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Characterization of pH-induced Transitions of β-Lactoglobulin: Ultrasonic, Densimetric, and Spectroscopic Studies

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Depending on solution conditions, β-lactoglobulin can exist in one of its six pH-dependent structural states. We have characterized the acid and basic-induced conformational transitions between these structural states over the pH range of pH 1 to pH 13. To this end, we have employed high-precision ultrasonic and densimetric measurements coupled with fluorescence and CD spectroscopic data. Our combined spectroscopic and volumetric results have revealed five pH-induced transitions of β-lacto-globulin between pH 1 and pH 13. The first transition starts at pH 2 and is not completed even at pH 1, our lowest experimental pH. This transition is followed by the dimer-to-monomer transition of β-lactoglobulin between pH 2.5 and pH 4. The dimer-to-monomer transition is accompanied by decreases in volume, $v°$ ($-0.008(\pm 0.003)$ cm^3 g^{-1}), and adiabatic compressibility, $k_S°$ ($-(0.7(\pm 0.4)) \times 10^{-6}$ cm^3 g^{-1} bar^{-1}). We interpret the observed changes in volume and compressibility associated with the dimer-to-monomer transition of β-lactoglobulin, in conjunction with X-ray crystallographic data, as suggesting a 7% increase in protein hydration, with the hydration changes being localized in the area of contact between the two monomeric subunits. The so-called N-to-Q transition of β-lactoglobulin occurs between pH 4.5 and pH 6 and is accompanied by increases in volume, $v°$ ($0.004(\pm 0.003)$ cm^3 g^{-1}), and compressibility, $k_S°$ ($(0.7(\pm 0.4)) \times 10^{-6}$ cm^3 g^{-1} bar^{-1}). The Tanford transition of β-lactoglobulin is centered at pH 7.5 and is accompanied by a decrease in volume, $v°$ ($-0.006(\pm 0.003)$ cm^3 g^{-1}), and an increase in compressibility, $k_S°$ ($(1.5(\pm 0.5)) \times 10^{-6}$ cm^3 g^{-1} bar^{-1}). Based on these volumetric results, we propose that the Tanford transition is accompanied by a 5 to 10% increase in the protein hydration and a loosening of the interior packing of β-lactoglobulin as reflected in a 12% increase in its intrinsic compressibility. Finally, above pH 9, the protein undergoes irreversible base-induced unfolding which is accompanied by decreases in $v°$ ($-0.014(\pm 0.003)$ cm^3 g^{-1}) and $k_S°$ ($-(7.0(\pm 0.5)) \times 10^{-6}$ cm^3 g^{-1} bar^{-1}). Combining these results with our CD spectroscopic data, we propose that, in the base-induced unfolded state of β-lactoglobulin, only 80% of the surface area of the fully unfolded conformation is exposed to the solvent. Thus, in so far as solvent exposure is concerned, the base-induced unfolded states of β-lactoglobulin retains some order, with 20% of its amino acid residues remaining solvent inaccessible.

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Introduction

β-Lactoglobulin is a major protein of the whey fraction of the milk of many mammals, including

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but not limited to the family Bovidae. At physiological conditions, bovine β-lactoglobulin forms a dimer, with each monomer consisting of 162 amino acid residues and characterized by a molecular mass of 18,350.[1] Below pH 3, the dimer dissociates into monomers which preserve their native con-

formation.[2−4] Genetically, β-lactoglobulin may exist as one of several variants, among which the variants A and B are the most abundant.[1] The A and B variants of the protein differ from each other by amino acid residues at positions Asp64 (Gly64 in variant B) and Val118 (Ala118 in variant B). These differences in primary structure render the two variants slightly different with respect to iso-electric point, solubility, self-association properties, as well as pressure and temperature stability.[5−7] However, the structural characteristics of the A and B variants of bovine β-lactoglobulin are virtually indistinguishable.[8] In its native state, β-lacto-globulin
is a predominantly β-sheet protein containing nine β-strands and three α-helices.[8−10] The core of the protein is formed by a flattened β-barrel (a calyx) composed of eight antiparallel β-strands (A to H).

Between pH 2 and pH 13, β-lactoglobulin exhibits a number of pH-induced, local and global structural transitions. Specifically, near pH 3, the protein dimerizes with little alteration in structure.[4,11] Between pH 4 and pH 5, β-lactoglobulin is reported to undergo the dimer-to-octamer transition as suggested by a variety of biophysical methods including optical rotation dispersion spectroscopy, ultracentrifugation, electrophoresis, light scattering, and NMR spectroscopy.[12−15] Significantly, Fourier transform infrared spectroscopic (FTIR) measurements performed by Casal et al.[16] have indicated that octamerization of the protein does not cause any appreciable change in its secondary structure. Between pH 4.5 and pH 6, β-lactoglobulin is converted from its expanded acidic Q-form to the more compact native N-form.[16,17] As suggested by Timasheff et al.,[17] this Q-to-N transition may involve changes in protein structure and hydration. This suggestion is consistent with the FTIR spectroscopic results of Casal et al.[16] Above pH 6.5, β-lactoglobulin undergoes the so-called Tanford transition which is triggered by protonation of Glu89 exhibiting an anomalously high pK_a value.[18] The Tanford transition involves displacement of the loop EF (residues 85 to 90) that acts as a lid which closes the protein interior/binding site below pH 7.3 and opens it at higher pH. The Tanford transition may involve some other structural changes as well. For example, the transition is accompanied by a change in the microenvironment of Tyr42[8] and causes an alteration in the relative orientation of monomers in the dimer by as much as 5°.[10] It should be noted that all transitions that take place between pH 2 and pH 9 do not cause any appreciable changes in the native-like β-barrel conformation of β-lactoglobulin.[3] By contrast, above pH 9, β-lactoglobulin undergoes an irreversible, base-induced unfolding transition with global disruption of both secondary and tertiary structures.[18−20]

The biological function of β-lactoglobulin is yet to be determined. Since the protein is capable of binding small hydrophobic substances (such as vitamin A and retinol) and exhibits remarkable resistance to acid-induced denaturation, it is thought that at least one function of β-lactoglobulin is to bind non-polar molecules and transport them through the acidic environment of stomach into the basic environment of intestine.[21] Consequently, an understanding of the pH-dependent behavior of β-lactoglobulin may shed light into its biological role. In addition, β-lactoglobulin is a widely used model system in protein folding studies.[22−25] Therefore, pH-dependent studies of β-lactoglobulin are also of independent biophysical importance and may have implications for protein folding. A survey of the literature reveals that despite the large number of solution studies, we know very little about the changes in volume and compressibility associated with each of the above-mentioned structural transitions of β-lactoglobulin. This deficiency is unfortunate since volumetric observables are sensitive to protein hydration and intrinsic packing which are likely to contribute to the conformation stability of each of the pH-specific structural states of the protein.

In this work, we employ volumetric and optical spectroscopic techniques to characterize the pH-induced transitions of β-lactoglobulin. Volumetric characteristics, such as partial specific volume and adiabatic compressibility measurements, have already proven useful for studying the hydration and intrinsic packing properties of proteins and other biologically relevant molecules.[26−32] In particular, volume and compressibility have been used for characterizing conformational transitions of proteins.[33−40] Here, we report the partial specific volume and adiabatic compressibility of β-lactoglobulin at 25 °C over the pH range of pH 1 to pH 13. We interpret our volumetric results in conjunction with the fluorescence anisotropy and circular dichroism (CD) spectroscopic data in terms of the hydration properties and intrinsic packing of the protein in each of its five pH-dependent structural states. In general, we discuss implications of our results for understanding forces stabilizing/destabilizing the native and locally and/or globally altered protein conformations.

Results

Circular dichroism

Figure 1 shows the far UV CD spectra of β-lactoglobulin at acidic and alkaline pH. In general, our far UV CD spectra of β-lactoglobulin are in good agreement with those reported by Dong et al.[41] and Barteri et al.[42] Inspection of Figure 1 reveals that, at neutral pH (pH 6.35 (□)), the protein exhibits a wide minimum around 218 nm which is a characteristics of β-rich proteins. We have used a previously described algorithm[43] to calculate the amounts of secondary structural elements of the protein from its far UV CD spectrum. At ∼pH 6, the relative amounts of α-helical, β-sheet, β-turn, and unordered secondary structural elements of β-lactoglobulin are equal to 19, 39, 12, and 30(±4)%,
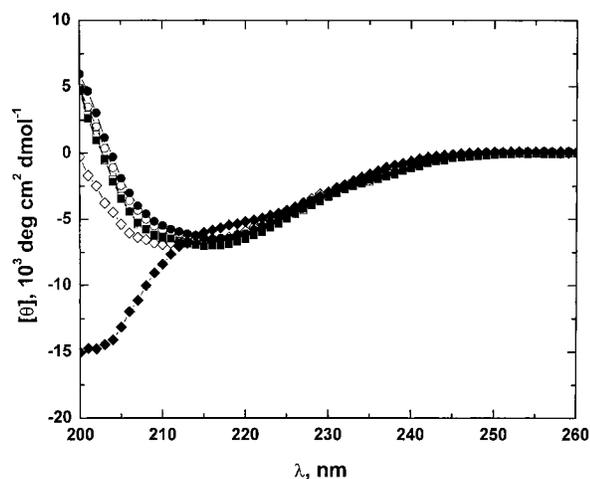
Figure 1. Far UV CD spectra of β-lactoglobulin at pH 0.92 (●), pH 2.30 (○), pH 5.00 (■), pH 6.35 (□); pH 9.16 (◇), and pH 12.35 (◆).

respectively. These values are in reasonable agreement with previous reports.[41]

Reinspection of Figure 1 reveals that the far UV CD spectrum of β-lactoglobulin weakly depends on pH over the entire range of acidic pH, yet, the difference between the far UV CD spectra of the protein above pH 4 (pH 5.00 (■)) and below pH 3 (pH 2.30 (○)) is statistically significant. This observation suggests that β-lactoglobulin undergoes a pH-induced transition between pH 3 and pH 4, which brings about a slight change in secondary structure. Figure 2(a) presents the profile of this transition as monitored by the pH-dependence of the protein ellipticity at 208 nm.

Further inspection of Figure 1 reveals that, at alkaline pH, the far UV CD spectrum of β-lactoglobulin undergoes significant alterations. In particular, the far UV CD spectrum of the protein at pH 12.35 (◆) resembles that of an unfolded polypeptide chain. Figure 2(b) presents the profiles of the base-induced transitions of β-lactoglobulin as monitored by the pH dependence of the protein ellipticity at 208 nm. As shown in Figure 2(b), there are two discernible sigmoidal transitions between pH 6 and pH 13. The midpoint of the first transition is around pH 7.5, while the second transition is centered at around pH 11.

Figure 3 shows the near UV CD spectra of β-lactoglobulin at acidic and alkaline pH. Inspection of Figure 3 reveals only slight acid-induced changes in the near UV CD spectrum of the protein, with the general shape of the native spectrum being preserved. In particular, note that the characteristic maximum at 288 nm and two minima at 285 and 293 nm are present in all the near UV CD spectra of β-lactoglobulin recorded between pH 0.9 and pH 5.7. Nevertheless, slight yet statistically significant acid-induced changes in the near UV CD spec-
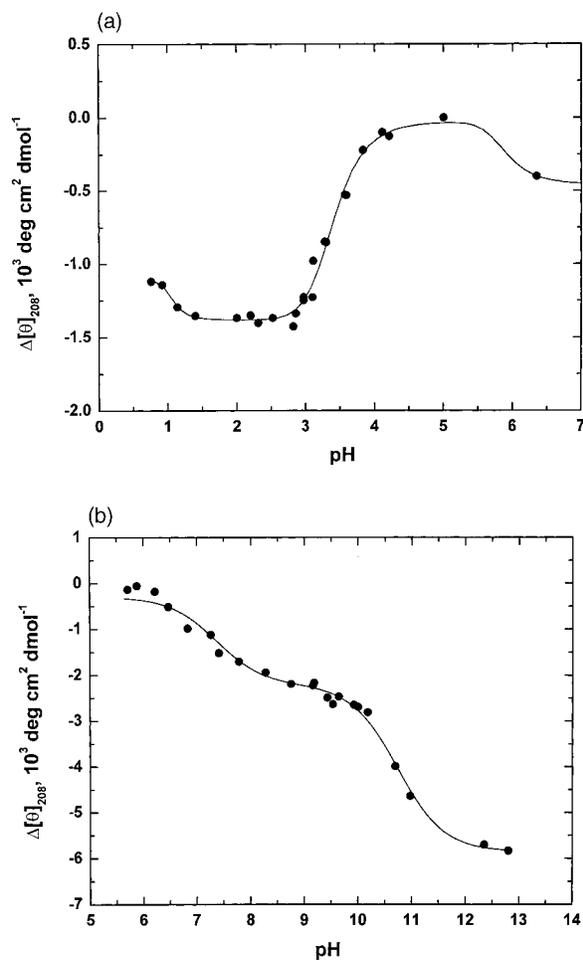

Figure 2. pH dependence for β-lactoglobulin of the molar ellipticity at 208 nm: (a) acidic range; (b) alkaline range. The fitting of experimental data (continuous lines) was performed using equation (1) as explained in the text.

trum of the protein are suggestive of some alterations in its tertiary structure. Figure 4(a) shows the pH-dependence of the protein ellipticity at 293 nm. Inspection of Figure 4(a) reveals that β-lactoglobulin undergoes an acid-induced transition between pH 3 and pH 4. Recall that this transition has also been reflected in the far UV CD spectrum of the protein (Figure 2(a)).

Further inspection of Figure 3 reveals that the base-induced changes in the near UV CD spectrum of β-lactoglobulin are much more drastic than the acid-induced changes. Judging by the near UV CD spectrum, β-lactoglobulin loses its rigid tertiary structure above pH 12 (pH 12.51 (◆)). Figure 4(b) shows the profile of the base-induced conformational transitions of β-lactoglobulin as monitored by the pH-dependence of the ellipticity at 293 nm. Inspection of Figure 4(b) reveals two pH-induced transitions with the midpoints at pH 7.5 and pH 11. Both transitions
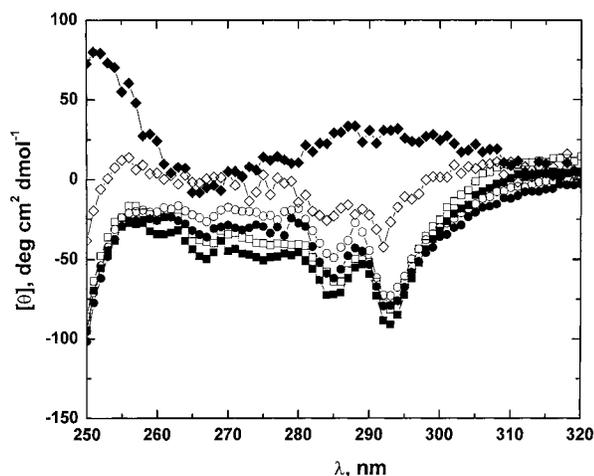
Figure 3. Near UV CD spectra of β-lactoglobulin at pH 0.90 (●), pH 2.19 (○), pH 4.59 (■), pH 5.72 (□), pH 9.23 (◇), and pH 12.51 (◆).
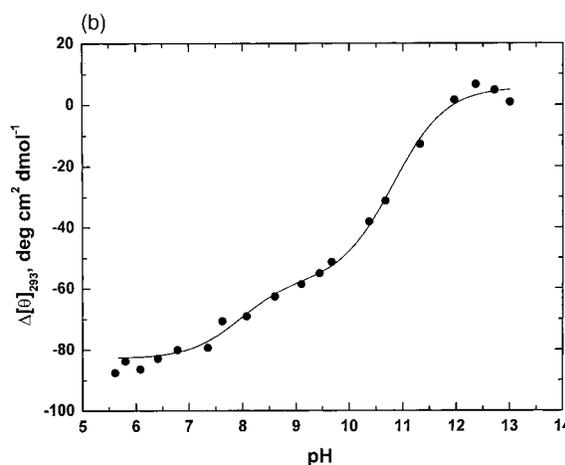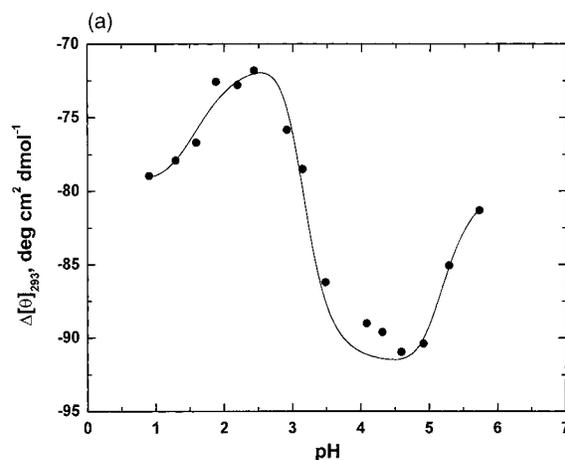

Figure 4. pH dependence for β-lactoglobulin of the molar ellipticity at 293 nm: (a) acidic range; (b) alkaline range. The fitting of experimental data (continuous lines) was performed using equation (1) as explained in the text.

have been reflected in the far UV CD spectra of the protein (Figure 2(b)).

Fluorescence anisotropy

Figure 5(a) and (b) present the pH-dependences of the fluorescence anisotropy of β-lactoglobulin over the acidic and alkaline pH range, respectively. Inspection of Figure 5(a) and (b) reveals that β-lactoglobulin undergoes pH-induced transitions with the midpoints at 3.5, 7.5 and 11. Significantly, the same transitions have been detected by CD (Figures 2(a), (b), 4(a) and (b)).

Sound velocity, volume, and compressibility

The relative specific sound velocity increment, $[u]$, partial specific volume, $v°$, and partial specific adiabatic compressibility, $k_S°$, of β-lactoglobulin in water at 10 mM NaCl (~pH 5.5) are equal to 0.197(±0.003) cm³ g⁻¹, 0.750(±0.003) cm³ g⁻¹, and (4.6(±0.5)) × 10⁻⁶ cm³ g⁻¹ bar⁻¹. These values are in good agreement with similar data reported in the literature.[40, 44-46]

Figures 6 and 7 present, the pH-dependences of the partial specific volume, $v°$, and partial specific adiabatic compressibility, $k_S°$, of the protein, respectively. Panels (a) and (b) of Figures 6 and 7 show the pH-dependences of $v°$ and $k_S°$ at acidic and alkaline pH, respectively. Our data on the pH-dependences of the relative specific sound velocity increment, $[u]$ (data are not shown), partial specific volume, $v°$ (Figure 6), and partial specific adiabatic compressibility, $k_S°$ (Figure 7), of β-lactoglobulin reveal that both acidification and alkalinization of the protein solution cause large and, generally, non-sigmoidal changes in all three volumetric observables studied here.

Discussion

Optical spectroscopy

As mentioned above, our CD spectroscopic and fluorescence anisotropy results suggest that β-lactoglobulin undergoes a number of pH-induced structural transitions between pH 1 and pH 13. Provided that these structural transitions are all two-state-like, the pH-dependence of an observable, X, can be presented as follows:

$$\Delta X(\text{pH}) = \sum_i \Delta X_i \frac{K_i(\text{pH})}{1 + K_i(\text{pH})} \qquad (1)$$

where $K_i(\text{pH})$ is the equilibrium constant of the ith transition; ΔX_i is the amplitude of the change in X accompanying the ith transition.

The effect of pH on the equilibrium constant, $K_i(\text{pH})$, can be analyzed using a simplified ver-
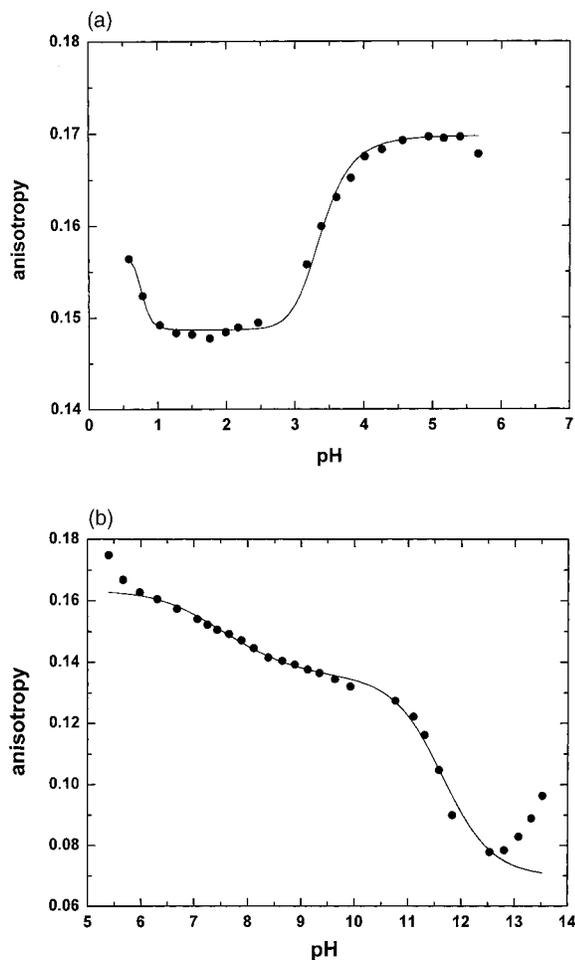
Figure 5. pH dependence for β-lactoglobulin of the fluorescence anisotropy at 340 nm: (a) acidic range; (b) alkaline range. The fitting of experimental data (continuous lines) was performed using equation (1) as explained in the text.
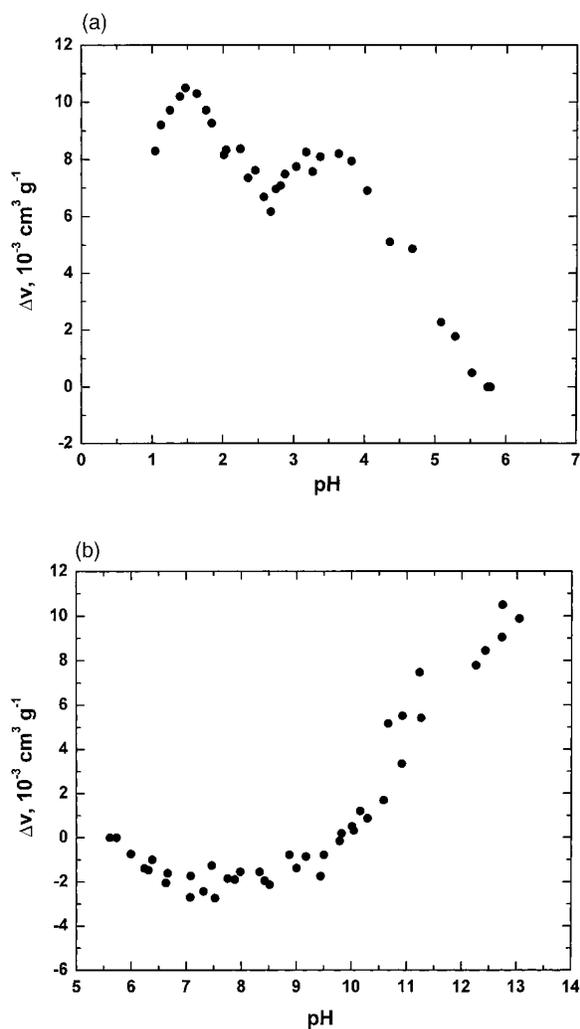

Figure 6. pH dependence for β-lactoglobulin of the partial specific volume, $v°$: (a) acidic range; (b) alkaline range.

sion of the procedure originally developed by Tanford.[47,48] Specifically, by simplifying the original equations described by Tanford,[47,48] one obtains the following relationship for acid (equation (2)) and base-induced (equation (3)) transitions:[34,49]

$$K_i = K_i^\circ (1 + 10^{pK_{aeff,i}-pH})^{\Delta v_i} \qquad (2)$$

$$K_i = K_i^\circ (1 + 10^{pH-pK_{aeff,i}})^{\Delta v_i} \qquad (3)$$

where K_i° is the equilibrium constant for the *i*th transition in the absence of protons (equation (2)) or hydroxyl ions (equation (3)); Δv_i is the difference in the numbers of protons (equation (2)) or hydroxyl ions (equation (3)) bound to the protein in its final and initial structural states; and $pK_{aeff,i}$ is the effective value of pK_a of abnor-

mally ionizable amino acid residues (residues that change their dissociation constants upon the protein transition).

We have used equations (1) to (3) to fit our experimental pH-dependent data on the protein CD (Figures 2(a), (b), 4(a) and (b)) and fluorescence anisotropy (Figure 5(a) and (b)). Table 1 presents the values of $pK_{aeff,i}$ (second row), Δv_i (third row), and K_i° (fourth row) for each pH-induced transition of β-lactoglobulin calculated from the combined fit of our spectroscopic and volumetric data (see below). However, it should be noted that our results for the base-induced denaturation of β-lactoglobulin (fifth column in Table 1) should be viewed with caution. Strictly speaking, since this transition is irreversible, it cannot be treated in terms of equilibrium thermodynamics.
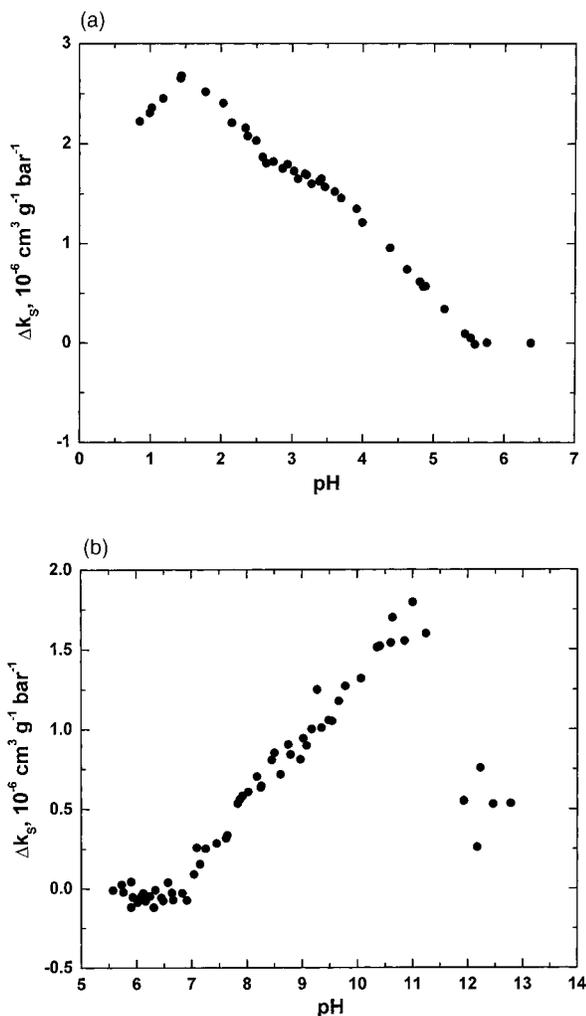
Figure 7. pH dependence for β-lactoglobulin of the partial specific adiabatic compressibility, k_S°: (a) acidic range; (b) alkaline range.

Partial specific volume

The pH-induced change in the partial specific volume, Δv, of a protein can be presented as a sum of two terms:[33, 46, 50-51]

$$\Delta v = \Delta v_{str} + \Delta v_{prot} \qquad (4)$$

where Δv_{str} is the structural change in volume, that is the change in volume associated with pH-induced alteration in protein structure/conformation; and Δv_{prot} is the protonation volume, that is the volume change associated with protonation/deprotonation of titrable groups. The protonation term can be analytically described by the following function:[33-35]

$$\Delta v_{prot} = M^{-1} \sum_j \Delta V_j (1 + 10^{\alpha(\text{pH}-\text{p}K_{aj})})^{-1} \qquad (5)$$

where M is the molecular mass of the protein; ΔV_j is the molar change in volume upon protonation (or deprotonation) of the jth group; α is a constant equal to 1 for acidic titration and -1 for basic titration; and $\text{p}K_{aj}$ is the dissociation constant of the jth titrable group.

For the acidic titration of β-lactoglobulin, the protonation term, Δv_{prot}, can be calculated based on the knowledge of all amino acid residues which are neutralized at acidic pH. In this respect, note that β-lactoglobulin has ten aspartic acid, 16 glutamic acid, two histidine residues, and a C-terminal carboxyl group, which are all neutralized (protonated) at acidic pH. Consequently, one derives the following relationship:

$$
\begin{aligned}
\Delta v_{prot} = M^{-1}[&10\Delta V_{Asp}(1 + 10^{\text{pH}-\text{p}K_{aAsp}})^{-1} \\
&+ 16\Delta V_{Glu}(1 + 10^{\text{pH}-\text{p}K_{aGlu}})^{-1} \\
&+ 2\Delta V_{His}(1 + 10^{\text{pH}-\text{p}K_{aHis}})^{-1} \\
&+ \Delta V_{Ct}(1 + 10^{\text{pH}-\text{p}K_{aCt}})^{-1}]
\end{aligned}
\qquad (6)
$$

where ΔV_{Asp}, ΔV_{Glu}, ΔV_{His}, and ΔV_{Ct} are the average changes in volume accompanying protonation of aspartic acid, glutamic acid, histidine, and the C-terminal carboxyl group, respectively; and $\text{p}K_{aAsp}$, $\text{p}K_{aGlu}$, $\text{p}K_{aHis}$, and $\text{p}K_{aCt}$ are the average $\text{p}K_a$ values of aspartic acid, glutamic acid, histidine, and C-terminal carboxyl group, respectively. In our analysis, we have used the values of $\text{p}K_{aAsp}$, $\text{p}K_{aGlu}$, $\text{p}K_{aHis}$, and $\text{p}K_{aCt}$ equal to 2.7 ± 0.5, 4.0 ± 0.9, 5.9 ± 0.8, and 2.8 ± 0.6, respectively.[52-55]

For the alkaline titration of β-lactoglobulin, the protonation term, Δv_{prot} (more precisely, the deprotonation term), can be calculated based on the knowledge that the protein contains 15 lysine,

Table 1. Parameters of the pH-induced transitions of β-lactoglobulin derived from the combined fit of spectroscopic and volumetric data

Transitions	Dimer-to-monomer transition	pH 5 transition	Tanford transition	Base-induced denaturation
$\text{p}K_{aeff}$	3.6 ± 0.6	5.5 ± 0.5	6.4 ± 0.5	8.7 ± 0.5
Δv	3 ± 1	2 ± 1	1 ± 0.4	1 ± 0.4
K° (M)	0.04 ± 0.02	0.18 ± 0.04	0.15 ± 0.02	0.005 ± 0.002
Δv_{str} (cm^3 g^{-1})	-0.008 ± 0.003	0.004 ± 0.003	-0.006 ± 0.003	-0.014 ± 0.003
Δk_{str} (10^{-6} cm^3 g^{-1} bar^{-1})	-0.7 ± 0.4	0.7 ± 0.4	1.5 ± 0.5	-7.0 ± 0.5

See the text for details.

three arginine, two histidine, three tyrosine, one free cysteine residue, and a N-terminal amino group, which are all deprotonated at alkaline pH. Consequently, one obtains the following relationship:

$$\Delta v_{\text{prot}} = M^{-1}[15\Delta V_{\text{Lys}}(1 + 10^{\text{p}K_{\text{aLys}}-\text{pH}})^{-1}$$

$$+ 3\Delta V_{\text{Arg}}(1 + 10^{\text{p}K_{\text{aArg}}-\text{pH}})^{-1}$$

$$+ 2\Delta V_{\text{His}}(1 + 10^{\text{p}K_{\text{aHis}}-\text{pH}})^{-1}$$

$$+ 3\Delta V_{\text{Tyr}}(1 + 10^{\text{p}K_{\text{aTyr}}-\text{pH}})^{-1} \quad (7)$$

$$+ \Delta V_{\text{Cys}}(1 + 10^{\text{p}K_{\text{aCys}}-\text{pH}})^{-1}$$

$$+ \Delta V_{\text{Nt}}(1 + 10^{\text{p}K_{\text{aNt}}-\text{pH}})^{-1}]$$

where ΔV_{Lys}, ΔV_{Arg}, ΔV_{Tyr}, ΔV_{Cys} and ΔV_{Nt} are the average changes in volume accompanying deprotonation of lysine, arginine, cysteine, tyrosine, and the N-terminal amino group, respectively; $\text{p}K_{\text{aLys}}$, $\text{p}K_{\text{aArg}}$, $\text{p}K_{\text{aTyr}}$, $\text{p}K_{\text{aCys}}$ and $\text{p}K_{\text{aNt}}$ are the average $\text{p}K_{\text{a}}$ values for lysine, arginine, cysteine, tyrosine, and N-terminal amino group, respectively. In our analysis, we used the values of $\text{p}K_{\text{aLys}}$, $\text{p}K_{\text{aArg}}$, $\text{p}K_{\text{aTyr}}$, $\text{p}K_{\text{aCys}}$ and $\text{p}K_{\text{aNt}}$ equal to 10.6 ± 0.4, 12.8 ± 0.5, 10.8 ± 1.5, 8.5 ± 0.5 and 7.9 ± 0.5, respectively.[52–55]

We approximate the values of ΔV_{Asp}, ΔV_{Glu}, and ΔV_{Ct} in equation (6) by 10.5 cm^3 mol^{-1}, the change in volume associated with protonation of the carboxyl terminus in triglycine at 25 °C ($-COO^{-}+H^{+} \leftrightharpoons -COOH$).[56] The value of ΔV_{His} for protonation of histidine in equation (6) can be approximated by -4.5 cm^3 mol^{-1}. This value can be calculated by subtracting 26.5 cm^3 mol^{-1}, the change in volume associated with deprotonation of the amino terminus in triglycine,[56] from 22.0 cm^3 mol^{-1}, the change in volume accompanying ionization of water ($H^{+} + OH^{-} \leftrightharpoons H_2O$).[36,57]

The values of ΔV_{Lys}, ΔV_{Arg}, ΔV_{His} and ΔV_{Nt} in equation (7) can be approximated by 26.5 cm^3 mol^{-1}, the change in volume associated with deprotonation of the amino terminus in triglycine ($-NH_3^{+} + OH^{-} \leftrightharpoons -NH_2 + H_2O$).[56] The value of ΔV_{Tyr} in equation (7) can be approximated by 11.5 cm^3 mol^{-1} which can be calculated by subtracting 10.5 cm^3 mol^{-1}, the change in volume associated with protonation of the carboxyl terminus in triglycine, from 22.0 cm^3 mol^{-1}, the volume change accompanying ionization of water.[58] The value of ΔV_{Cys} is difficult to estimate directly in the absence of experimental data. In our analysis, we set the value of ΔV_{Cys} equal to ΔV_{Tyr}. While tyrosine is likely a poor quantitative approximation for cysteine, any error caused by this approximation in equation (7) should be negligible, since there is only a single free cysteine residue in β-lactoglobulin.

We have used the pH-dependences of the protonation term, Δv_{prot}, analytically described by equations (6) and (7) to calculate the structural con-
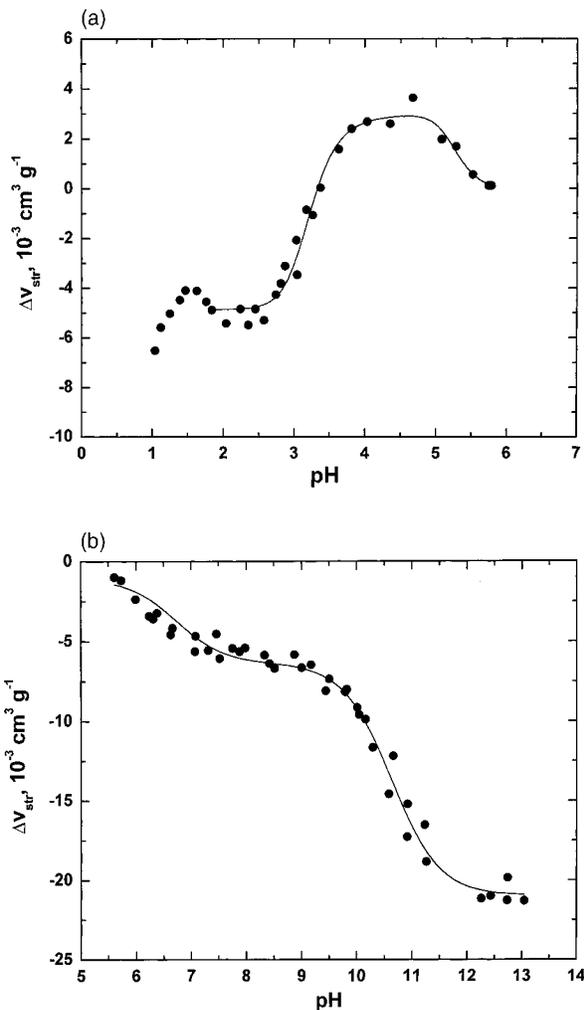

Figure 8. pH dependence for β-lactoglobulin of the structural change in volume, Δv_{str}: (a) acidic range; (b) alkaline range. The fitting of experimental data (continuous lines) was performed using equation (1) as explained in the text.

tribution, Δv_{str}, in equation (4) as a function of pH. Results of these calculations are graphically presented in Figure 8(a) (acidic range) and (b) (alkaline range). The pH-dependences of Δv_{str} shown in Figure 8(a) and (b) have been fitted using equation (1) and the structural changes in volume, $\Delta v_{\text{str},i}$, associated with each transition of β-lactoglobulin that has been calculated. The resulting data on $\Delta v_{\text{str},i}$ are presented in Table 1 (fifth row).

Partial specific adiabatic compressibility

The pH-induced change in the partial specific adiabatic compressibility, k_{S}^{0}, of a protein can be presented as a sum of three terms:[33,34]

$$\Delta k_{\text{S}}(\text{pH}) = \Delta k_{\text{str}} + \Delta k_{\text{prot}} + \Delta k_{\text{rel}} \quad (8)$$

where Δk_{str} is the structural change in compressibility, that is the change in compressibility associ-

ated with the pH-induced alteration in protein structure/conformation; Δk_{prot} is the protonation compressibility, that is the compressibility change due to protonation/deprotonation of titrable groups; and Δk_{rel} is the relaxation compressibility, that is the compressibility change due to ultrasonic relaxation.[59]

The term Δk_{rel} in equation (8) mainly originates from proton transfer reactions associated with neutralization of titrable groups and has been shown to be relatively small as compared with the Δk_{str} and Δk_{prot} terms.[34] Consequently, as a first approximation, the relaxation term, Δk_{rel}, can be neglected. The protonation term, Δk_{prot}, can be described analytically by a function similar to that given by equation (5):[33–35]

$$\Delta k_{prot} = M^{-1} \sum_j \Delta K_j (1 + 10^{\alpha(pH - pK_{aj})})^{-1} \quad (9)$$

where ΔK_j is the molar change in adiabatic compressibility accompanying protonation/deprotonation of the jth group.

For protonation of titrable groups of β-lactoglobulin at acidic pH, equation (9) can be rewritten as follows:

$$\begin{aligned} \Delta k_{sprot} = M^{-1}[&10\Delta K_{Asp}(1 + 10^{pH - pK_{aAsp}})^{-1} \\ &+ 16\Delta K_{Glu}(1 + 10^{pH - pK_{aGlu}})^{-1} \\ &+ 2\Delta K_{His}(1 + 10^{pH - pK_{aHis}})^{-1} \\ &+ \Delta K_{Ct}(1 + 10^{pH - pK_{aCt}})^{-1}] \end{aligned} \quad (10)$$

where ΔK_{Asp}, ΔK_{Glu}, ΔK_{His}, and ΔK_{Ct} are the average changes in adiabatic compressibility accompanying complete protonation of aspartic acid, glutamic acid, histidine, and the C-terminal carboxyl group, respectively.

For deprotonation of titrable groups of β-lactoglobulin at alkaline pH, equation (9) can be rewritten as follows:

$$\begin{aligned} \Delta k_{sprot} = M^{-1}[&15\Delta K_{Lys}(1 + 10^{pK_{aLys} - pH})^{-1} \\ &+ 3\Delta K_{Arg}(1 + 10^{pK_{aArg} - pH})^{-1} \\ &+ 2\Delta K_{His}(1 + 10^{pK_{aHis} - pH})^{-1} \\ &+ 3\Delta K_{Tyr}(1 + 10^{pK_{aTyr} - pH})^{-1} \\ &+ \Delta K_{Cys}(1 + 10^{pK_{aCys} - pH})^{-1} \\ &+ \Delta K_{Nt}(1 + 10^{pK_{aNt} - pH})^{-1}] \end{aligned} \quad (11)$$

where ΔK_{Lys}, ΔK_{Arg}, ΔK_{Tyr}, ΔK_{Cys} and ΔK_{Nt} are the average changes in adiabatic compressibility accompanying deprotonation of lysine, arginine, tyrosine, cysteine, and the N-terminal amino group, respectively.

We approximate the values of ΔK_{Asp}, ΔK_{Glu}, and ΔK_{Ct} in equation (10) by 18.4×10^{-4} cm^3 mol^{-1}

bar^{-1}, the change in compressibility associated with protonation of the carboxyl terminus in triglycine at 25 °C (-COO$^-$ + H$^+$ ⇌ -COOH).[56] The value of ΔK_{His} for protonation of histidine in equation (10) can be approximated by -18.5×10^{-4} cm^3 mol^{-1} bar^{-1} that can be calculated by subtracting 67.5×10^{-4} cm^3 mol^{-1} bar^{-1}, the change in compressibility associated with deprotonation of the amino terminus in triglycine,[56] from 51.3×10^{-4} cm^3 mol^{-1} bar^{-1}, the compressibility change accompanying ionization of water (H$^+$ + OH$^-$ ⇌ H$_2$O).[36,57] The value of ΔK_{Lys}, ΔK_{Arg}, ΔK_{His}, and ΔK_{Nt} in equation (11) can be approximated by 67.5×10^{-4} cm^3 mol^{-1} bar^{-1}, the change in compressibility associated with deprotonation of the amino terminus in triglycine (-NH3$^+$ + OH$^-$ ⇌ -NH$_2$ + H$_2$O).[56] The value of ΔK_{Tyr} in equation (11) can be approximated by 32.9×10^{-4} cm^3 mol^{-1} bar^{-1} that can be calculated by subtracting 18.4×10^{-4} cm^3 mol^{-1} bar^{-1}, the compressibility change associated with protonation of the carboxyl terminus in triglycine, from 51.3×10^{-4} cm^3 mol^{-1} bar^{-1}, the compressibility change accompanying ionization of water.[58] Analogous to volume, we treat the values of ΔK_{Cys} and ΔK_{Tyr} as equal.

We now use equations (10) and (11) in conjunction with equation (8) to calculate the structural term, $\Delta k_{str,i}$, in equation (8). Figure 9(a) and (b) present the pH-dependences of Δk_{str} at acidic and alkaline pH, respectively. We have fitted these pH-dependences using equation (1). Table 1 presents the resulting changes in adiabatic compressibility, $\Delta k_{str,i}$ (sixth row), for each of the pH-induced transitions of β-lactoglobulin.

Intrinsic and hydration contributions to the structural change in volume and compressibility

The structural change in volume, Δv_{str}, associated with each of the five pH-induced transitions of β-lactoglobulin can be presented as a sum of two contributions:

$$\Delta v_{str} = \Delta v_M + \Delta\Delta v_h \quad (12)$$

where Δv_M is the change in the intrinsic volume of the protein; and $\Delta\Delta v_h$ is the change in the hydration contribution to volume.

Analogously, the structural change in compressibility, Δk_{str}, can be presented as the sum of the intrinsic, Δk_M, and hydration, $\Delta\Delta k_{Sh}$, contributions:

$$\Delta k_{str} = \Delta k_M + \Delta\Delta k_h \quad (13)$$

The value of Δv_M for each transition can be calculated if the X-ray crystallographic structures of the protein are known "before" and "after" the transition. The intrinsic compressibility, k_M, of globular protein is equal to $\beta_M v_M$, where $\beta_M = 25 \times 10^{-6}$ bar^{-1} is the average coefficient of
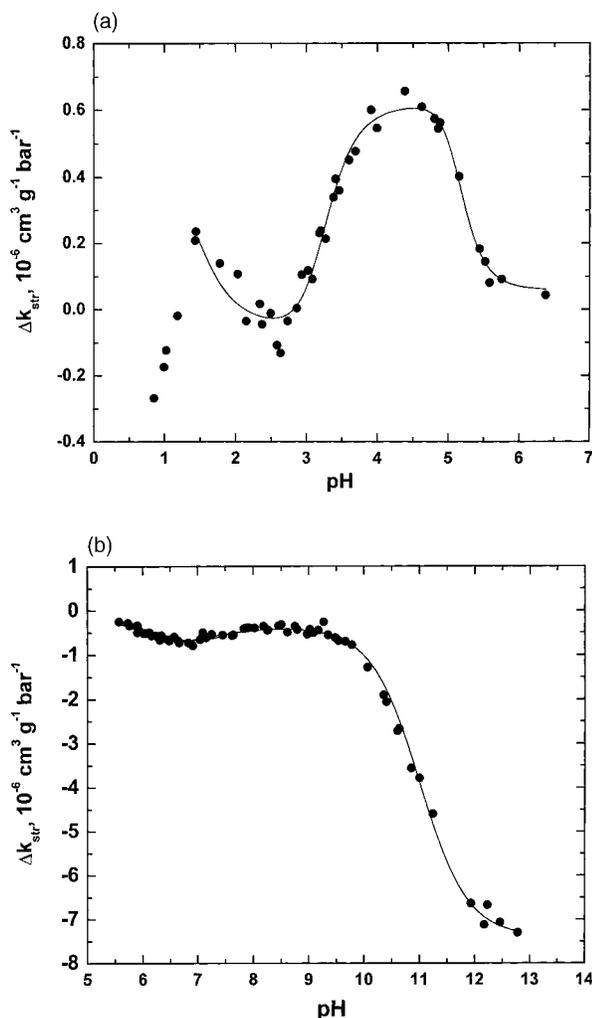
Figure 9. pH dependence for β-lactoglobulin of the structural change in compressibility, $\bar{k}_{str}$: (a) acidic range; (b) alkaline range. The fitting of experimental data (continuous lines) was performed using equation (1) as explained in the text.

adiabatic compressibility of the protein interior.[46] Consequently, as a first approximation, the value of Δk_M is related to Δv_M through $\Delta k_M = \beta_M \Delta v_M$. In this scenario, it is assumed that the protein transition, while causing a change in the intrinsic volume, v_M, does not result in any appreciable change in β_M. This simplification becomes invalid if the transition brings about an alteration in the interior packing of the protein, an event which is likely to cause a change in β_M. In this case, the change in β_M should be taken into account when calculating the change in the intrinsic compressibility of the protein: $\Delta k_M = \beta_M \Delta v_M + \Delta \beta_M v_M$.

Identification of transitions

One aggregate result of our spectroscopic and volumetric data is that β-lactoglobulin undergoes five pH-induced transitions that can be identified between pH 1 and pH 13. The first transition starts at pH 2 and is not completed even at pH 1, our lowest experimental pH. The beginning of this transition has been detected by our far and near UV CD data (Figures 2(a) and 4(a)), fluorescence anisotropy data (Figure 5(a)), as well as volume (Figure 8(a)) and compressibility (Figure 9(a)) data. The second transition occurs between pH 2.5 and pH 4 as suggested by all of our spectroscopic (Figures 2(a), 4(a), and 5(a)) and volumetric (Figures 8(a) and 9(a)) data. The third transition starts at pH 4.5 and is completed at around pH 6 as suggested by our far and near UV CD spectroscopic (Figures 2(a) and 4(a)) and volumetric (Figures 8(a) and 9(a)) results. Fluorescence anisotropy is not sensitive to this transition. The fourth transition, which has been detected by all of our spectroscopic (Figures 2(b), 4(b) and 5(b)) and volumetric (Figures 8(b) and 9(b)) observables, occurs between pH 6.5 and pH 8.5. Finally, the fifth transition occurs between pH 9 and pH 12.5. This transition has also been detected by all of our spectroscopic (Figures 2(b), 4(b) and 5(b)) and volumetric (Figures 8(b) and 9(b)) observables.

The pH 2 transition

This transition occurs below pH 2 and has never been reported. As seen from Figure 1, β-lactoglobulin retains most of its secondary structure even at pH 0.92 (●). However, some changes in tertiary structure at this low pH are evident from the near UV CD spectra of the protein presented in Figure 3. Inspection of Figures 8(a) and 9(a) reveals that both the volume and compressibility of the protein increase between pH 2 and pH 1.5 and then decrease sharply below pH 1.5. At ~pH 1, the transition is far from complete.

It is difficult to speculate about the origins of this transition, since, at pH 2, all ionizable groups should have already been protonated. Perhaps, the protein undergoes some electrostatically driven structural changes in response to an increased concentration of Cl^- anions. Further salt-dependent studies are required to prove or refute this possibility.

The dimer-to-monomer transition

This transition, which occurs between pH 2.5 and pH 4, has been previously identified as a dimer-to-monomer transition.[60,61] Our far UV CD spectroscopic data (Figure 1) suggest that the dimer-to-monomer transition of β-lactoglobulin is accompanied by a slight increase in its secondary structure (the far UV CD spectrum of the protein at pH 2.30 (○) is slightly more negative compared with that at pH 5.00 (■)). In addition, our near UV CD data (Figure 3) suggest that the transition causes a change in tertiary structure of the protein as well. Thus, we propose that while the general conformation of β-lactoglobulin is retained, some changes in secondary and tertiary structures

accompany the dimer-to-monomer transition. This conclusion is consistent with the structural results of Fogolari et al.,[11] Uhrínová et al.,[4] and Kuwata et al.[2] These authors compared their NMR data on β-lactoglobulin obtained between pH 2 and pH 2.7 with the X-ray crystallographic results of Qin et al.[9] obtained at ~pH 6 and found a number of structural differences, particularly, in the main α-helix (residues 129 to 142). Specifically, at low pH, the main α-helix adopts a slightly different orientation with respect to the protein calyx.[4] Fogolari et al.[11] have shown that these structural differences modify the surface electrostatic properties of β-lactoglobulin. The importance of electrostatic interactions in the dimerization of β-lactoglobulin was first emphasized by Townend et al.[60]

Our combined fit of the profiles of the dimer-to-monomer transition of β-lactoglobulin, as monitored by the CD spectroscopic (Figures 1 and 3), fluorescence anisotropy (Figure 5(a)), and volumetric (Figures 8(a) and 9(a)) observables using equation (1) reveals a dissociation constant of $0.04(\pm0.02)$ M. We have also determined that the transition is accompanied by protonation of 3 ± 1 ionizable groups with average pK_{aeff} value of 3.6 ± 0.6.

It is reasonable to expect that dissociation of a dimeric protein should bring about a significant increase in solvent accessible surface area, S_A. To estimate the change in S_A associated with the dimer-to-monomer transition of β-lactoglobulin, we have performed solvent accessible surface area calculations based on the X-ray crystallographic structure of the dimer at pH 6.5 presented by Brownlow et al.[10] (RCSB PDB entry 1beb). Our calculations reveal that the solvent accessible surface area, S_A, of the dimer is 14,723 Å^2. Assuming that the global structure of β-lactoglobulin is preserved, the value of S_A for the monomer can be calculated from the X-ray crystallographic structure of the dimer. In this approach, the structure of each monomer can be generated by eliminating the other monomer from the structure of the dimer. Using this approach, one can generate two monomeric structures of β-lactoglobulin, for which we calculate the values of S_A equal to 7934 Å^2 and 7812 Å^2. Thus, we estimate that the dimer-to-monomer transition of β-lactoglobulin results in an increase in S_A of 1023 Å^2 per dimer $(14,723 - (7934 + 7812))$. This is a quite substantial increase which amounts to $\sim 7\%$ of the original value of S_A.

Such a large increase in S_A should bring about an enhancement of protein hydration which, in turn, should be reflected in the values of Δv_{str} and Δk_{str}. Inspection of Table 1 (second column) reveals that, for the dimer-to-monomer transition, Δv_{str} and Δk_{str} are equal to $-0.008(\pm0.003)$ cm^3 g^{-1} and $-(0.7(\pm0.4)) \times 10^{-6}$ cm^3 g^{-1} bar^{-1}, respectively. Recall that Δv_{str} and Δk_{str} contain the intrinsic and hydration contributions (see equations (12) and (13)). In our analysis below, we assume that the intrinsic terms Δv_M and Δk_M in equations (12) and

(13) are small (close to 0) and, therefore, can be neglected. This assumption is reasonable in view of only very modest structural changes of the protein upon its dimer-to-monomer transition. With this assumption, our measured values of Δv_{str} and Δk_{str} solely reflect the transition-induced changes in the hydration contributions to volume, $\Delta\Delta v_h$, and compressibility, $\Delta\Delta k_h$. Thus, the values of $\Delta\Delta v_h$ and $\Delta\Delta k_h$ are equal to -0.008 cm^3 g^{-1} and -0.7×10^{-6} cm^3 g^{-1} bar^{-1}, respectively. The negative sign of Δv_{str} associated with the dimer-to-monomer transition of β-lactoglobulin is in qualitative agreement with dissociation volumes of dimeric and tetrameric proteins previously determined from pressure-related studies.[62,63]

For interpreting $\Delta\Delta v_h$ in terms of hydration, it is instructive to recall that the partial specific volume, $v°$, of a protein represents a sum of three terms:[64]

$$v° = v_M + v_T + v_I \qquad (14)$$

where v_T is the thermal volume; and v_I is the interaction volume which represents a decrease in the solvent volume under the influence of solute-solvent interactions.

The thermal volume, v_T, originates from thermally activated mutual vibrational motions of solute and solvent molecules and, as a first approximation, is proportional to the solvent accessible surface area of a protein, S_A: $v_T \approx N_A S_A \delta/M$, where N_A is Avogadro's number, δ is the thickness of the thermal volume, and M is the molecular weight of the protein. For globular proteins, the thickness of the thermal volume, δ, has been estimated to be ~ 1 Å.[46] Differentiating equation (14), one obtains the following relationship for the structural change in volume, Δv_{str}, associated with a protein transition:

$$\Delta v_{str} = \Delta v_M + \Delta v_T + \Delta v_I \qquad (15)$$

Comparison of equation (15) and (12) reveals that:

$$\Delta\Delta v_h = \Delta v_T + \Delta v_I \qquad (16)$$

Recall that our estimate of ΔS_A, the change in S_A associated with the dimer-to-monomer transition of β-lactoglobulin, is 1023 Å^2. Using this value, we calculate Δv_T of 0.017 cm^3 g^{-1} ($\Delta v_T = N_A \Delta S_A \delta/M$). Hence, the value of Δv_I, as calculated from equation (16) is equal to -0.025 cm^3 g^{-1} ($-0.008 - 0.017$). The negative sign of Δv_I is consistent with a net enhancement of the protein hydration upon dissociation. To estimate the relative magnitude of Δv_I, we compare it with the overall value of v_I of the dimer which can be calculated from equation (14). The intrinsic volume, v_M, of the dimer is 44,151 Å^3 (0.724 cm^3 g^{-1}) as can be determined from the structural data of Brownlow et al.[10] The thermal volume, v_T, of the dimer is equal to 0.242 cm^3 g^{-1} ($N_A S_A \delta/M$). Armed with the values of v_M and v_T, we now use equation (14) to calculate the interaction volume, v_I, of dimeric

β-lactoglobulin: $v_I = v° - v_M - v_T = -0.216$ cm^3 g^{-1} (0.750 − 0.724 − 0.242).

If one assumes that the change in hydration of β-lactoglobulin associated with its dissociation into monomers is proportional to an increase in solvent accessible surface area, then a 7% increase in the absolute value of v_I should be expected: $\Delta v_I \approx -0.015$ cm^3 g^{-1} (−0.216 × 0.07). Taking into account all the assumptions and approximations of our analysis, the expected value of Δv_I (−0.015 cm^3 g^{-1}) is in reasonable agreement with the observed value of −0.025 cm^3 g^{-1}. Judging by this qualitative agreement, our volume data are suggestive of the changes in protein hydration being mostly localized in the area of contact between the two monomers. As explained below, our measured change in the hydration contribution to compressibility, $\Delta\Delta k_h$, is also close to the value expected on the basis of a 7% increase in S_A.

Recall that for dimeric β-lactoglobulin at neutral pH, the partial specific adiabatic compressibility, $k°_S$, equals $(4.6(\pm 0.5)) \times 10^{-6}$ cm^3 g^{-1} bar^{-1}. This value is the sum of the intrinsic, $k_M = \beta_M v_M$, and hydration, Δk_h, contributions.[30,31,45,46,65] The value of k_M of β-lactoglobulin is equal to 18.1×10^{-6} cm^3 g^{-1} bar^{-1} (0.724 × 25 × 10^{-6}). Consequently, the hydration contribution, Δk_h equals -13.5×10^{-6} cm^3 g^{-1} bar^{-1} (4.6 × 10^{-6} − 18.1 × 10^{-6}). Note that 7% of this value amounts to -1×10^{-6} cm^3 g^{-1} bar^{-1} (−13.5 × 10^{-6} × 0.07), which is in good agreement with our measured value of -0.7×10^{-6} cm^3 g^{-1} bar^{-1}. Hence, based on our compressibility results, we conclude that the dimer-to-monomer transition of β-lactoglobulin is accompanied by an increase in hydration that could be anticipated based on a 7% increase in the solvent accessible surface area.

In the aggregate, our volume and compressibility data suggest that no unusual changes in the average protein hydration result from the acid-induced dissociation of dimeric β-lactoglobulin into monomers. Most probably, the hydration changes are localized in the area of contact between the two monomeric subunits and correlate with an increase in the solvent accessible surface area accompanying the protein dissociation.

The pH 5 transition

This transition, which has been reported by Timasheff et al.,[17] and Casal et al.,[16] occurs between pH 4.5 and pH 6 as detected by our near UV CD data (Figures 3 and 4(a)) and volumetric results (Figures 8(a) and 9(a)). As seen from Figure 3, a slight change in the near UV CD spectra of β-lactoglobulin accompanies acidification of the solution from pH 5.72 (□) to pH 4.59 (■). Specifically, at pH 4.59, the near UV CD spectrum of the protein is shifted to slightly more negative values compared to that at pH 5.72. This observation suggests that the pH 5 transition of β-lactoglobulin results in a slight change in its tertiary structure. By contrast, this transition does not cause any significant

alteration in secondary structure, even though a slight change in the far UV CD spectrum of β-lactoglobulin (practically, within experimental error) is evident from Figures 1 and 2(a). This observation is consistent with the FTIR spectral data of Casal et al.,[16] who observed only a very small increase in intensity of the protein bands at 1636 and 1681 cm^{-1}.

Based on optical rotatory dispersion and hydrodynamic data, Timasheff et al.[17] qualified this transition as a pH-induced conformational change from the native form (N) of β-lactoglobulin at pH 6 to the acidic form (Q) at ∼pH 4.5 (N-to-Q transition). These authors arrived at the conclusion that the N form, exhibiting the hydrodynamic radius of 25.8 Å, is more compact than the acidic Q form with the hydrodynamic radius of 27.2 Å. In addition, Timasheff et al.[17] proposed that the N state may be less ordered, have more solvent-exposed groups, and exhibit a reduced amount of β-structure.

Our volumetric data graphically presented in Figures 8(a) and 9(a) suggest that the N-to-Q transition results in increases in the values of Δv_{str} and Δk_{str}. Inspection of Table 1 (third column) reveals that Δv_{str} and Δk_{str} are equal to 0.004(±0.003) cm^3 g^{-1} and (0.7(±0.4)) × 10^{-6} cm^3 g^{-1} bar^{-1}, respectively. In the absence of reliable structural data on this transition, it is difficult to estimate the changes in the protein intrinsic volume, Δv_M, and compressibility, Δk_M. However, our near UV CD spectroscopic data and the hydrodynamic results of Timasheff et al.[17] suggest that some changes in the intrinsic volume and compressibility of β-lactoglobulin are likely to accompany its N-to-Q transition.

Given the difficulty with reliable estimates of Δv_M and Δk_M, we are currently unable to separate the hydration contributions $\Delta\Delta v_h$ and $\Delta\Delta k_h$ from our determined structural changes in volume, Δv_{str}, and compressibility, Δk_{str}, associated with the N-to-Q transition of β-lactoglobulin. Consequently, it is impossible to judge if the transition causes any significant changes in protein hydration as suggested by Timasheff et al.[17] For the moment, our measured values of Δv_{str} and Δk_{str} should be viewed as a signature of the transition and a basis for future investigations.

It should be noted that, around pH 4.5, β-lactoglobulin is prone to form octameric structures.[15] In particular, Timasheff et al.[17] proposed that the N-to-Q transition facilitates octamer formation. In contrast to these reports, our fluorescence anisotropy and volumetric data do not exhibit any changes that can be ascribed to octamerization of the protein at ∼pH 4.5. Note that these characteristics (fluorescence anisotropy and volumetric data) are known to be exquisitely sensitive to protein association/dissociation equilibria. Hence, we conclude that, under the experimental conditions of this study, no significant octamerization of the protein occurs around ∼pH 4.5. This conclusion is consistent with the results of McKenzie & Sawyer,[12] who found that, around room temperature, the

population of octamers is small for β-lactoglobulin A and negligible for β-lactoglobulin B. In addition, the NMR data of Pessen *et al.*[14] suggest that, even though octamerization of β-lactoglobulin A takes place around pH 4.5, the dimer remains the persisting unit.

Our combined fit of the N-to-Q transition profiles of β-lactoglobulin as monitored by the near UV CD, volume, and compressibility data (Figures 2(a), 8(a) and 9(a)) using equation (1) reveals an equilibrium constant, K°, of 0.18 ± 0.04. The transition involves protonation of $2(\pm 1)$ ionizable groups with an effective pK_a value of 5.5 ± 1. These numbers are in reasonable agreement with the previous estimates of Timasheff *et al.*[17]

The Tanford transition

A pH-induced transition of β-lactoglobulin, which occurs between pH 6 and pH 8, was first described 40 years ago by Tanford and collaborators.[18,66] Since then, the transition is commonly referred to as the Tanford transition. As mentioned above, the Tanford transition of β-lactoglobulin has been detected by all of the spectroscopic and volumetric observables employed in this work. Our combined fit of the transition profiles as monitored by the far and near UV CD (Figures 2(b) and 4(b)), fluorescence anisotropy (Figure 5(b)), volume (Figure 8(b)), and adiabatic compressibility (Figure 9(b)) using equation (1) reveals an equilibrium constant, K°, of 0.15 ± 0.05, an effective pK_{aeff} of 6.4 ± 0.5, the differential number of bound protons, Δv, of 1 ± 0.4. These estimates are in good agreement with similar results previously reported by Tanford *et al.*[18]

The main structural change of the protein is related to the displacement of the loop EF which opens the interior of the calyx above pH 7.5.[9] As a consequence, the carboxyl group of Glu89, previously buried and, therefore, exhibiting an anomalously high pK_a value, becomes accessible to the solvent. Protonation of this residue is thought to trigger the Tanford transition.[9] Out data presented in Figures 1 and 3 reveal that the Tanford transition is accompanied by alterations in secondary and tertiary structures of β-lactoglobulin, with these alterations being rather local and causing no global change in protein conformation.

Inspection of Figure 5(b) reveals that the Tanford transition of β-lactoglobulin causes a decrease in fluorescence anisotropy which is, most likely, related to the transition-induced change in local microenvironment of aromatic residues and their increased rotational motions.[67] In general, the reduced anisotropy of the protein is consistent with transition-induced loosening of the interior packing with concomitant increase in the magnitude of rotational motions of aromatic fluorophores. As discussed below, we arrive at the same conclusion based on our compressibility data.

The data presented in Table 1 (fourth column) reveal that the structural changes in volume, Δv_{str},

and compressibility, Δk_{str}, associated with the Tanford transition are equal to $-0.006(\pm 0.003)$ cm^3 g^{-1} and $(1.5(\pm 0.5)) \times 10^{-6}$ cm^3 g^{-1} bar^{-1}, respectively. Using the X-ray crystallographic data on the protein reported by Qin *et al.*[9] ''before'' (pH 6.2) and ''after'' (pH 8.2) the Tanford transition, we have calculated the changes in the solvent accessible surface area, S_A, and intrinsic volume, v_M, of the protein accompanying the transition. Our calculations reveal that the Tanford transition results in an increase in S_A from 8240 Å^2 (per monomer) at pH 6.2 to 8670 Å^2 (per monomer) at pH 8.2. Hence, the change in S_A equals 430 Å^3 (per monomer) which is $\sim 5\%$ of the solvent accessible area of the protein. The intrinsic volume, v_M, of β-lactoglobulin increases from 22,530 Å^3 (0.737 cm^3 g^{-1}) at pH 6.2 to 22,684 Å^3 (0.742 cm^3 g^{-1}) at pH 8.2. Based on a 5% increase in S_A, some enhancement of protein hydration is to be expected to accompany the Tanford transition. This change in hydration should be reflected in the hydration components of the protein volume, $\Delta\Delta v_h$, and compressibility, $\Delta\Delta k_h$ (see equations (12) and (13)).

To calculate the values of $\Delta\Delta v_h$ and $\Delta\Delta k_h$ from equations (12) and (13), one needs to estimate the values of Δv_M and Δk_M. Based on the foregoing discussion, the change in the intrinsic volume, Δv_M, is equal to 0.005 cm^3 g^{-1} (0.742 −0.737). Using equation (12), we calculate $\Delta\Delta v_h$ of -0.011 cm^3 g^{-1} ($-0.006 - 0.005$). This value represents the sum of the changes in the thermal volume, Δv_T, and interaction volume, Δv_I (see equation (16)) The value of Δv_T can be estimated by multiplying the change in the solvent accessible surface area, ΔS_A, of β-lactoglobulin associated with the Tanford transition by 1 Å, the thickness of the thermal volume. We calculate the value of Δv_T to be 0.014 cm^3 g^{-1} ($N_A\Delta S_A\delta/M$). The change in the interaction volume, Δv_I, can be evaluated from equation (16) to be -0.025 cm^3 g^{-1} ($-0.011 - 0.014$). This value roughly equals 10% of the interaction volume, v_I, of β-lactoglobulin (-0.216 cm^3 g^{-1}) and correlates with a 5% increase in S_A. This correlation is rather satisfactory in view of the numerous assumptions and approximations used in our analysis. Thus, judging by the solvent contraction in the vicinity of the protein molecule, the Tanford transition results in an overall increase in protein hydration, with this increase correlating with the transition-induced change in the solvent accessible surface area, ΔS_A, of β-lactoglobulin.

Based on the foregoing discussion, one may reasonably expect that the change in the hydration contribution to compressibility, $\Delta\Delta k_h$, accompanying the Tanford transition should be negative and correlate with a 5% increase of S_A. As a first approximation, $\Delta\Delta k_h$ should be roughly equal to 5% of the total value of Δk_h of β-lactoglobulin: $\Delta\Delta k_h = 0.05 \quad \Delta k_h = -0.7 \times 10^{-6}$ cm^3 g^{-1} bar^{-1} ($-13.5 \times 10^{-6} \times 0.05$). In contrast to this expectation, our determined structural change in compressibility, Δk_{str}, is positive and equal to $(1.5(\pm 0.5)) \times 10^{-6}$ cm^3 g^{-1} bar^{-1}. Based on

equation (13), the observed disparity can be explained by proposing that the Tanford transition results in a significant increase in the intrinsic compressibility, k_M, of β-lactoglobulin: $\Delta k_M = \Delta k_{str} - \Delta\Delta k_h = 2.2 \times 10^{-6}$ cm^3 g^{-1} bar^{-1} $(1.5 \times 10^{-6} + 0.7 \times 10^{-6})$. This increase, which constitutes 12 % of the intrinsic compressibility, k_M, of β-lactoglobulin (18.1×10^{-6} cm^3 g^{-1} bar^{-1}), cannot be accounted for by a 0.005 cm^3 g^{-1} ($\sim$0.7 %) increase in the intrinsic volume, v_M. Recall that a change in the intrinsic compressibility, Δk_M, of a globular protein is equal to $\beta_M \Delta v_M + \Delta\beta_M v_M$. Consequently, a 12 % increase in k_M suggests a 12 % increase in β_M which amounts to 3×10^{-6} bar^{-1} $(25 \times 10^{-6} \times 0.12)$. Judging by this result, we propose that the Tanford transition of β-lactoglobulin is accompanied by a loosening of its intrinsic packing which is reflected in a 12 % increase in the intrinsic compressibility. Furthermore, this quantitative estimate is consistent with the observed decrease in fluorescence anisotropy, which is also suggestive of a loosening of the packing of the protein interior.

In the aggregate, our combined volume and compressibility data suggest that the Tanford transition of β-lactoglobulin is accompanied by a 5 to 10 % increase in hydration and a loosening of its interior packing as reflected in a 12 % increase in the intrinsic compressibility. The latter result is supported by our fluorescence anisotropy data.

The base-induced denaturation

This transition, which occurs between pH 9 and pH 13, has been previously identified as the base-induced denaturation of β-lactoglobulin.[16,18,20] It should be noted that the transition also results in final disruption of any remaining native-like dimeric structures into unfolded monomers. The near UV CD spectra of the protein (Figure 3) suggest that the base-induced denatured state of β-lactoglobulin at pH 12.51 (◆) lacks rigid tertiary structure present in its native state (below pH 9). On the other hand, the far UV CD spectra of the protein (Figure 1) suggest that the base-induced denaturation of β-lactoglobulin proceeds with a significant decrease in secondary structure. However, the base-induced denatured state of β-lactoglobulin does not lose all of its secondary structure. Using the far UV CD spectrum of β-lactoglobulin at pH 12.35 (◆), we calculate that $\sim$20 % of β-sheets and $\sim$10 % of α-helices are preserved in the base-induced denatured state. This observation is in agreement with the FTIR spectroscopic results of Casal *et al.*[16] who have proposed that the protein retains some of its secondary structure even at highly alkaline pH.

Inspection of the data in Table 1 (fifth column) reveals that the values of Δv_{str} and Δk_{str} associated with the transition are equal to $-0.014(\pm 0.003)$ cm^3 g^{-1} and $-(7.0(\pm 0.5)) \times 10^{-6}$ cm^3 g^{-1} bar^{-1}, respectively. The absolute value of Δv_{str} (-0.014 cm^3 g^{-1}) is small. It constitutes only 1.8 % of the partial

specific volume, v°, of β-lactoglobulin in its native state $(0.750(\pm 0.003)$ cm^3 g$^{-1})$. This observation is in accord with other reports of transition volumes for protein denaturation events.[64] As previously discussed, near zero values of Δv_{str} originate from compensation between transition-induced changes in the intrinsic volume, Δv_M, thermal volume, Δv_T, and interaction volume, Δv_I.[64] Because of these compensatory effects, decomposition of the transition volume, Δv_{str}, of the base-induced denaturation of β-lactoglobulin in terms of intrinsic and hydration contributions is not a simple matter.

The structural change in compressibility, Δk_{str}, of $-(7.0(\pm 0.5)) \times 10^{-6}$ cm^3 g^{-1} bar^{-1} associated with the base-induced denaturation of β-lactoglobulin is typical for native-to-partially unfolded transitions of globular proteins.[65] Recall that native-to-partially unfolded transitions of globular proteins are accompanied by a moderate decrease in compressibility ranging from -3×10^{-6} to -7×10^{-6} cm^3 g^{-1} bar^{-1}. This range of Δk_{str} values reflects a decrease in the size of solvent-inaccessible core and an increase in hydration associated with native-to-partially unfolded transitions of globular proteins. However, our measured value of Δk_{str} $(-(7.0(\pm 0.5)) \times 10^{-6}$ cm^3 g^{-1} bar$^{-1})$ is significantly smaller in magnitude than -20×10^{-6} cm^3 g^{-1} bar^{-1}, the value of Δk_{str} expected for a transition from the native to fully unfolded, random coil-like state of a globular protein.[65] The observed disparity suggests that the base-induced denatured state of β-lactoglobulin is not random coil-like and retains some water-inaccessible core of amino acid residues. This conclusion is in agreement with our far UV CD data and FTIR spectroscopic results of Casal *et al.*[16]

To evaluate the size of the retained solvent-inaccessible core of the base-induced denatured state of β-lactoglobulin, we employed the assumptions and analysis previously described for characterizing base, acid, and heat-induced denaturation of other globular proteins.[33,35,36] Using this analysis and the value of Δk_{str} of $-(7.0(\pm 0.5)) \times 10^{-6}$ cm^3 g^{-1} bar^{-1}, we estimate that roughly 80 % of the total surface of β-lactoglobulin becomes exposed to the solvent in its base-induced denatured state. Hence, about 20 % of amino acid residues ($\sim$30) of β-lactoglobulin are still buried from the solvent in the base-induced denatured state. This result is numerically similar to our previous estimates of the size of solvent-inaccessible cores of other globular proteins in their partially unfolded conformational states[33,35,36] and consistent with theoretical predictions of Lee.[68]

Our combined fit of the base-induced denaturation profiles of β-lactoglobulin as monitored by the far and near UV CD (Figures 2(b) and 4(b)), fluorescence anisotropy (Figure 5(b)), volume (Figure 8(b)), and compressibility (Figure 9(b)) data reveals that the transition involves deprotonation of a single residue ($\Delta v = 1 \pm 0.4$) with pK_{aeff} of 8.7 ± 0.5. The apparent equilibrium constant, K°, of the transition is 0.005 ± 0.002 (see the fifth column

in Table 1). However, these results should be viewed with caution, since they may be misleading. The base-induced denaturation of β-lactoglobulin is irreversible. Consequently, interpretation of its transition profiles in terms of equilibrium thermodynamics parameters may yield erroneous results. For the moment, these results should be viewed merely as a basis for future investigations.

Materials and Methods

Materials

The mixture of variants A and B of β-lactoglobulin was purchased from Sigma-Aldrich Canada (Mississauga, Ontario, Canada) and used without further purification. NaCl, HCl, and NaOH were purchased from EM Science (Gibbstown, NJ, USA), BDH Inc. (Toronto, Ontario, Canada), and Fisher Scientific Canada (Mississauga, Ontario, Canada), respectively. β-Lactoglobulin was dissolved and dialyzed against unbuffered 10 mM NaCl in aqueous solution. All solutions were prepared using doubly distilled water. The protein concentration was determined spectrophotometrically using the extinction coefficient $\varepsilon_{278} = 0.960 \, \text{l g}^{-1} \, \text{cm}^{-1}$.[69]

All of our acoustic, densimetric, and optical spectroscopic pH-titration experiments were performed using Hamilton syringes equipped with Chaney adaptors (Fisher Scientific Canada, Mississauga, Ontario, Canada) as described.[33,36] NaOH and HCl were used for adjusting the solution pH. The pH values for protein solutions were measured separately using a VWR brand Benchtop model 8015 pH meter and an Accumet Ag/AgCl combination microelectrode (VWR Canada, Mississauga, Ontario, Canada). The absolute error of our pH-measurements was ± 0.01 unit.

Optical spectroscopy

Optical absorbance spectra of the protein were measured at 25 °C using an AVIV model 14 DS spectrophotometer (Aviv Associates, Lakewood, NJ. USA). CD spectra were recorded at 25 °C using an AVIV model 62A DS spectropolarimeter (Aviv Associates, Lakewood, NJ. USA). The far UV CD spectroscopic measurements were carried out in a 1 mm path-length cuvette, while, for the near UV CD spectroscopic measurements, a 10 mm path-length cuvette was employed. For all far UV CD spectroscopic measurements, the protein concentration was on the order of ~0.3 g l⁻¹, while, for all near UV CD spectroscopic measurements, the protein concentration was ~1.5 g l⁻¹. The secondary structure contents of β-lactoglobulin were calculated from its far UV CD spectra using the CONTIN software package.[43]

Fluorescence anisotropy measurements were performed in a 10 mm path-length cuvette at 25 °C using an Aviv model ATF 105 spectrofluorometer (Aviv Associates, Lakewood, NJ, USA). The protein samples were excited at 295 nm with the excitation polarizer oriented in the vertical position, and vertical and horizontal components of the polarized emission light were recorded through a monochromator at 340 nm. The anisotropy was calculated using the standard relationship:[67]

$$r = \frac{I_{VV} - GI_{VH}}{I_{VV} + 2GI_{VH}} \tag{17}$$

where I_{VV} and I_{VH} are the emission intensities when the

excitation light is vertically polarized and the emitted light is polarized vertically and horizontally, respectively; G is the ratio of the sensitivities of the detection system for vertically and horizontally polarized light. The value of G was determined for each experimental point separately. For all fluorescence anisotropy measurements, protein concentration was about ~1 g l⁻¹.

Densimetry

All densities were measured at 25 °C with a precision of $\pm 1.5 \times 10^{-6}$ g cm⁻³ using a vibrating tube densimeter (DMA-60/602, Anton Paar, Austria). The partial specific volume, $v°$, of β-lactoglobulin was calculated from density values using the relationship:[70]

$$v° = \frac{1}{\rho_o} - \frac{\rho - \rho_o}{\rho_o c} \tag{18}$$

where ρ and ρ_o are, the densities of the protein solution and the solvent, respectively; c is the specific protein concentration.

Densimetric acid and base titrations of β-lactoglobulin were performed in a specially designed vial connected *via* Tygon tubings to the densimeter as described.[36] For all density measurements, the protein concentration was between 1 and 2 g l⁻¹.

Ultrasonic velocimetry

Solution sound velocity measurements were carried out at 7.2 MHz by analyzing the amplitude-frequency characteristics of an ultrasonic resonator as described.[71–74] We employed an ultrasonic resonator cell with lithium niobate piezotransducers and a minimum sample volume of 0.8 ml.[72] For this type of acoustic resonators, the relative precision of sound velocity measurements at frequencies near 7 MHz is at least ± 0.15 cm s⁻¹.[75,76] The analysis of the frequency characteristics of the resonator was performed by a Hewlett Packard model HP4195A network/spectrum analyzer (Mississauga, Ontario, Canada).

The key characteristic of a solute directly derived from ultrasonic velocimetric measurements is the relative specific sound velocity increment, [u]:

$$[u] = \frac{U - U_o}{U_o c} \tag{19}$$

where U and U_o are the sound velocities in the protein solution and the neat solvent, respectively. Protein concentrations between 1 and 2 g l⁻¹ were used for these ultrasonic experiments.

Acoustic pH titration experiments were performed at 25 °C by adding to both the sample and the reference cells, each containing 0.9 ml of the protein solution and the solvent, respectively, an equal amount of salt solution (HCl or NaOH). When calculating [u], we took into account the changes in sound velocity in the solvent, U_o, and in the specific protein concentration, c, that result from the addition of the salt solution.

Determination of the partial specific adiabatic compressibility

Values of the relative specific sound velocity increment, [u], were used in conjunction with the measured

partial specific volume data, $v°$, to calculate the partial specific adiabatic compressibility, $k_S°$, of β-lactoglobulin using the following relationship:[77,78]

$$k_S° = \beta_{S_o}\left(2v° - 2[u] - \frac{1}{\rho_o}\right) \qquad (20)$$

where β_{S_o} is the coefficient of adiabatic compressibility of the solvent. The volumetric and ultrasonic velocimetric experiments have been performed at least three times with the average values of $[u]$ and $v°$ being used in equation (20).

Determination of intrinsic volume and solvent-accessible surface area

The atomic coordinate sets of β-lactoglobulin needed for calculating its intrinsic Voronoi volume and solvent-accessible surface area at several pH were obtained from the RCSB Protein Data Bank.[79] Specifically, the following PDB entries were used in our calculations: 3blg for pH 6.2,[9] 1beb for pH 6.5,[10] and 2blg for pH 8.2.[9] For calculating the protein Voronoi volume and solvent accessible area, we used the most recent version of the original Lee & Richards algorithm[68,80] developed by Gerstein and co-workers.[81–83]

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