Structural Features and Reversible Association of Different Quaternary Structures of β-Lactoglobulin

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Structural and functional features were studied on the native dimeric form of β-lactoglobulin at neutral pH and on the monomeric forms obtained either by raising the pH to 9.0 or by blocking the thiol group of Cys121 with iodoacetamide under bland dissociating conditions. The thiol blocked monomer did not reassociate to native-like dimers, it retained retinol-binding ability, and it was found to display many of the structural features of the monomer obtained at pH 9, but differed in several structural features from the native dimer. The supramolecular associative properties of the proteins were studied by measuring concentration dependence of the accessibility of the backbone exchangeable amide protons in ¹H NMR H/D exchange experiments, of their ligand-binding properties, and of their intrinsic fluorescence features. Evidence of reversible association was found for all the proteins with a very similar concentration dependence, indicating that the weak forces involved in this association were different from those stabilizing the native dimer.

The globular protein β-lactoglobulin (BLG) is found in the whey fraction of the milk of many mammals. Despite numerous physical and biochemical studies, its function is still not clearly understood (Hambling et al., 1992; Papiz et al., 1986). The crystalline structure of bovine BLG has been determined, showing a similarity with the plasma retinol-binding protein and with the odorant-binding protein, which all belong to the lipocalin superfamily (Pervaiz and Brew, 1985; Papiz et al., 1986; Monaco et al., 1987; Brownlow et al., 1997). This suggests that the role of BLG may be connected with transport or accumulation of lipid-soluble biological components, including retinol, fatty acids, and vitamin D (Robillard and Wishnia, 1972a,b; Wang et al., 1997).

Refolding of the tertiary structure of BLG from the chaotrope-denatured form has been investigated extensively at low pH, where the association of monomers into multimeric forms is negligible (Kuwajima et al., 1987; Ptitsyn and Semisotnov, 1991), and was framed in the now current "molten globule" hypothesis of intermediate formation in protein folding/unfolding (Ptitsyn, 1992). The remarkable stability of BLG at low pH has been explained by the strong stabilizing action of the two disulfide bonds present in its tertiary structure (Papiz et al., 1986; DeWit and Klarenbeek, 1984), which allows the preservation of several features of the secondary structure present at physiologic pH (Molinari et al., 1996).

The free, highly reactive thiol group of Cys121 in each monomer has been shown to be involved in intramolecular and intermolecular disulfide interchange with other thiol groups upon physical treatment of the protein at high temperature (Shimada and Cheftel, 1989; Griffin et al., 1993; Roefs and De Kruif, 1994) or at high hydrostatic pressures (Tanaka et al., 1996; Iametti et al., 1997; Funtenberger et al., 1997).

Despite the wealth of structural information available, little is known about the monomer/monomer interface in the freely associating BLG dimer, at least for what concerns its modification during the folding/ unfolding processes at neutral pH. Our group studied the thermal sensitivity of BLG at neutral pH, by focusing on the mechanism and the nature of structural protein modifications occurring in the initial steps of exposure to heat. These modifications were found to affect subsequent macroscopic changes, such as loss of solubility, and to result in the exposure of structural regions of BLG suitable for different kinds of interaction with other protein components in complex systems (Watanabe and Klostermeyer, 1976; Haque et al., 1987). Monomer dissociation was found to be a necessary step for subsequent polymerization, which occurred through adhesion of exposed hydrophobic regions to give aggregates that were then stabilized, at least above a certain temperature threshold, by intermolecular disulfide exchange. The modifications in the exposure to the solvent of hydrophobic residues and in their organization into surface hydrophobic patches upon thermal treatment at neutral pH and at low concentration were studied along with nonreversible alterations in the association equilibrium of BLG. Details on the mechanism of heat-induced polymerization of the protein

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were provided, along with insights on the nature of the chemical bonds involved (Cairoli et al., 1994; Iametti et al., 1995; Iametti et al., 1996), and were in good agreement with those proposed by others (Griffin et al., 1993; Roefs and De Kruif, 1994; Hoffmann and Van Mil, 1997).

In the course of several of the studies mentioned above, different results were obtained when treating protein solutions of different concentration, suggesting that some of the different individual events of the overall unfolding/aggregation phenomena were affected in a peculiar fashion by the protein concentration (Cairoli et al., 1994; Iametti et al., 1995; Hoffmann et al., 1996, 1997). These observations, as well as circumstantial evidences accumulated in other molecular characterization and in limited proteolysis studies (Reddy et al., 1988), suggest that BLG could undergo reversible association in solution beyond the dimeric state. This process produces supramolecular associated species and is favored by increased protein concentrations. In this context, it seems reasonable to hypothesize that the intermolecular forces responsible for concentration-dependent association of BLG should be different (in their chemical nature and/or in their strength) from those stabilizing the structure of the native BLG dimer itself.

Concentration-dependent formation of supramolecular aggregates in solution is not restricted to BLG, as many proteins of industrial interest display "functional properties" (such as foaming, gelling, binding of hydrophobic molecules), or stability features (resistance to thermal denaturation or to enzyme degradation, to name a few) that are concentration-dependent. Such effects are less appreciated in studies on proteins featuring enzymatic activities, where the protein concentration is typically in the nanomolar to micromolar range, but become appreciable when these proteins are studied by techniques that require very concentrated samples, such as NMR.

To approach the issue of concentration-dependent association in BLG, we investigated the associative behavior of the native dimer and of monomers that were chemically modified to make them unable of reassociating at the same interface used in the native dimer. A multipronges approach was used, which combined protein chemistry methodologies with spectroscopic and separation techniques, on the premises that the different association mechanisms revealed by different methodologies should be related to the nature of the physicochemical interactions among the involved BLG molecules.

MATERIALS AND METHODS

Chemicals were reagent or HPLC grade. Bovine β-lactoglobulin (BLG) was from Sigma. Each protein batch was tested as received for the absence of multimeric forms or of disulfide-linked dimers, by using HPLC gel-permeation and SDS−PAGE under nonreducing conditions. Unless otherwise specified, buffer was 50 mM phosphate, pH 6.8, or 50 mM TAPS, pH 9.0 as indicated.

In the experiments involving blocking of the protein thiol groups with iodoacetamide, a weighed amount of the solid reagent was added anaerobically in a 20-fold molar excess to a protein solution in 50 mM phosphate, pH 6.8, and the mixture was stirred under Ar for 2 h at room temperature. Excess iodoacetamide and other reagents or byproducts were then removed by ultrafiltration on an Amicon YM5 membrane. The yield of the reaction in blocked monomers was monitored

by gel permeation HPLC, and was routinely > 95% blocked monomer, the remainder being dimeric BLG.

Gel permeation HPLC was performed in 50 mM phosphate, 0.1 M NaCl, pH 7.5, on a Superdex G75 10/30 column fitted to a Waters 625 chromatograph equipped with a Waters 490 variable-wavelength detector set at 280 nm.

Spectrofluorometric studies were carried out in a Perkin-Elmer LS 50 fluorometer, equipped with an electronic temperature controller. Tryptophan emission fluorescence spectra were recorded by using λ_{ex} = 298 nm. Titration studies were carried out as reported elsewhere (Eynard et al., 1992; Fugate and Song, 1980; Cogan et al., 1976), by using λ_{ex} = 390 nm and λ_{em} = 480 nm for the binding of 1,8-anilinonaphthalen-sulfonate (ANS) and λ_{ex} = 334 nm and λ_{em} = 479 nm for the binding of retinol, which was added from a concentrated stock solution in absolute ethanol. The stock solutions of retinol were prepared under strict anaerobiosis in the dark, kept in a light-tight vial under an Ar atmosphere and used within the same day. Binding data for either titrant were analyzed according to standard algorithms (Ruzic, 1982).

Far- and near-UV CD spectra were recorded at 25 °C in thermostated cells of suitable path length (0.1 cm for the far-UV spectral region and 1 cm for the near-UV region) on a Jasco J500 A spectropolarimeter and were analyzed by means of the Jasco J700 software.

^{1}H NMR spectra were recorded at 25 °C on a Bruker AMX-600 spectrometer operating at 600 MHz, by using a 5-mm reverse probe. Chemical shifts in ppm were referred to an external probe (DSS; 3-(trimethylsilyl)propane-1-sulfonate). Spectra were acquired on protein solutions of different concentration, all in aqueous buffers (50 mM phosphate, pH 6.8, or 50 mM TAPS, pH 9.0 as appropriate), containing 10% D_2O. Suppression of water signals was achieved by presaturation. For D/H exchange time-course experiments; solutions of native BLG at the required concentration in aqueous 50 mM phosphate, pH 6.8, were diluted with 10 volumes of D_2O in the NMR tube immediately before recording of the spectra.

RESULTS

Structural Characterization of Different Monomeric Species of BLG. Most studies on the solution structure of BLG were carried out on the monomeric form that is predominant at very low pH ($\leq$3) (Kella and Kinsella, 1988; Molinari et al., 1996). However, these conditions are far from the physiological ones and from those encountered in most industrial processes involving whey or whey proteins. In former studies carried out at neutral pH, formation of aggregates and extensive protein modification was shown to be consequent to (and depending upon) dissociation of the native dimer (Roefs and De Kruif, 1994; Iametti et al., 1996). Dimer dissociation at neutral pH occurs as a consequence of minimal structural changes, breaking the H-bonds network at the dimer interface (Brownlow et al., 1997). These apparently modest modifications result in increased exposure of the thiol group of Cys121 and of adhesive hydrophobic surfaces, and these latter events represent one of the earliest steps in thermal modification of BLG (Iametti et al., 1996; Funtenberger et al., 1997; Prabakaran and Damodaran, 1997). Thus, for studies aimed at understanding the structural features of the "active" BLG monomer involved in BLG denaturation under conditions typical of milk and sweet whey, preparation on a suitably large scale of a monomeric form of BLG stable at neutral pH was necessary.

Monomer BLG stable at neutral pH was prepared by trapping the exposed thiol group in transiently dissociated monomers with iodoacetamide (IA). As shown for other sulfhydryl reagents (Iametti et al., 1996; Funtenberger et al., 1997), the covalently modified BLG
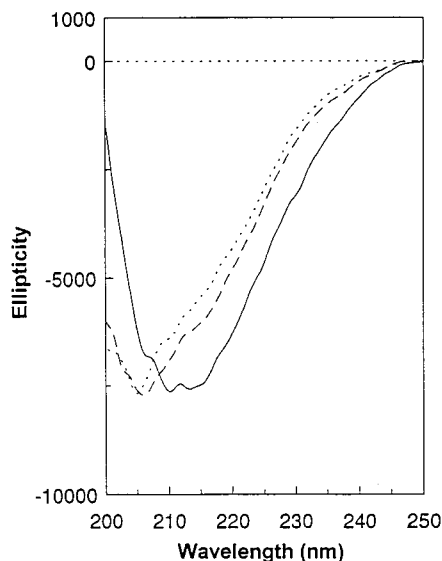
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Figure 1. Far-UV CD spectra of different BLG forms. Untreated BLG was dissolved in 50 mM phosphate, pH 6.8 (full line), or in 50 mM TAPS, pH 9.0 (dots). Blocked monomer (dashes) was dissolved in 50 mM phosphate, pH 6.8. For all samples, protein concentration was 3.8 mg/mL.

Figure 2. Near-UV CD spectra of different BLG forms. Untreated BLG wa dissolved in 50 mM phosphate, pH 6.8 (full line), or in 50 mM TAPS, pH 9.0 (dots). Blocked monomer was dissolved in 50 mM phosphate, pH 6.8 (dashes). For all samples, protein concentration was 3.8 mg/mL.

monomers did not reassociate, because of the steric hindrance of the modifying group. However, the Ellman's reagent (dithiobis-*p*-nitrobenzoate; Ellman, 1959) used in former studies by our group (Iametti et al., 1996) was not suitable for these structural investigations. Indeed, the strong UV absorbance of the protein-bound nitrobenzoate function impaired spectroscopic characterization of the product, and the occurrence of disulfide exchange reactions made the products of the reaction with DTNB unstable upon long-term storage at neutral pH. The use of iodoacetamide (IA) as the thiol blocking reagent called for neutral to slightly alkaline reaction conditions, thus ruling out the use of low pH values for achieving dimer dissociation, as is done in most structural studies on BLG.

Several physical and chemical dissociating agents were tested for monomer dissociation prior to blocking with IA. Selection was made on the basis of full reversibility of the modifications leading to monomer dissociation. Among the tested agents were pH, chaotropes at different concentration, and moderate heating of the protein at temperatures where irreversible modifications did not occur at neutral pH (Cairoli et al., 1994). The highest yields in modified monomer (>95% as detected by gel permeation chromatography) were obtained by carrying out the reaction with IA for at room temperature either in the presence of 2 M guanidine hydrochloride at pH 6.8, or at pH 9.0, in the absence of other chemicals. Control experiments showed that the modifications induced by 2 M guanidine hydrochloride in the CD and fluorescence spectra of the native protein were fully reversible upon removal of the denaturant (not shown).

The species obtained through reaction with IA in the presence of 2 M guanidine hydrochloride at pH 6.8, and subsequent removal of the denaturant is thereon referred to as blocked monomer. Structural features of the blocked monomer were compared with those of the native protein, as well as of the monomer obtained by dissolving BLG at pH 9.0.

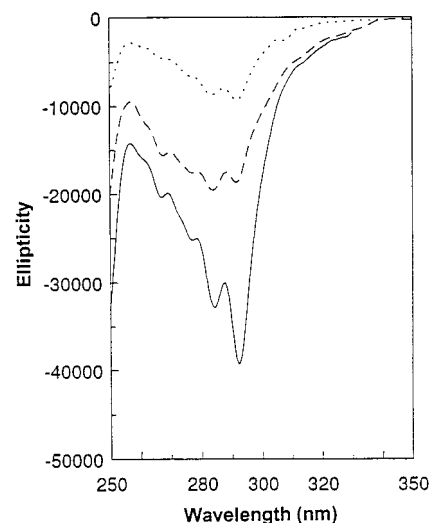As shown in Figure 1, the features of the far-UV CD spectrum of the native dimer are not conserved in the blocked monomer and in the monomer obtained by dissolving native BLG at pH 9 (Townend et al., 1962). Alkaline dissociation of the native protein was confirmed by gel permeation HPLC. Both monomeric forms appear to share a very similar distribution of secondary structure elements. Although the predominance of β-structure in the BLG folding does not facilitate a quantitative estimate of the structural changes introduced by either mode of monomer dissociation, the blue shift of the negative peak in our far-UV CD spectra indicates some conversion of α-helix and β-sheet to aperiodic structures (Prabakaran and Damodaran, 1997).

Figure 2 compares features in the near-UV region of the CD spectrum for different proteins. The features of the native dimer at pH 6.8 almost disappear when the same protein is dissolved at pH 9.0, indicating (Strickland, 1974) that the asymmetry in the chemical environment of aromatic residues that characterizes the tertiary structure of interacting monomers in the native dimer is lost concomitantly to monomer dissociation at pH 9.0.

On the other hand, CD spectral modifications in the near-UV region for the blocked monomer suggested that a significant fraction of the original features in the BLG dimer were retained in this form of the protein. Near-UV CD spectra of the blocked monomer are similar to those recorded on native BLG heated at 60−65 °C at neutral pH (Cairoli et al., 1994). At these temperatures, lower than the temperature required for the occurrence of irreversible thermal modifications, a significant fraction of the BLG molecules is present in solution as dissociated monomers (Iametti et al., 1996), and the observed spectroscopic changes are fully reversible upon cooling (Cairoli et al., 1994). On this basis, we believe that the tertiary structure of the blocked monomer is representative of that of monomers obtained through thermal dissociation, while that of the monomer obtained at alkaline pH differs from that of the "active" monomer responsible of irreversible association in heated BLG at neutral pH (Iametti et al., 1996; Prabakaran and Damodaran, 1997).

Further details on the nature of the modifications induced by monomerization of BLG came from intrinsic
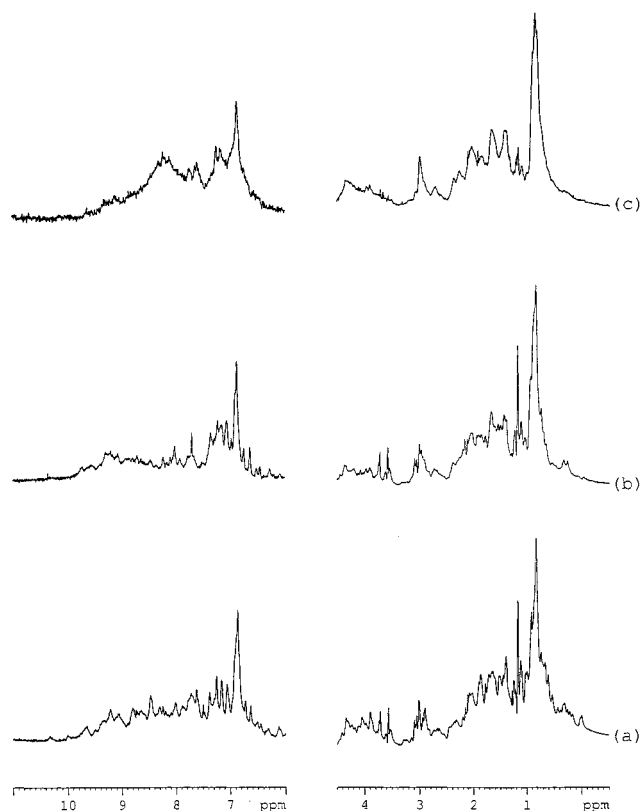
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Figure 3. ^{1}H NMR spectra of different BLG forms. Untreated BLG was dissolved in 50 mM phosphate, pH 6.8 (a), or in 50 mM TAPS, pH 9.0 (b). Blocked monomer was dissolved in 50 mM phosphate, pH 6.8 (c). All samples contained 10% D_2O (v/v) and had the same protein concentration (3.8 mg/mL).

fluorescence studies. The tryptophan fluorescence spectra of the blocked monomer (not shown) had an emission maximum at the same wavelength to those of the native protein and of the monomer formed at pH 9.0, but both displayed a slightly higher emission intensity. In analogy to what observed in similar studies (Stapelfeldt and Skibsted, 1997) and according to available details on the solution and crystal structure of BLG (Molinari et al., 1996; Brownlow et al., 1997), we are inclined to attribute this increase in the fluorescence yield of tryptophan to a decreased interaction between Trp19 and quenching side chains in the protein structure. Arg124 was indicated as the residue responsible for this quenching (Brownlow et al., 1997). This interaction is removed also upon heat treatment of BLG, which leads to an increase in the intensity of Trp fluorescence without affecting the emission wavelength (Cairoli et al., 1994). This could be taken as further evidence of possible structural similarities between the monomeric forms obtained through pH treatment or thiol blockage and those transiently generated by heating the protein below the temperature threshold for irreversible structural modification.

The role of aromatic residues in establishing the structural differences among the investigated forms of BLG, already made evident from the near-UV CD spectra in Figure 2, may be better appreciated in the ^{1}H NMR spectra of Figure 3. As reported in a number of other studies, the flexibility of the BLG molecule at neutral pH resulted in a relatively poor overall quality of the ^{1}H NMR spectra of the protein, making it unfeasible the straightforward attribution of most signals. Nevertheless, the spectra in Figure 3 indicate that

signals in the spectral region encompassing aromatic (6−7 ppm) and amide protons (5−10 ppm) are by far more sensitive to changes in the quaternary structure of the protein than those in the region were signals stemming from aliphatic side chains are dominant (0−5 ppm).

This confirms what observed in the near-UV CD studies discussed above, and suggests that different (and limited) regions of the protein tertiary structure are modified upon dissociation of the native dimer in the conditions used here. Modified regions apparently involve aromatic side chains, which may be relevant to the binding properties of BLG either toward small molecules or toward other macromolecules.

Retinol-Binding Ability of Chemically Modified BLG Monomer. From a structural standpoint, BLG belongs to the class of retinol-binding proteins, and it is known to bind tightly retinol and several of its derivatives. Therefore, functional characterization of the blocked monomer was carried out by comparing its retinol-binding ability with that of the native dimer at neutral pH. This comparison should address also the issue of whether the changes observed upon formation of the monomer in the previous paragraph, and involving regions of tertiary structure rich in aromatic residues, were of possible relevance for the specific binding of hydrophobic molecules of physiological meaning.

The spectrofluorometric titration of the two different protein forms with retinol gave similar figures as for the number of retinol binding sites for each polypeptide chain (1.31 and 1.06 for the dimer and blocked monomer, respectively). The blocked monomer had a slightly lower affinity for retinol than the native dimer (K_d^{app} were 23.9 and 12.8 μM, respectively). Literature reports on the affinity of retinol for BLG gave figures that ranged above 1 order of magnitude (Fugate and Song, 1980; Cogan et al., 1976). Thus, it seems safe to conclude that structural modifications resulting from production of the blocked monomer did not affect the single retinol-binding site present on individual polypeptides. This confirms that the free thiol group of Cys121 has no spatial relationship with the retinol-binding region of the protein, as suggested by crystallographic data (Brownlow et al., 1997; Monaco et al., 1987; Papiz et al., 1986; Pervaiz and Brew, 1985) and by recent studies on the binding of retinol to BLG treated at high pressure in the presence of N-ethylmaleimide as a thiol blocking agent (Tanaka, 1996).

When taking into account the CD and ^{1}H NMR evidences presented in the previous paragraph, our binding data confirm that also in the isolated blocked monomer the hydrophobic calyx responsible for the binding of retinol-like molecules is mostly lined with aliphatic hydrophobic side chains.

^{1}H NMR Proton Exchange Rate Studies on Self-Association in Native BLG. There are a number of literature reports and of circumstantial evidences suggesting that the regions involved in dimerization and in the binding of retinol are not the only "active surfaces" in the BLG molecule. Observations indicating supramolecular aggregation of BLG at neutral pH can be found in the literature reports concerning light-scattering studies (Hoffmann and Van Mil, 1996), ligand-binding studies (Cairoli et al., 1994), limited proteolysis studies (Reddy et al., 1988), and the concentration-dependence of thermally induced aggregation (Iametti et al., 1995; Hoffman et al., 1996).

In an attempt to elucidate through a direct approach the nature of the residues involved in the supramolecular aggregation discussed above, ^{1}H NMR spectra were recorded on solutions of different forms of the protein, each dissolved at different concentrations. Unfortunately, concentration-dependent modifications in the spectra of all protein forms were minor and difficult to interpretate, also because of the absence of accurate assignment of specific resonances in ^{1}H NMR spectra of BLG at neutral pH. An indirect approach was therefore developed, with the aim of confirming inter-protein association and to discriminate between groups contributing to the structure of individual proteins and groups relevant to concentration-dependent intermolecule interactions.

Rates of exchange with deuterium for amide and other exchangeable protons in protein structures can be easily determined through ^{1}H NMR spectroscopy, and they are one of the most reliable indicators of the accessibility of these groups to the solvent and of the strength of their interaction with neighboring residues.

We used this approach to monitor the time course of the disappearance of selected signals in the amide region of the ^{1}H NMR spectra of BLG as a function of the protein concentration. The background assumption in these experiments was that some of those signals would undergo slower exchange at higher protein concentrations, where they are more likely to be buried inside the hypothetical supramolecular aggregates discussed in the above paragraphs. At neutral pH, the rate of H/D exchange is higher than under acidic conditions, making it easier to detect even those slowly exchanging protons that are more deeply buried inside the protein structure.

Native BLG was chosen for these studies because of the relatively better quality of signals from this protein in the investigated region (7−10 ppm, see Figure 3) with respect to those obtained from the blocked and the free monomer. As will be discussed in the next section, all the forms of BLG showed similar aggregation behavior in ligand binding and intrinsic fluorescence studies, and therefore it seemed reasonable to assume that exchange studies carried out on the native protein dimer are representative of changes occurring also for the other forms of the protein.

The ^{1}H NMR spectra in Figure 4 were taken at the beginning and at the end of a D/H exchange experiment, and allowed the identification of a number of signals coming from exchangeable protons in the amide region of the spectrum. These experiments also allowed identification of signals from slowly exchangeable or non-exchangeable protons in amino acid side chains in the same region, such as those of the indole ring NH of Trp at 10.5 ppm, or those likely stemming from aromatic rings at 9.4 ppm that were unaffected even by the longest exposure to D₂O.

The dependence on the protein concentrations of the exchange rates for a few selected signals is shown in Figure 5. The vast majority of the amide protons showed a pronounced decrease in the exchange rates with increasing protein concentration, as shown in the bottom panel of Figure 5 for the signal at 9.38 ppm. However, exchange rates for a few of the investigated signals were not affected by protein concentration (as shown in the top panel of Figure 5 for the signal at 7.75 ppm). These latter signals likely originate from amide groups located in the interior of individual BLG mol-
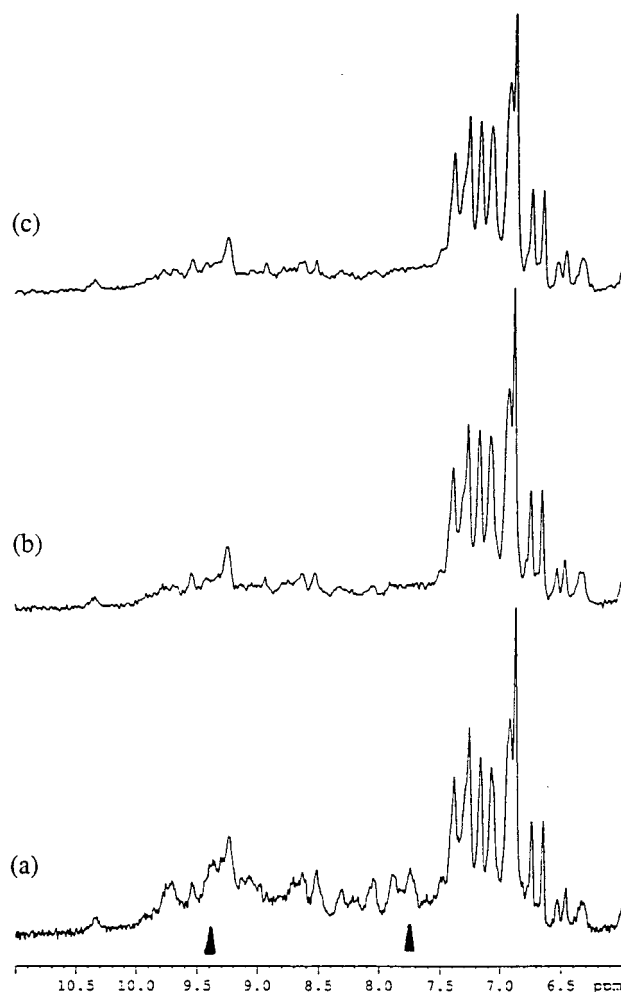
Figure 4. ^{1}H NMR spectra of native, dimeric BLG before and after D/H exchange. The amide and aromatic region of ^{1}H NMR spectra is shown for native BLG (0.05 mL, 42 mg/mL in aqueous 50 mM phosphate, pH 6.8) added to 0.45 mL of H₂O/D₂O (9/1, v/v; a) or to 0.45 mL of D₂O. The spectra of the latter mixture, were recorded 330 and 1100 min after dilution of the protein (b and c, respectively). No further changes were evident in spectra taken after ≈36 h from protein dilution. Arrows indicate the position of signals considered in Figure 5.

ecules, whose accessibility does not depend on the formation of aggregates.

The majority of exchangeable amide groups in BLG, at least on the time scale of our experiments, was located in protein regions that were shielded from the solvent by interaction with other protein molecules.

Self-Association of Chemically Modified BLG Monomers and of Native BLG Dimers. Availability of the blocked monomer made it possible to investigate whether the supramolecular aggregation studied in the former section was a feature restricted to the native dimer or was a feature common to all the BLG forms. If the latter were true, it seemed interesting to study whether the protein surfaces involved in this particular associative behavior bore any relationship to those involved in dimerization of native BLG at neutral pH.

A first set of experiments addressed the concentration dependence of intrinsic features of the protein, and in particular the fluorescence of Trp61, which has been established as a very sensitive parameter for determining the exposure of surface hydrophobic regions in the protein structure (Iametti et al., 1996; Stapelfeldt and Skibsted, 1997).
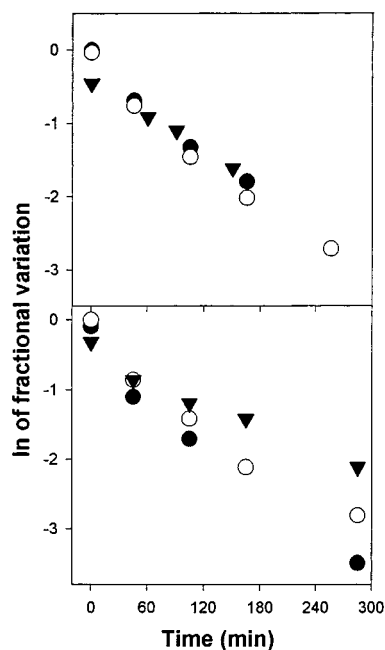
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Figure 5. Time course for the disappearance of selected ^{1}H NMR signals in D/H exchange experiments carried out on BLG solutions at different concentration. An aliquot (0.05 mL) of BLG solutions of appropriate concentration in aqueous 50 mM phosphate, pH 6.8, was diluted with 0.45 mL of D$_2$O. Spectra were taken at the given times. The natural logarithm fractional variation in the intensity of signals at 7.75 ppm (top) and at 9.38 ppm (bottom) is plotted as a function of time for BLG solutions having a final protein concentration in the NMR tube of 9.8 (triangles), 3.8 (circles), and 1.0 mg/mL (dots).
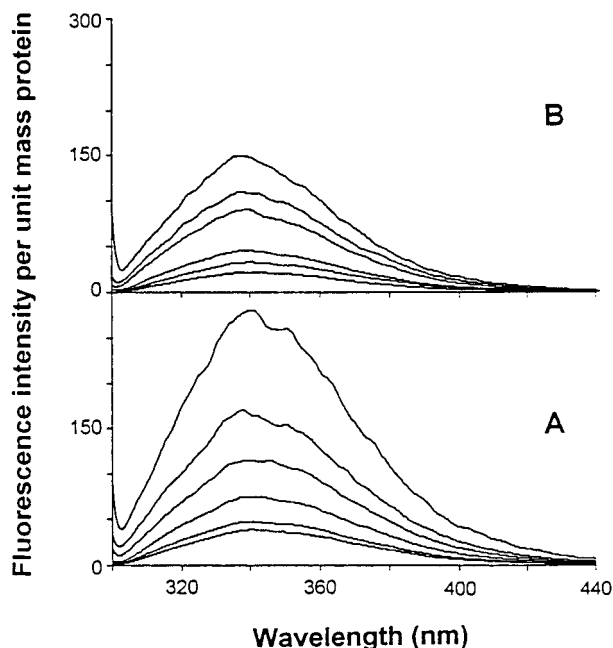

Figure 6. Concentration-dependent modification in tryptophan fluorescence emission spectra of blocked BLG monomer (A) and native BLG (B) at pH 6.8. The proteins were dissolved as required in 50 mM phosphate, pH 6.8. In both panels, from top to bottom, protein concentrations were 0.5, 1, 2.5, 5, 8, and 10 mg/mL. Excitation was at 298 nm.

Figure 6 shows the concentration dependence of signal intensity and of emission maxima for the tryptophan fluorescence in solutions of native BLG and of blocked monomer at neutral pH. Specific fluorescence intensity (emission per unit mass protein) decreases with increas-

ing concentration for either protein species, that both showed a very similar trend. As determined by taking fluorescence spectra in cells with a different path length and by changing the width of the excitation slit (not shown), only a minor portion of the observed decrease may be ascribed to increased absorption of the excitation/emission radiation in solutions of increasing concentration. In our estimate, self-absorption accounts for 10−20% of the observed decrease in fluorescence intensity at the highest protein concentration.

Thus, most of the observed decrease should be ascribed to progressive quenching of the tryptophan fluorescence in solutions of increasing BLG concentration. As for the position of the emission maximum of tryptophan fluorescence, which is dependent on the physicochemical environment of the indole ring in the tryptophan side chain, it was not modified to a significant extent as a function of protein concentration, suggesting that tryptophan residues were not present at the interface(s) between interacting molecules in superaggregate forms of BLG or that their environment was not significantly modified by supramolecular association.

Transient association of different BLG forms was also studied by carrying out titrations with the hydrophobic fluorescent probe ANS of protein solutions of increasing concentration. As reported in Table 1, the number of surface sites available for ANS binding in the blocked monomer for the probe is lower than that of the native dimer or of the monomer obtained at pH 9.0. For all proteins, affinity toward the probe was essentially independent of protein concentration, and was decreasing in the order: blocked monomer > monomer at pH 9.0 > native dimer.

For all proteins, the number of surface sites available for ANS binding decreased with increasing protein concentration, with a very similar trend.

These results may be explained by assuming that monomer formation leads to a decrease in the total number of surface hydrophobic sites. The ones left, however, display an increased affinity toward the probe.

DISCUSSION

The evidence we present here indicates that the monomer obtained at pH 9 or by blocking the thiol group of Cys121 is modified at the dimer interface, but not in the hydrophobic calyx region responsible of retinol binding.

Starting from general structural features, far-UV CD spectra indicate that dimer dissociation, however induced, involves a rearrangement of the secondary structure elements in BLG, without randomization of any of them. The intergroup distances are also modified, as shown by the partial loss of the quenching effect of Arg124 on Trp19. However, the chemical environment of Trp19 and Trp61 is not modified upon monomerization, at least in what is reflected by fluorescence spectra. The Trp residues remain buried in the monomers as well, as indicated by their fluorescence emission maxima and also by the fact that the secondary amine of Trp19 remains protonated in both monomers even after the longest exposure to D$_2$O at neutral pH.

However, monomerization introduces relevant changes in the spatial relationships between hydrophobic amino acids and surrounding residues, as made evident by NMR and near-UV CD spectra. It may be of interest to note that the "backside" of the putative region

Table 1. Concentration-Dependent Changes in Surface Hydrophobicity Parameters for Native BLG and Blocked BLG Monomer at Neutral pH[a]

		protein concentration, mg/mL							
		1.0	1.5	2.5	3.5	5.0	6.5	8.0	10
BLG dimer at pH 6.8	n	1.18	0.88	0.57	0.42	0.26	0.21	0.15	0.11
	K_f	0.056	0.060	0.052	0.048	0.043	0.045	0.051	0.053
BLG monomer at pH 9.0	n	1.16	0.89	0.62	0.49	0.29	0.22	0.18	0.19
	K_f	0.040	0.042	0.042	0.041	0.047	0.050	0.052	0.058
blocked BLG monomer	n	0.78	0.58	0.38	0.26	0.16	0.14	0.11	0.11
	K_f	0.082	0.092	0.087	0.085	0.083	0.090	0.089	0.093

[a] Binding of ANS to native BLG (in 50 mM phosphate, pH 6.8 or in 50 mM TAPS, pH 9.0, as indicated) or to the blocked BLG monomer (in 50 mM phosphate, pH 6.8) was monitored through spectrofluorometric titration experiments on protein solutions of different concentration. The number of binding sites per polypeptide chain (n) and the apparent average formation constant for the ANS–protein complex (K_f, L/mol) were calculated by using the Ruzic algorithm (Ruzic, 1992).

involved in the stabilization of the dimer interface (strand I, according to Brownlow et al., 1997) presents an hydrophobic motif (Ile147; Leu149; Phe151) opposed to the hydrophilic one that was shown by the same authors to form H-bonds with the antiparallel I strand of the interacting monomer (residues His146; Arg148; Ser150).

When considering that the nearby α-helix region presents a hydrophobic "zipper-like" motif made of residues Leu133, Phe136, and Leu140, it is tempting to speculate that monomerization affects the hydrophobic pocket formed by the two nearby regions. This could explain the results obtained in ANS titration of the different protein forms, that is, a decrease in the apparent number of surface hydrophobic sites with a concomitant increase of their affinity toward the probe, when going from the dimer to the monomer.

Near-UV CD spectra indicated that the asymmetry in the environment of aromatic residues (in the dimerization region or elsewhere) appear more conserved in the monomer generated by blocking Cys121 than in the one obtained at alkaline pH. This could be consistent with deprotonation of some groups at the dimer interface, or somewhere else in the protein structure, where they are in close proximity to aromatic side chains.

The surface regions modified upon dimer dissociation are not the ones involved in the spontaneous, concentration-dependent association of BLG in solution. The nature of the regions involved in this association process remains elusive, although fluorescence data apparently indicate a possible involvement of an hydrophobic region containing Trp residues in this phenomenon. The relatively mobile CD loop after Trp61 appears as a suitable candidate, along with the nearby strand C, rich in hydrophobic residues. The hypothetical involvement of hydrophobic regions in concentration-dependent association is supported by the decrease of the number of ANS-binding sites when the protein concentration is increased.

The fact that all monomeric forms of BLG showed the same concentration-dependent changes could be taken as a further evidence that the regions involved in supramolecular association are different from those involved in the stabilization of the native, dimeric form of BLG at neutral pH.

The presence of these associated form may be of practical relevance. Besides the obvious consequences for the design and operation of membrane processes, the association effects reported here may be relevant, among others, to the rate of aggregate formation upon thermization of BLG. Concentration-dependent changes in the apparent reaction order have been reported for denaturative insolubilization of BLG in heat-treated milk, whey, and whey protein isolates (Watanabe and Klostermeyer, 1976). Irreversible stabilization of covalent aggregates formed by purified BLG through disulfide exchange in heat treatment of the protein was also shown to depend markedly on protein concentration (Iametti et al., 1995).

The irreversible aggregation phenomena that result from severe heat treatment of BLG require interprotein interactions, which could be greatly facilitated as a consequence of the self-association events discussed above. The early steps of irreversible aggregation involves the release of "activated monomers", which represent necessary intermediates for subsequent reactions involving hydrophobic surfaces of the protein and newly exposed reactive groups (Griffin et al., 1993; Roefs and De Kruif, 1994; Iametti et al., 1996; Hoffmann and Van Mil, 1997).

As pointed out in the early sections of this paper, the blocked monomer shares many of the structural features of the one obtained in the course moderate thermal treatment. This suggests that the monomers transiently produced during thermal treatment retain important features, such as the ability at binding retinol and other lipophilic molecules, a feature of interest when using BLG as a food ingredient for its aroma-binding properties.

In addition, isolated monomers have been shown here to show the same supramolecular associative behavior displayed by the native dimer, a feature that may be relevant to their participation to irreversible aggregation phenomena. Upon association, the number of exposed surface hydrophobic sites per unit mass protein decreases considerably. The ones left, however, display an increased affinity toward the hydrophobic probe used in this study. Both these events may be relevant to those physicochemical transformation, such as formation of gels and interaction with other proteins in food systems, in which surface hydrophobic regions and exposed reactive groups in heated BLG play a prominent role.

ABBREVIATIONS USED

LITERATURE CITED

Brownlow, S.; Morais Cabral, J. H.; Cooper, R.; Flower, D. R.; Yewdall, S. J.; Polikarpov, I.; North, A. C. T.; Sawyer, L. Bovine β-lactoglobulin at 1.8 Å resolution—still an enigmatic lipocalin. *Structure* **1997**, *5*, 481–495.

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