on the surface, and hence tryptophan-19 in the Papiz et al. (1986) structure may be responsible for the strong CD signals at 293 nm. Thus, it can probably be assumed that tryptophan-19 is more important than tryptophan-61 for the observed changes in both fluorescence and CD intensities.

Both β-sheet and α-helix secondary structures show CD peaks at about 200 nm and troughs in the 210−235 nm region while "random" and turn structures give rise to deep troughs near 200 nm (Johnson, 1990). The present result (Figure 5) in the absence of urea is similar to those previously reported and consistent with a 50% sheet and 10−15% helix assignment for secondary structure (e.g., Timasheff et al., 1967; Creamer et al., 1983) and the crystal structures (Papiz et al., 1986; Monaco et al., 1987). Urea also diminished the far-UV CD intensity at 220 nm (Figures 5 and 8). Thus, it is likely that the main decrease centered at 4.4 M urea is related to loss of β-sheet structure, although the loss of α-helix structure would also make a contribution to this decrease. These results, including the small increase in CD at urea concentrations up to 3 M, are similar to those reported by Lapanje and Kranjc (1982) for β-lactoglobulin unfolding with urea at pH 2.0. By contrast, Kella and Kinsella (1988) state that there was no change in the near- or far-UV CD spectra at pH 6.85 between 0 and 3 M urea.

Bovine β-lactoglobulin has been shown to bind ANS (Mills & Creamer, 1975; Laligant et al., 1991) with an increase in ANS fluorescence. In the present study, ANS extrinsic fluorescence increased slightly as urea concentration increased to about 3 M followed by a major decrease centered on a urea concentration of about 4.4 M (Figure 9). Hattori et al. (1993) noted a similar occurrence with retinol and guanidine hydrochloride while Müller and Skerra, (1993) showed a similar shift with the analogous protein, porcine RBP, and retinoic acid. It has been shown recently (Creamer, unpublished result) that both retinol and ANS appear to bind to the same binding site on bovine β-lactoglobulin. Thus, in both cases it is likely that the integrity of this binding site is lost as a consequence of protein unfolding. The structural change that precedes the unfolding step, and gave rise to the small increase in fluorescence, was also evident in the 220 nm CD results (Figure 8), suggesting that there could possibly be some increases in secondary structure at low denaturant concentrations, although it is equally possible that some of the loosely structured surface loops changed conformation. One possible explanation for these various results is that the dimeric protein dissociated to monomers under these conditions, possibly allowing better access of ANS and retinol to the binding site. Kella and Kinsella (1988) showed that the apparent elution position of β-lactoglobulin from a Sephadex G-100 column depended on urea concentration between 0 and 3 M at pH 6.85 in 0.01 M phosphate buffer, and suggested that the dimeric protein was completely dissociated to monomer in 3 M urea solution. Thus, at low urea concentrations and neutral pH, dissociation of the dimer of β-lactoglobulin is the major conformational change taking place. On this assumption, and placing tryptophan-19 within the calyx, it is not surprising that the various indices of tryptophan environmental exposure (Figures 2, 4, and 6) did not change at urea concentrations between 0 and 3 M.

Effect of SDS and Palmitate on β-Lactoglobulin Unfolding. SDS had a profound effect on the equilibrium unfolding of bovine β-lactoglobulin as shown in Figures 2, 4, 6, and 8 by apparently maintaining the protein in the native conformation to higher urea concentrations. Unfortunately, the hydrophobic binding site could not be probed with ANS or laurodan as SDS competes and displaces them from the binding site (Creamer, unpublished results). SDS itself binds strongly to a small number of sites on β-lactoglobulin at low SDS concentrations (Ray & Chatterjee, 1967; Jones & Wilkinson, 1976; Lamiot et al., 1994) and to a greater number at concentrations approaching the critical micelle concentration (CMC) of about 2.5 mM in aqueous buffers. It can be seen from Figure 7 that the major change in CD at 293 nm occurs at SDS:β-lactoglobulin ratios below 1:1, which suggests that the effect is complete with the formation of a 1:1 complex between SDS and β-lactoglobulin and that the protein can be refolded by the addition of SDS to the unfolded protein in urea solution. Zanotti et al. (1993) determined the structures of both holo- and apo-RBP and found that the region occupied by retinol in the holo structure was occupied by water molecules in the apo structure. Similar results were obtained when other apo and holo lipid-binding proteins were examined (Banaszak et al., 1994). In these latter cases, it was concluded that the presence of strong interactions between the amino acid side chains within the β-barrels held the barrels open and the presence of some hydrophilic side chains lowered the energy change that could have occurred with the loss of the retinol or fatty acid from the respective binding sites. It seems likely that similar considerations apply to RBP and β-lactoglobulin. Thus, it is likely that when SDS or palmitate and possibly retinol binds to bovine β-lactoglobulin, solvent water is displaced. The acquisition of a refined high-resolution X-ray structure of β-lactoglobulin should clarify these issues.

It has been reported that porcine β-lactoglobulin, which has only one tryptophan (Conti et al., 1986), does not bind fatty acids although it does bind retinol (Pérez et al., 1993; Frapin et al., 1993). Similarly, bovine β-lactoglobulin at low pH does not bind fatty acids but does bind retinol (Spector & Fletcher, 1970; Frapin et al., 1993; Lamiot et al., 1994). Cho et al. (1994) have implicated lysine-70 as an important amino acid in binding retinoic acid to β-lactoglobulin, and examination of the structures of a FABP (Benning et al., 1992) and RABPs (Banaszak et al., 1994) indicates that an arginine or lysine is generally present within the binding pocket of the protein and interacts with the ligand and that mutating the charged residues in these proteins can alter the specificity for retinoic or fatty acid to/from retinol (Jakoby et al., 1993; Winter et al., 1993; Zhang et al., 1992). Porcine β-lactoglobulin has no such amino acid in an appropriate position within the putative binding pocket. [Lysine-69 and lysine-70 in the bovine protein become glutamic acid-69 and valine-70 in the porcine (Conti et al., 1986).] The lack of fatty acid binding at low pH, where these acids would be present in the protonated form and not the anion, also suggests an important role for a basic residue, which could be lysine-70, in bovine β-lactoglobulin as a fatty acid and SDS binder but relatively less important for retinol, laurodan, or ANS.

Effect of Ligand Binding on the Stability of Lipid-Binding Proteins. Despite the structural differences between β-lactoglobulin or RBP (*e*-LBPs) and CRABP, FABP, etc. (*e*-LBPs), there are a number of similarities in the effect of ligand binding on stability to urea or thermal unfolding or hydrolysis. For example, McMeekin et al. (1949) reported that the SDS complex with β-lactoglobulin has a greater heat stability than the apo-protein, and Puyol et al. (1994) have reported that β-lactoglobulin with bound palmitic acid is more stable to thermal denaturation than the apo-protein or the retinol−β-lactoglobulin complex, while Zhang et al. (1992) showed that the CRABP−retinoic acid complex was more stable (by 17 °C) to thermal denaturation than the uncomplexed protein. Using the approach of limited proteolysis, Jamison et al. (1994) showed that cellular retinol-binding protein (CRBP) and cellular retinol-binding protein type II (CRBP-II) were more resistant to hydrolysis at an arginine bond near the β-barrel opening in the holo form than in the apo form. They also showed that holo forms of the related CRABP and heart FABP were more resistant to proteolysis. This compares with similar results obtained using bovine β-lactoglobulin where trypsin hydrolysis is modified by the presence of palmitic acid but not retinol (Puyol et al., 1993). It has been reported (Müller & Skerra, 1993) that for a porcine RBP mutant the midpoint in the equilibrium unfolding in guanidine hydrochloride was higher for the holo-protein (with retinoic acid) than for the apo-protein. They also found that the unfolding was reversible and appeared to be an example of the classic two-state process. This is comparable to the results of the present

study and also the study by Griffin et al. (1989) using guanidine hydrochloride as the unfolding agent.

From these considerations, it appears that there are functional as well as structural similarities between β-lactoglobulin and the retinoid- and fatty acid-binding proteins but these are unlikely to be genetically based (Flower et al., 1993; Banaszak et al., 1994).

CONCLUSIONS

It can be concluded that at pH 6.72 bovine β-lactoglobulin unfolds in urea solution and this is possibly preceded by dissociation of the dimer to monomer. The unfolding resembles a two-state process in which only fully-folded monomer protein and completely unfolded monomer protein participate. The addition of low concentrations of SDS or palmitate (but not ANS or laurodan) shifts the midpoint in the equilibrium unfolding from about 4.4 M urea to about 5.8 M urea. These results are consistent with β-lactoglobulin binding SDS within the β-barrel of the protein at a site that overlaps the site that has been suggested for the binding site of retinol. Although there was no evidence for sequential loosening of the protein structure (e.g., the α-helix moving, or unfolding, to expose the hydrophobic, disulfide-containing, external face of the β-barrel), the parameters examined in this study were unlikely to vary with such structural changes, and thus sequential loosening could not be excluded.

ACKNOWLEDGMENT

I am very grateful for discussions with Carl Batt, Thomasz Haertlé, William and Mary Griffin, and Carl Holt about their unpublished studies.

REFERENCES

Banaszak, L., Winter, N., Xu, Z., Bernlohr, D. A., Cowan, S., & Jones, T. A. (1994) *Adv. Protein Chem. 45*, 89−151.
Bell, K., & McKenzie, H. A. (1967) *Biochim. Biophys. Acta 147*, 109−122.
Benning, W. M., Smith, A. F., Wells, M. A., & Holden, H. M. (1992) *J. Mol. Biol. 228*, 208−219.
Cho, Y., Batt, C. A., & Sawyer, L. (1994) *J. Biol. Chem. 269*, 11102−11107.
Conti, A., Godovac-Zimmermann, J., Pirchner, F., Liberatori, J., & Braunitzer, G. (1986) *Biol. Chem. Hoppe-Seyler 367*, 871−878.
Creamer, L. K., Parry, D. A. D., & Malcolm, G. N. (1983) *Arch. Biochem. Biophys. 227*, 98−105.
Demchenko, A. P. (1992) in *Topics in Fluorescence Spectroscopy* (Lakowicz, J. R., Ed.) Vol. 3, pp 65−111, Plenum Press, New York.
Eftink, M. R., & Ghiron, C. A. (1981) *Anal. Biochem. 114*, 199−227.
Flower, D. R., North, A. C. T., & Attwood, T. K. (1993) *Protein Sci. 2*, 753−761.
Franklin, J. G., & Leslie, J. (1968) *Biochim. Biophys. Acta 160*, 333−339.
Frapin, D., Dufour, E., & Haertlé, T. (1993) *J. Protein Chem. 12*, 443−449.
Futterman, S., & Heller, J. (1972) *J. Biol. Chem. 247*, 5168−5172.
Griffin, W. G., Griffin, M. C. A., & Martin, S. R. (1989) Conformational Studies of the β-Lactoglobulin/Retinol Complex. Poster presentation at IUPAC Conference, Stockholm, Sweden.
Hambling, S. G., McAlpine, A. S., & Sawyer, L. (1992) in *Advanced Dairy Chemistry −1: Proteins* (Fox, P. F., Ed.) pp 141−190, Elsevier Science Publishers, London.
Hattori, M., Ametani, A., Katakura, Y., Shimizu, M., & Kaminogawa, S. (1993) *J. Biol. Chem. 268*, 22414−22419.
Jakoby, M. G., IV, Miller, K. R., Toner, J. J., Bauman, A., Cheng, L., Li, E., & Cistola, D. P. (1993) *Biochemistry 32*, 872−878.
Jamison, R. S., Newcomer, M. E., & Ong, D. E. (1994) *Biochemistry 33*, 2873−2879.
Johnson, W. C., Jr. (1990) *Proteins: Struct., Funct., Genet. 7*, 205−214.
Jones, M. N., & Wilkinson, A. (1976) *Biochem. J. 153*, 713−718
Kahn, P. C. (1979) *Methods Enzymol. 61*, 339−378.
Kella, N. K. D., & Kinsella, J. E. (1988) *Int. J. Pept. Protein Res. 32*, 396−405.
Laligant, A., Dumay, E., Valencia, C. C., Cuq, J.-L., & Cheftel, J.-C. (1991) *J. Agric. Food Chem. 39*, 2147−2155.
Lamiot, E., Dufour, E., & Haertlé, T. (1994) *J. Agric. Food Chem. 42*, 695−699.
Lapanje, S., & Kranjc, Z. (1982) *Biochim. Biophys. Acta 705*, 111−116.
McKenzie, H. A. (1971) in *Milk Proteins* (McKenzie, H. A., Ed.) Vol. 2, pp 257−330, Academic Press, New York.
McKenzie, H. A., Ralston, G. B., & Shaw, D. C. (1972) *Biochemistry 11*, 4539−4547.
McMeekin, T. L., Polis, B. D., DellaMonica, E. S., & Custer, J. H. (1949) *J. Am. Chem. Soc. 71*, 3606−3609.
Mills, O. E. (1976) *Biochim. Biophys. Acta 434*, 324−332.
Mills, O. E., & Creamer, L. K. (1975) *Biochim. Biophys. Acta 379*, 618−626.
Monaco, H. L., Zanotti, G., Spadon, P., Bolognesi, M., Sawyer, L., & Eliopoulos, E. E. (1987) *J. Mol. Biol. 197*, 695−706.
Müller, H. N., & Skerra, A. (1993) *J. Mol. Biol. 230*, 725−732.
Pace, C. N. (1986) *Methods Enzymol. 131*, 266−280.
Pace, C. N., & Tanford, C. (1968) *Biochemistry 7*, 198−208.
Papiz, M. Z., Sawyer, L., Eliopoulos, E. E., North, A. C. T., Findlay, J. B. C., Sivaprasadarao, R., Jones, T. A., Newcomer, M. E., & Kraulis, P. J. (1986) *Nature 324*, 383−385.
Pérez, M. D., Sanchez, L., Aranda, P., Ena, J. M., Oria, R., & Calvo, M. (1992) *Biochim. Biophys. Acta 1123*, 151−155.
Pérez, M. D., Puyol, P., Ena, J. M., & Calvo, M. (1993) *J. Dairy Res. 60*, 55−63.
Pervaiz, S., & Brew, K. (1985) *Science 228*, 335−337.
Puyol, P., Pérez, M. D., Mata, L., Ena, J. M., & Calvo, M. (1993) *Int. Dairy J. 3*, 589−597.
Puyol, P., Pérez, M. D., Peiro, J. M., & Calvo, M. (1994) *J. Dairy Sci. 77*, 1494−1502.
Ralston, G. B. (1973) *Compt. Rend. Trav. Lab. Carlsberg 38(26)*, 499−512.
Ray, A., & Chatterjee, R. (1967) in *Conformation of Biopolymers* (Ramachandran, G. N., Ed.) pp 235−252, Academic Press, London.
Seibles, T. S. (1969) *Biochemistry 8*, 2949−2954.
Slavík, J. (1982) *Biochim. Biophys. Acta 694*, 1−25.
Spector, A. E., & Fletcher, J. E. (1970) *Lipids 5*, 403−411.
Tanford, C., & De, P. K. (1961) *J. Biol Chem. 236*, 1711−1715
Timasheff, S. N., Susi, H., Townend, R., Stevens, L., Gorbunoff, M. J., & Kumosinski, T. (1967) in *Conformations of Biopolymers* (Ramachandran, G. N., Ed.) Vol. 1, pp 173−196, Academic Press, London.
Winter, N. S., Bratt, J. M., & Banaszak, L. J. (1993) *J. Mol. Biol. 230*, 1247−1259.
Wu, P., & Brand, L. (1994) *Anal. Biochem. 218*, 1−13.
Zanotti, G., Ottonello, S., Berni, R., & Monaco, H. (1993) *J Mol. Biol. 230*, 613−624
Zhang, J., Liu, Z.-P., Jones, T. A., Gierasch, L. M., & Sambrook, J. F. (1992) *Proteins: Struct., Funct., Genet. 13*, 87−99.

BI942479B