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Conformation and stability of thiol-modified bovine β-lactoglobulin

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Abstract

Bovine β-lactoglobulin A assumes a dimeric native conformation at neutral pH, while the conformation at pH 2 is monomeric but still native. β-Lactoglobulin A has a free thiol at Cys121, which is buried between the β-barrel and the C-terminal major α-helix. This thiol group was specifically reacted with 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) in the presence of 1.0 M Gdn-HCl at pH 7.5, producing a modified β-lactoglobulin (TNB-blg) containing a mixed disulfide bond with 5-thio-2-nitrobenzoic acid (TNB). The conformation and stability of TNB-blg were studied by circular dichroism (CD), tryptophan fluorescence, analytical ultracentrifugation, and one-dimensional ^{1}H-NMR. The CD spectra of TNB-blg indicated disordering of the native secondary structure at pH 7.5, whereas a slight increase in the α-helical content was observed at pH 2.0. The tryptophan fluorescence of TNB-blg was significantly quenched compared with that of the intact protein, probably by the energy transfer to TNB. Sedimentation equilibrium analysis indicated that, at neutral pH, TNB-blg is monomeric while the intact protein is dimeric. In contrast, at pH 2.0, both the intact β-lactoglobulin and TNB-blg were monomeric. The unfolding transition of TNB-blg induced by Gdn-HCl was cooperative in both pH regions, although the degree of cooperativity was less than that of the intact protein. The ^{1}H-NMR spectrum for TNB-blg at pH 3.0 was native-like, whereas the spectrum at pH 7.5 was similar to that of the unfolded proteins. These results suggest that modification of the buried thiol group destabilizes the rigid hydrophobic core and the dimer interface, producing a monomeric state that is native-like at pH 2.0 but is molten globule-like at pH 7.5. Upon reducing the mixed disulfide of TNB-blg with dithiothreitol, the intact β-lactoglobulin was regenerated. TNB-blg will become a useful model to analyze the conformation and stability of the intermediate of protein folding.

Keywords: β-lactoglobulin; CD; DTNB; fluorescence; molten globule; monomer–dimer

Bovine β-lactoglobulin (blg) is a major whey protein abundant in cow's milk (Godovac-Zimmermann, 1988; Hambling et al., 1992). It consists of 162 amino acid residues and contains two disulfide bonds (Cys66–Cys160 and Cys106–Cys119) and a free thiol (Cys121). It is a predominantly β-sheet protein consisting of nine β (A-I) strands and one major α-helix at the C-terminal end of the molecule (Fig. 1) (Brownlow et al., 1997; Kuwata et al., 1998, 1999; Qin et al., 1998a, 1998b). A-H β-strands form an up-and-down β-barrel with a hydrophobic cavity inside, where hydrophobic compounds such as retinol bind. Bovine blg forms a dimer at neutral pH, while a monomeric native state is stable at pH values below 3. The dimer is stabilized by hydrogen bonds distributed between the surface AB loop and the antiparallel β-sheet between the βI strands, and by tight packing of the residues in the interface.

We have been studying the folding mechanism of bovine blg (Hamada et al., 1995, 1996; Shiraki et al., 1995; Hamada & Goto, 1997; Kim et al., 1997; Kuwata et al., 1998, 1999; Forge et al., 2000). Although it is a predominantly β-sheet protein, it has a markedly high helical propensity. During its refolding reaction, an intermediate with nonnative α-helical structure accumulates because the local interactions between neighboring amino acid residues favor the α-helical structure. Thus, blg is an intriguing model for studying the interplay between local and nonlocal interactions during protein folding. In addition, it may provide clues for clarifying the α-to-β-transition (Dalal et al., 1997; Mihara & Takahashi, 1997) suggested for several biologically important processes, such as the conformational transition of prion protein (Cohen, 1999; Jackson et al., 1999).

A free thiol of bovine blg at Cys121 of βH strand is completely buried under the C-terminal α-helix (Fig. 1). However, it has been known that, under certain conditions, this thiol group can be chemically modified by thiol-specific reagents such as 2-mercaptoethanol, mercaptopropionic acid, 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB)

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Abbreviations: blg, β-lactoglobulin; CD, circular dichroism; C_L fragment, isolated constant domain of the immunoglobulin light chain; DTT, dithiothreitol, Gdn-HCl, guanidine hydrochloride; TNB-blg, β-lactoglobulin modified with DTNB; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); HPLC, high-performance liquid chromatography; 1D, one-dimensional; TNB, 2-nitro-5-thiobenzoic acid; TMR, tetramethylrhodamine; UV, ultraviolet.

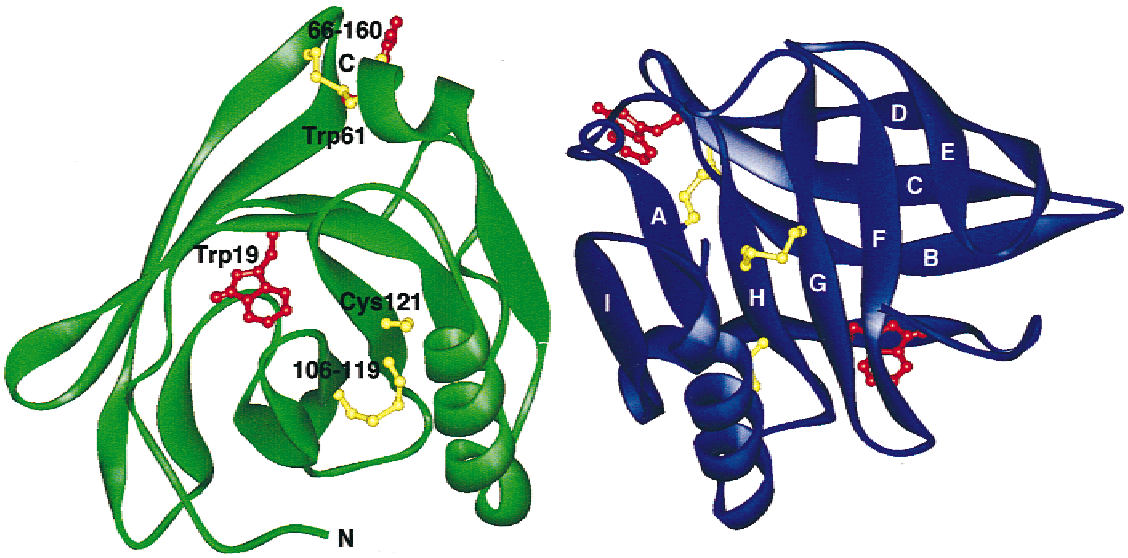


Fig. 1. Schematic picture of dimeric β-lactoglobulin A. Locations of disulfide bonds, the thiol group, and two tryptophan residues are indicated. The picture was drawn by WebLabxViewer Lite 3.2 with a Protein Data Bank file 1BS0.

or 4,4′-dithiopyridine (Cupo & Pace, 1983; Kella & Kinsella, 1988; Apenten, 1998; Burova et al., 1998; Narayan & Berliner, 1998). The modification of blg by relatively small reagents (i.e., 2-mercaptoethanol, mercaptopropionic acid) results in destabilization of the native structure, while retaining its native fold (Cupo & Pace, 1983; Burova et al., 1998). Intriguingly, the thiol modification improves the reversibility from denaturation because the thiol-disulfide exchange reaction after unfolding is suppressed (Burova et al., 1998). On the other hand, modification with a bulky fluorescent reagent such as tetramethylrhodamine (TMR) maleimide results in significant denaturation of the molecule, probably due to the bulkiness of the TMR group, and the modified blg becomes bound to a molecular chaperone, GroEL (Yamasaki et al., 1999). Because GroEL recognizes the denatured state of proteins, the binding of blg labeled with TMR is consistent with the denaturation of blg by modification.

We expected that detailed analysis of the conformation and stability of thiol-modified blg would be useful for improving our understanding of the mechanism of protein folding. In addition, we noticed that blg modified with TMR assumes a monomeric state, when measured by size exclusion gel chromatography, distinct from the dimeric intact blg (Yamasaki et al., 1999). In fact, dissociation of blg into monomer by modification of the thiol group has been reported for several reagents including 2-mercaptoethanol and mercaptopropionic acid (Burova et al., 1998) and a bulky mercurial dye (Zimmerman et al., 1970). Analysis of the monomer–dimer equilibrium, which is dependent on modification of the thiol group, may give insight into the mechanism of blg dimer formation.

DTNB consists of two molecules of 2-nitro-5-thiobenzoic acid (TNB) (Riddles et al., 1983). Reaction of a protein thiol with DTNB by a thiol-disulfide exchange reaction produces one free TNB molecule showing a strong absorption at 412 nm and a protein derivative in which the thiol group forms a mixed disulfide bond with TNB. The formation of the mixed disulfide bond can be detected from the absorption of bound TNB with a maximum at 325 nm. This mixed disulfide bond is easily cleaved by a reducing reagent such as dithiothreitol (DTT), regenerating the intact blg. In this study, we characterized the conformation and stability of bovine blg labeled with TNB at Cys121 (TNB-blg). The results suggest that the modification of the buried thiol group destabilizes the rigid hydrophobic core and the dimer interface, producing a monomeric state. Intriguingly, the modified blg is native-like at pH 2.0 while it is molten globule-like at pH 7.5.

Results

Titration of the buried thiol group

The free thiol of β-lactoglobulin was titrated with DTNB by manually adding the DTNB solution to the protein solution. While the titration reaction of the buried thiol of blg with 0.4 mM DTNB at pH 7.5 and 20 °C was very slow, the reaction was accelerated by the addition of Gdn-HCl (Fig. 2A). In the presence of 1.0 M Gdn-HCl, the reaction followed first-order kinetics with an apparent rate constant of 0.0023 s^{-1}. In the presence of 1.5 M Gdn-HCl, the burst-phase, which occurred within a dead time of about 10 s, appeared and the rate of the slow phase increased to 0.0036 s^{-1}. In the presence of Gdn-HCl at concentrations higher than 2.0 M, the titration reaction was completed within the dead time.

The titration of the buried thiol with DTNB can be represented by the following scheme:

$$\text{blg-SH(buried)} \rightleftarrows \text{blg-SH(exposed)} \xrightarrow{\text{DTNB}} \text{blg-SSTNB} + \text{TNB} \quad (1)$$

where blg-SH(buried) and blg-SH(exposed) are the unreactive and reactive conformations of blg, respectively, and blg-SSTNB is TNB-blg, a reaction product with a mixed disulfide bond. The reaction is analogous to the ^1H–^2H exchange reaction of amide protons (Li & Woodward, 1999; Milne et al., 1999) in a context that the equilibrium of the closed and open conformations precedes the chemical reaction. The reaction produces 1 mol of the TNB molecule with an absorption maximum at 412 nm and a TNB-blg with

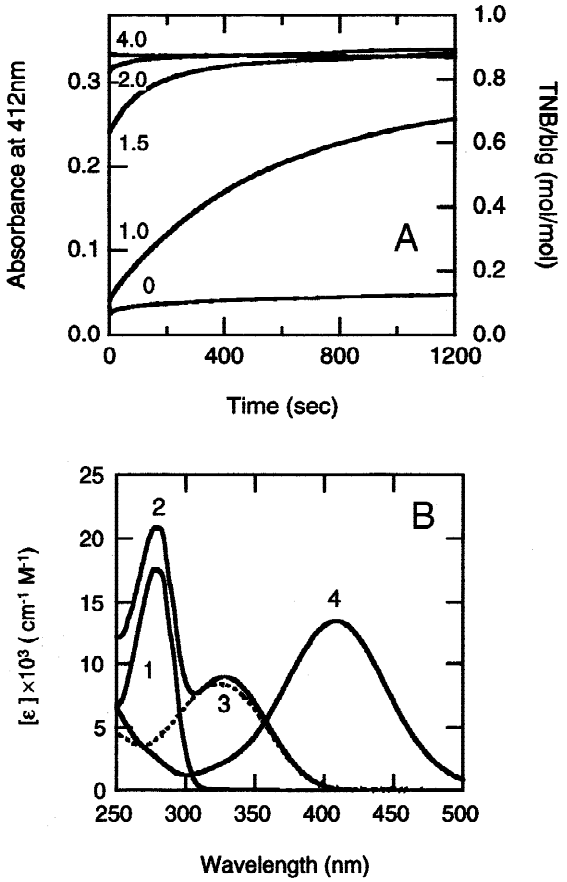


Fig. 2. Titration of the thiol group of blg with DTNB at pH 7.5 and 20 °C. **A:** Kinetics of the titration reaction. The figures indicate the final Gdn-HCl concentration. Concentrations of TNB-blg and DTNB were 0.4 mg mL^{-1} (20 μM) and 0.4 mM, respectively. In 1.0 M Gdn-HCl, more than 90% of the reaction followed first-order kinetics with an apparent first-order rate constant of 0.0023 s^{-1}. In the presence of 1.5 M Gdn-HCl, the amplitude and the rate constant of the slow phase were 0.3 and 0.0036 s^{-1}, respectively. **B:** Absorption spectra of intact blg (1) and TNB-blg (2). For comparison, the absorption spectra of the oxidized (3) and reduced (4) forms of DTNB are included, in which the spectrum of the oxidized DTNB represents one molecule of TNB.

1 mol of TNB bound that has an absorption maximum at 325 nm (Riddles et al., 1983) (Fig. 2B).

Although the exact conformation of blg-SH(exposed) is unknown, it is likely to be a substantially disordered structure of blg, since the thiol group is completely buried in the native state (Fig. 1), and a bulky and hydrophilic DTNB molecule has to attack the thiol group. In the absence of denaturant, the unfolding reaction by which the thiol group becomes reactive occurs rarely, so that the titration reaction is very slow. In the presence of a low concentration of denaturant (e.g., 1.0 M Gdn-HCl), where blg still assumes the native structure, the unfolding reaction becomes significant and DTNB reacts more frequently with Cys121. The increase in the reactivity of the thiol at Cys121 toward DTNB by the addition of urea was reported by Kella and Kinsella (1988). They suggested that the dissociation into monomer in the presence of urea is an important factor determining the reactivity. In the presence of high concentrations of Gdn-HCl, where the protein is unfolded, the reaction is rate-limited by the intrinsic thiol-disulfide exchange reaction, which is very fast under the conditions used.

From the absorption spectrum of TNB-blg with a peak at 325 nm, it was confirmed that 1 mol of the TNB molecule was bound to 1 mol of blg (Fig. 2B). Later, we show that the attached TNB molecule can be removed by reduction of the disulfide bond with DTT, reversibly yielding the intact blg.

CD spectrum

At either pH 7.5 or pH 2.0, the far-UV CD spectrum of the intact blg had a minimum at around 218 nm, consistent with the predominantly β-sheet conformation (Fig. 3A,C). The near-UV CD showed negative peaks at 295 and 285 nm representing the fixed aromatic side chains (Fig. 3B,D).

The far-UV CD spectrum of TNB-blg at pH 7.5 with a minimum at around 205 nm was distinct from that of the native state, although it was also different from the spectrum in 4 M Gdn-HCl (Fig. 3A). This suggests extensive disordering of the native structures with retaining some secondary structures. The absence of sharp peaks in the aromatic region confirmed the disordering of the rigid native structure. On the other hand, the near-UV CD showed a broad positive maximum at around 300 nm, probably arising from the loosely fixed TNB group. These results are distinct from the modification of blg by mercaptoethanol or mercaptopropionic acid (Burova et al., 1998), in which only slight changes in the far- and near-UV CD spectra have been reported. This difference probably arises from the difference in the size of the reagent molecules: mercaptoethanol and mercaptopropionic acid are smaller than TNB.

In contrast, the far-UV CD spectrum of TNB-blg at pH 2.0 was not so different from that of the native spectrum, although the intensity below 220 nm was notably increased. This suggests that TNB-blg retains significant native-like secondary structures at pH 2.0, more than at pH 7.5. The aromatic CD spectrum of TNB-blg at pH 2.0 with sharp peaks is consistent with this interpretation, indicating the presence of rigid tertiary structures. The broad and large positive peak with a maximum at 350 nm probably represents the CD of the tightly fixed TNB group.

At both pH 7.5 and pH 2.0, these characteristics of the CD spectra disappeared upon addition of 4.0 M Gdn-HCl. This indicated that the conformation of TNB-blg is unique at both pH values, although the conformation at pH 7.5 seemed more disordered than that at pH 2.0. We tried to estimate the secondary structure contents of the modified species using a method reported by Chen et al. (1994). However, the fitting was poor, probably because of the low CD intensities in the far-UV region. Therefore, only the α-helical content was estimated from the ellipticity value at 222 nm, using another method reported by Chen et al. (1972). The estimated α-helical content was 12.2 and 13.2% for the intact blg at pH 7.5 and 2.0, respectively, and 9.6 and 14.7% for TNB-blg at pH 7.5, and 2.0, respectively. The secondary structure contents on the basis of the X-ray structure are 16.7% for α-helix, 41.4% for β-sheet, and 42.0% for other structures (Qin et al., 1998a). The α-helix content of TNB-blg at pH 2.0 is slightly higher than that of the intact blg, suggesting the formation of a small amount of nonnative helical structure as observed for the kinetic intermediate (Hamada et al., 1996; Forge et al., 2000).

Fluorescence spectrum

Blg has two Trp residues at positions 19 on the βA strand and 61 on the βD strand (Fig. 1). While Trp19, facing into the base of the hydrophobic pocket before the bend of the βA strand, is fully

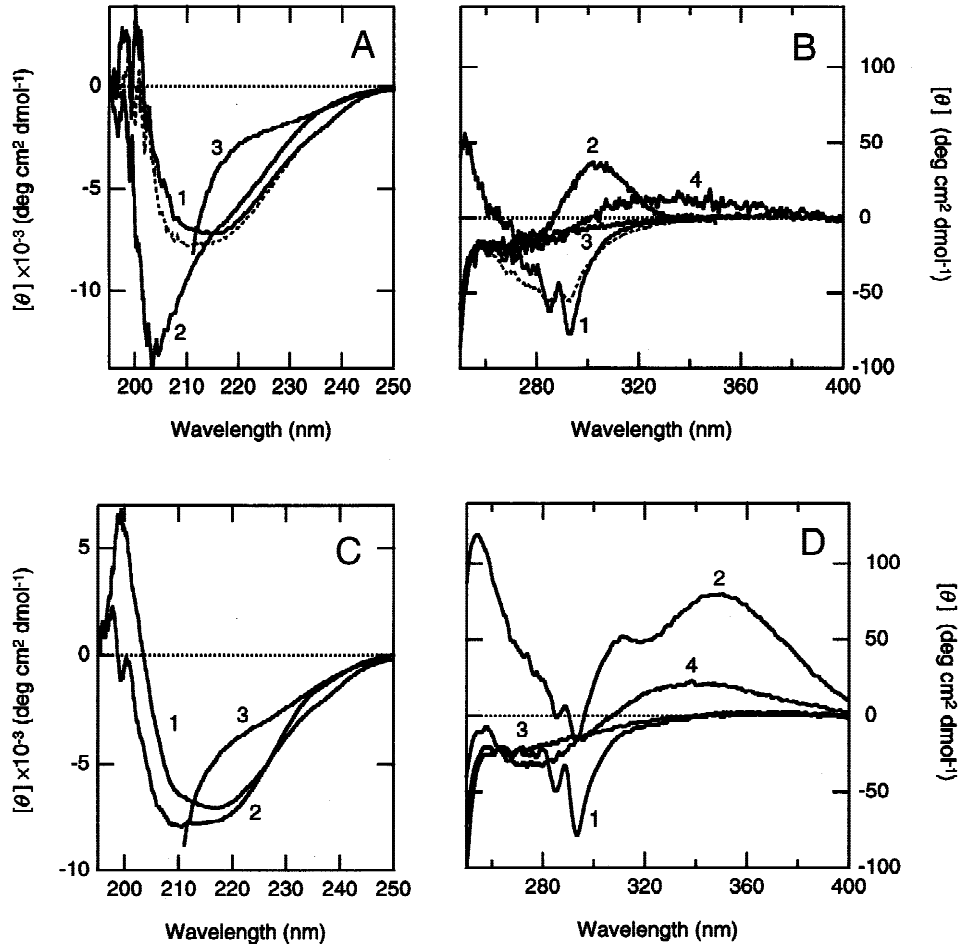


Fig. 3. (**A, C**) Far-UV and (**B, D**) near-UV CD spectra of intact blg and TNB-blg (**A, B**) at pH 7.5 and (**C, D**) at pH 2.0. (1) Intact blg, (2) TNB-blg, (3) intact blg in 4 M Gdn-HCl, (4) TNB-blg in 4 M Gdn-HCl. Buffers used were 10 mM Tris-HCl (pH 7.5) and 10 mM HCl (pH 2.0). The spectra described by broken lines in **A** and **B** show the spectra of TNB-blg after reaction with DTT, indicating that the intact blg was regenerated from TNB-blg.

buried, Trp61, at the end of the βC strand, is relatively exposed to the solvent (Brownlow et al., 1997; Qin et al., 1998a). The fluorescence spectrum of the native state showed a maximum at 335 nm at pH 7.5 (Fig. 5). The guanidino group of Arg124, located only 3.0–4.0 Å distant from Trp19 indole ring, has been suggested to be a strong quencher of Trp19 fluorescence (Brownlow et al., 1997). At pH 2.0, the maximum was 330 nm and, as reported previously (Mills & Creamer, 1975; Renard et al., 1998), the fluorescence intensity was about 1.5-fold higher than that at pH 7.5. This suggests that the tryptophan residues are more buried at pH 2.0 than at pH 7.5, or that the pH-dependent conformational transition is accompanied by dislocation of the quencher Arg124. Upon denaturation by 7.0 M Gdn-HCl, the maximal wavelength shifted to 350 nm for both pH states. It is notable that, at pH 7.5, the maximal fluorescence intensity increased upon unfolding by Gdn-HCl, whereas it decreased at pH 2.0. The maximal intensity of the intact blg in the unfolded state at pH 2.0 was slightly lower than that at pH 7.5.

The reaction with DTNB resulted in a dramatic decrease of fluorescence intensity, which was accompanied by a red shift in the maximal emission wavelength (Fig. 4). The maximal fluorescence wavelength of TNB-blg was 345 nm at both pH 7.5 and pH 2.0, and the maximal intensity at pH 2.0 was about a half of that at pH 7.5. The absorption of TNB group with a maximum at 325 nm exactly overlaps the tryptophan fluorescence. Therefore, the quenching of tryptophan fluorescence of both Trp19 and Trp61 is probably caused by transfer of the fluorescence energy to the TNB group. Quenching of the fluorescence by the newly created mixed disulfide at Cys121 is also likely to be involved because the disulfide bond is one of the most important quenchers of tryptophan fluorescence, as observed for the immunoglobulin domains (Goto & Hamaguchi, 1982; Tsunenaga et al., 1987). The red shift of the emission maximum in both pH regions suggests that the buried tryptophan (Trp19) is preferentially quenched, consistent with the proximity of Cys121 and Trp19 (Fig. 1).

Upon unfolding by 7.0 M Gdn-HCl, the fluorescence intensity increased and the values at pH 7.0 and pH 2.0 were similar (Fig. 4). This also indicates that TNB-blg in the absence of denaturant assumes a compactly folded structure in which the tryptophan residues and quenchers are adjacent. Nevertheless, the fluores-

cence intensities in the unfolded state were less than those of the intact blg in the unfolded state, indicating that the TNB group still quenches the tryptophan fluorescence.

Sedimentation equilibrium

It has been shown that intact blg assumes a dimeric structure at pH 7, whereas the monomer is predominant at regions below pH 3 (Zimmerman et al., 1970; Hambling et al., 1992; Joss & Ralston, 1996; Burova et al., 1998). We examined the monomer–dimer equilibrium of TNB-blg at pH 6.5 and pH 3.0 by sedimentation equilibrium. The plots of concentration vs. radius data clearly indicated that, whereas the profile at pH 3.0 is independent of thiol group modification, the profile at pH 6.5 is dependent (Fig. 5). We obtained the dimerization constant K by fitting the theoretical curves (see Equation 5 in Materials and methods) to the plots (Fig. 5; Table 1).

At pH 6.5, the intact blg had a dimerization constant of $5.4 \times 10^4\ M^{-1}$. This value corresponds to a dimer fraction of 66% at a protein concentration of 1 mg mL^{-1} and is similar to that ($4.9 \times 10^4\ M^{-1}$) reported previously (Burova et al., 1998). This dimerization constant is not so high, indicating that a substantial proportion of the molecules exist as a monomer at low protein concentrations (Table 1). The dimerization constant of TNB-blg was $2.8 \times 10^3\ M^{-1}$, one-twentieth of the value for the intact blg, and the dimer fraction was calculated to be 19% at 1 mg mL^{-1}, much less than that of the intact blg. In contrast, at pH 3.0 under conditions of low salt, the dimerization constants of the intact blg and TNB-blg were $9.6 \times 10^2\ M^{-1}$ and $2.0 \times 10^2\ M^{-1}$, respectively. Both the intact blg and TNB-blg were calculated to be practically monomeric even at a protein concentration of 1 mg mL^{-1}. The pH-dependent change in the dimerization constant of intact blg is consistent with previous reports (Zimmerman et al., 1970; Hambling et al., 1992). The present results show that modification of the thiol group by DTNB results in destabilization of the dimer at neutral pH.

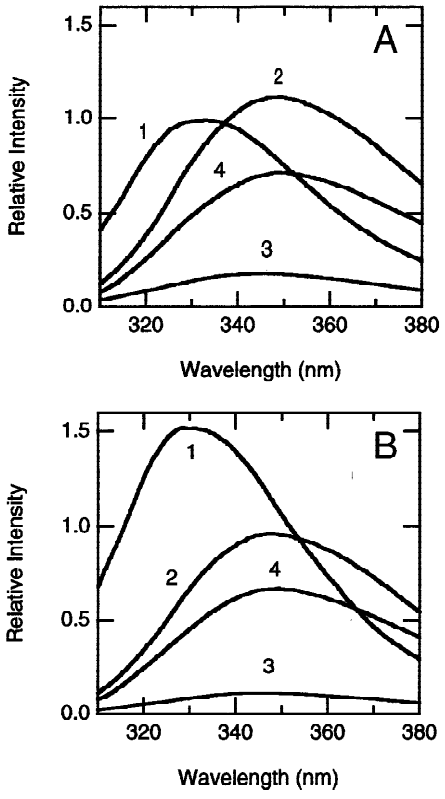


Fig. 4. Fluorescence spectra of intact blg and TNB-blg (**A**) at pH 7.5 and (**B**) at pH 2.0. (1) Intact blg, (2) intact blg in 7.0 M Gdn-HCl, (3) TNB-blg, (4) TNB-blg in 7.0 M Gdn-HCl. Buffers used were 10 mM Tris-HCl (pH 7.5) and 10 mM HCl (pH 2.0). Excitation wavelength was 295 nm, and the emission intensity is relative to that of the maximum of the native state at pH 7.5.

NMR spectra

Whereas we can obtain clear NMR spectra of the monomeric native blg at pH 2 (Kuwata et al., 1998, 1999; Ragona et al., 1999), detailed NMR analysis of the dimer at neutral pH has been difficult because of its large size (molecular weight 36,000). We expected that monomeric TNB-blg would be useful for NMR measurements at neutral pH as well as at acidic pH.

Figure 6 shows the 1D ^{1}H-NMR spectra of the intact blg and TNB-blg at 25 °C. At pH 3.0, TNB-blg exhibited a native-like NMR spectrum with a high dispersion of the sharp peaks. The 1D spectrum of TNB-blg at pH 3.0 was similar to that of the intact blg at the same pH. In contrast, the 1D spectrum of TNB-blg at pH 7.5 was typical for a denatured protein or a molten globule state (Ptitsyn, 1995), indicating that the side chains are largely fluctuating. In comparison, the NMR spectrum of the intact blg at pH 7.5 was dispersed although the peaks were broad (Fig. 6).

Unfolding transition

Unfolding transitions of the intact blg and TNB-blg induced by Gdn-HCl at pH 7.5 and 2.0 were measured using tryptophan fluorescence at a protein concentration of 0.2 mg mL^{-1} (Fig. 7). Because the monomer–dimer equilibrium might affect the unfolding transitions at pH 7.5, measurements at protein concentrations of 0.1 and 0.5 mg mL^{-1} were also made with the intact blg at pH 7.5. However, the transition curve was independent of the protein concentration, suggesting that the dimers, if present, dissociate into monomers before the global unfolding.

As described before, the fluorescence intensity of the native blg at pH 2.0 was higher than that at pH 7.5, so that a drastic decrease in intensity upon unfolding was observed at pH 2.0. It was obvious that the intact protein is more stable at pH 2.0 than at pH 7.5. Because the modification of thiol group quenched the tryptophan fluorescence, the unfolding of TNB-blg by Gdn-HCl resulted in an increase of fluorescence intensity at both pH values (see also Fig. 4). Apparently, the unfolding transitions of TNB-blg occurred at Gdn-HCl concentrations similar to those of the intact blg, and TNB-blg was more stable at pH 2.0 than at pH 7.5. For both pH values, the unfolding transition of TNB-blg was less cooperative than that of the intact blg. For intact blg at pH 2.0, a slight but evident increase in intensity was observed below 0.5 M Gdn-HCl. This increase in the fluorescence of intact blg was suggested to be due to dimer formation in the presence of a low concentration of Gdn-HCl at pH 2.0, and the details of this will be described elsewhere.

The unfolding curves were analyzed assuming a two-state transition mechanism between the folded and unfolded states (Pace, 1990; Myers et al., 1995). Because slight accumulation of the equilibrium intermediate has been demonstrated for the unfolding

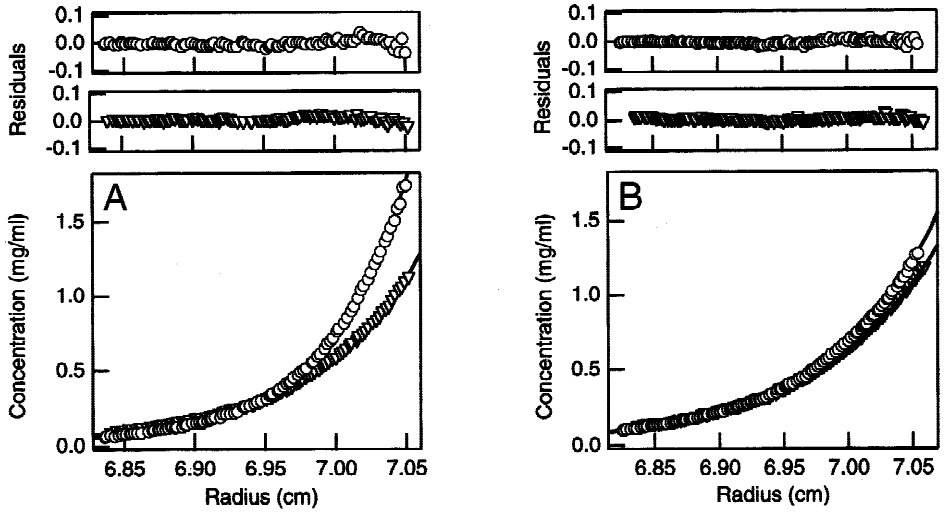


Fig. 5. Sedimentation equilibrium analysis of intact blg (○) and TNB-blg (▽) (**A**) at pH 6.5 and (**B**) at pH 3.0. Bottom panels show the concentration vs. radius plot. The solid lines are theoretical curves based on Equation 1. Upper panels show the residuals from the theoretical curves. Although it is difficult to interpret these profiles intuitively, the data with a larger inclination in the outer region have a higher association constant.

of blg by Gdn-HCl (Hamada & Goto, 1997), the two-state transition should be considered an approximation for quantitatively comparing the unfolding curves. The free energy change of unfolding (ΔG_U) decreases with an increase in the concentration of Gdn-HCl ([Gdn-HCl]), as described by the relation

$$\Delta G_U = \Delta G_U(H_2O) - m[\text{Gdn-HCl}] \quad (2)$$

where $\Delta G_U(H_2O)$ is the free energy change in the absence of denaturant and m represents a measure of the cooperativity of unfolding. The unfolding curves were fitted to Equation 2 by the least-squares curve fitting program using the Igor software package, in which $\Delta G_U(H_2O)$, m, and the baselines for the folded and unfolded states, respectively, were adjustable parameters. The baselines and theoretical curves are indicated in Figure 7, and the values of the midpoint Gdn-HCl concentration (C_m), m, and $\Delta G_U(H_2O)$ are summarized in Table 2. It will be noted that, for the intact blg at pH 2.0, the data points below 0.5 M Gdn-HCl were not included in the fitting. The m values of TNB-blg were smaller than those of the intact blg, confirming the decreased cooperativity. At both pH regions, the C_m value of TNB-blg was slightly higher than that of the intact blg. However, because the m values of TNB-blg were much less than those of the intact blg, the $\Delta G_U(H_2O)$ values of TNB-blg were correspondingly smaller.

Table 1. *Sedimentation equilibrium analysis of β-lactoglobulin A at 20 °C*

Conditions	Species	K (M^{-1})[a]	Fraction of dimer (%) Protein concentration (mg mL^{-1}) 0.1	0.5	1.0
pH 6.5	Intact blg	$5.36\ (\pm 1.32) \times 10^4$	29.2	56.1	66.3
	TNB-blg	$2.76\ (\pm 0.86) \times 10^3$	2.8	11.6	19.3
pH 3.0	Intact blg	$9.56\ (\pm 4.68) \times 10^2$	1.0	4.7	8.6
	TNB-blg	$1.96\ (\pm 0.48) \times 10^2$	0.2	1.0	2.0

[a]The values in parentheses are standard deviation of 3–14 measurements.

Reduction of TNB-blg by DTT

The TNB group of TNB-blg was removed by reduction with DTT. The removal of the TNB group was followed by an increase in absorption at 412 nm due to the formation of a free TNB group (Fig. 8) or by an increase in tryptophan fluorescence (data not

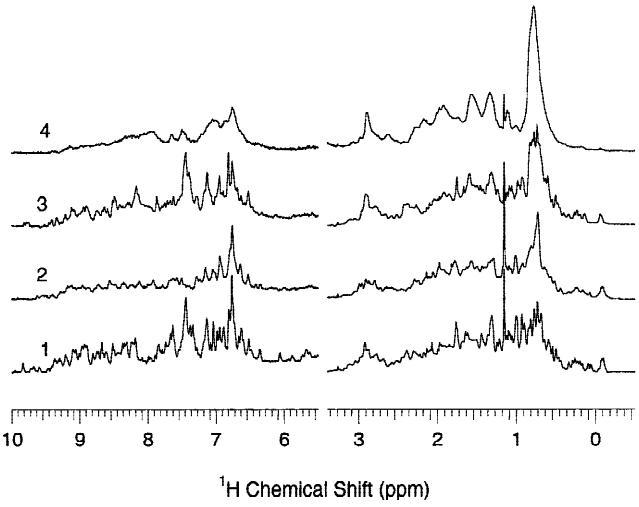


Fig. 6. 1D ^{1}H-NMR spectra of intact blg and TNB-blg at 25 °C. (1) Intact blg at pH 3.0, (2) intact blg at pH 7.5, (3) TNB-blg at pH 3.0, and (4) TNB-blg at pH 7.5.

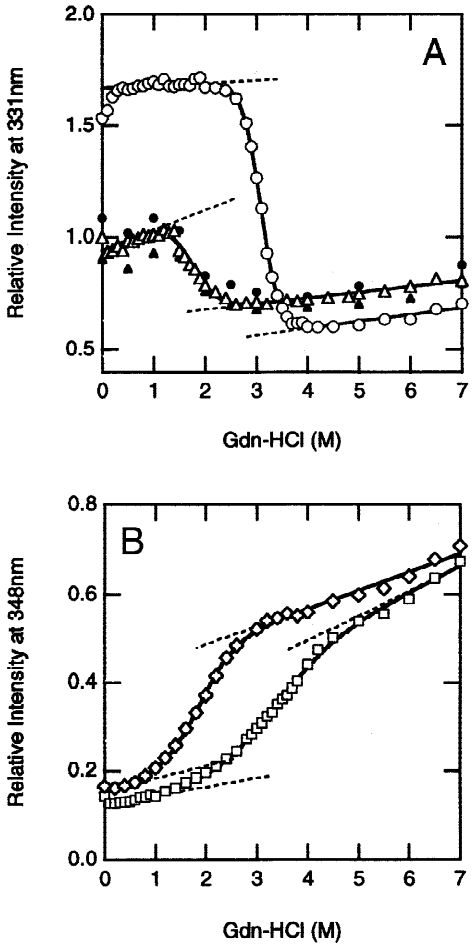


Fig. 7. Unfolding transitions of (**A**) intact blg and (**B**) TNB-blg at 20 °C measured by the fluorescence intensity. Excitation was 295 nm, and emissions were 331 and 348 nm for (**A**) intact blg and (**B**) TNB-blg, respectively. The protein concentration was 0.2 mg mL^{-1}. (△) Intact blg at pH 7.5, (○) intact blg at pH 2.0, (◇) TNB-blg at pH 7.5, and (□) TNB-blg at pH 2.0. Solid symbols indicate the data for TNB-blg at pH 7.5 at protein concentrations of (●) 0.1 and (▲) 0.5 mg mL^{-1}. The ordinates are relative to the intact blg in the absence of Gdn-HCl at pH 7.5. The theoretical transition curves were drawn on the basis of Equation 2 and the parameters shown in Table 2, and the baselines are indicated.

shown, see Fig. 4). The CD (Fig. 3) and tryptophan fluorescence (data not shown) spectra indicated that the intact blg was regenerated. The rate of reduction at a constant concentration of DTT (e.g., 0.2 mM) increased with an increase in Gdn-HCl concentration. This indicated that the mixed disulfide bond of the protein-bound TNB group is buried inside the protein molecule and the reduction with DTT is rate-limited by the conformational transition that exposes the TNB group. Thus, like the intact blg, TNB-blg shows an equilibrium between the open and closed conformations with respect to the reactivity of the TNB group.

$$\begin{array}{c}\text{DTT (reduced)}\\ \searrow\\ \text{blg-SSTNB (buried)} \rightleftarrows \text{blg-SSTNB (exposed)} \rightarrow \text{blg-SH + TNB + DTT (oxidized).}\end{array} \quad (3)$$

Table 2. *Unfolding equilibria of β-lactoglobulin A by Gdn-HCl at 20 °C*

Conditions	Species	C_m (M)	m[a] (kJ/M/mol)	$\Delta G_U(H_2O)$[a] (kJ/mol)
pH 7.5	Intact blg	1.74	10.1 ± 1.2	17.6 ± 2.4
	TNB-blg	1.85	6.8 ± 0.8	12.6 ± 1.9
pH 2.0	Intact blg	3.08	13.7 ± 0.4	42.2 ± 1.3
	TNB-blg	3.15	3.9 ± 0.5	12.3 ± 1.6

[a]Errors are fitting errors.

Discussion

Dissociation of dimer

We indicated that modification of the buried Cys121 by TNB decreases the dimerization constant at neutral pH, resulting in dissociation into monomer (Table 1). The dissociation induced by the thiol-modification was previously demonstrated by Zimmerman et al. (1970) using a mercurial dye, 4-(p-dimethylaminobenzeneazo)-phenylmercuric acetate. They measured the sedimentation coefficient as a function of protein concentration at pH 6.9 and indicated that the protein is converted fully to the monomer by modification of the thiol. We previously reported that modification of the thiol group by TMR maleimide results in dissociation into monomer, which can be bound to GroEL (Yamasaki et al., 1999). The present results showed that TNB, a reagent with a molecular weight much smaller than 4-(p-dimethylaminobenzeneazo)-phenylmercuric acetate or TMR, can induce the dissociation of blg dimer into monomer. Burova et al. (1998) reported that modification of the thiol group by 2-mercaptoethanol or mercaptopropionic acid, which are smaller molecules than DTNB, resulted in dimer destabilization. Therefore, the thiol modification-induced dissociation of blg dimer is likely to be a common phenomenon independent of the reagent.

X-ray crystallographic studies (Brownlow et al., 1997; Qin et al., 1998a, 1998b) have characterized several dimeric structures of bovine β-lactoglobulin, in which βI strands join two monomers by

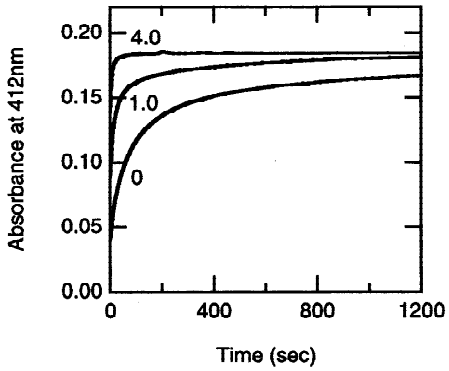


Fig. 8. Reduction of TNB-blg with 0.2 mM DTT at pH 7.5 and 20 °C. The figures indicate the final concentration of Gdn-HCl. Concentrations of TNB-blg and DTNB were 0.2 mg mL^{-1} (10 μM) and 0.2 mM, respectively. Buffer used was 10 mM Tris-HCl (pH 7.5).

forming an antiparallel β-sheet (Fig. 1). The monomers are associated by 12 hydrogen bonds distributed between AB loops (eight side- chain bonds) and βI strands (four main-chain bonds) (Brownlow et al., 1997). The dimer interface buries about 1,000 Å^2 of the accessible surface area per dimer, corresponding to about 6% of the total surface area of the isolated native structures (Brownlow et al., 1997; Qin et al., 1998a). This value lies at the lower end of the range for strongly associated dimers (Qin et al., 1998a), suggesting that this dimeric interaction is not very tight. Consistent with this, the dimerization constant was only 5×10^4 M^{-1} at pH 6.5, and a significant fraction of the molecules existed as a monomer at low protein concentrations (Table 1). The X-ray structure indicates that the thiol group is completely buried under the C-terminal helix and located at a site not far from the dimer interface (Fig. 1). Modification of the buried thiol group will therefore disturb the hydrophobic core. This may affect the nearby dimer interface, consequently destabilizing the dimer.

The dissociation of the intact blg into monomer is also observed at low pH regions. A decrease of pH easily destabilizes the dimer, forming a monomeric native state at pH values below 3.0 under conditions of low salt. In this case, an increase in the net charge repulsion is the major driving force because the dimer is again stabilized by addition of salt even at low pH (Joss & Ralston, 1996). In a context of the monomer–dimer equilibrium, it is intriguing that many nonruminant blgs are monomeric even at neutral pH. Consideration of sequence variation suggests that Ala34 or Arg40 in the AB loop is important for stabilizing the dimer (Brownlow et al., 1997). Recently, the conformation and folding of horse blg have been characterized in detail (Ikeguchi et al., 1997; Fujiwara et al., 1999).

Conformation of TNB-blg at pH 7

While the thiol modification destabilizes the structure around the thiol, it is anticipated that such destabilizing effects will depend on the size and properties of the group introduced. Modification by 2-mercaptoethanol or mercaptopropionic acid, which are smaller molecules, resulted in destruction of the dimer without destroying the rigid native structure, although the stability of the native stricture was decreased significantly (Burova et al., 1998). In the case of DTNB or TMR (Yamasaki et al., 1999), the dissociation into monomer was coupled with significant disordering of the protein structure. The NMR spectrum of TNB-blg at pH 7.5 was similar to that of the unfolded protein or molten globule state (Ptitsyn, 1995). The conformation of blg modified with 4-(p-dimethylaminobenzeneazo)-phenylmercuric acetate was not examined (Zimmerman et al., 1970). Considering its size, the modified blg would assume a largely denatured state.

Destruction of the hydrophobic core by modification of the buried thiol group with a bulky group has been demonstrated for several cases including the disulfide-reduced immunoglobulin domain (Goto & Hamaguchi, 1979, 1981). The isolated constant domain of the immunoglobulin light chain (C_L fragment) has been used for studying of the role of the disulfide bond in protein folding and stability, because it contains only one intramolecular disulfide bond buried inside the protein molecule. The disulfide-reduced C_L fragment assumed a tertiary structure very similar to the disulfide-bonded intact C_L fragment, although the stability was decreased. Alkylation of the thiol groups of the reduced C_L fragment by iodoacetamide resulted in cooperative unfolding of the protein conformation.

Nevertheless, the monomeric TNB-blg at pH 7.5 is not a fully unfolded state. While the ^{1}H-NMR and far-UV CD spectra suggested substantial disordering of TNB-blg at pH 7.5, its near-UV CD spectrum exhibited the clear peak for TNB, implying that the TNB group is fixed loosely in the molecule. The reduction of TNB by DTT at pH 7.5 was dependent on Gdn-HCl concentration, suggesting that the TNB group is buried in the protein molecule. The extensively quenched fluorescence spectrum of TNB-blg indicated that the tryptophan residues are close to the quenchers in the compactly folded conformation. The unfolding transition induced by Gdn-HCl was cooperative at pH 7.5, although the cooperativity was less than that of the intact dimeric state. The *m* value is considered to be proportional to the difference in the accessible surface area between the folded and unfolded states (Myers et al., 1995). The buried surface area of blg may be decreased by modification of the thiol group. All these results suggest that TNB-blg at pH 7.5 adopts a unique compact denatured structure with fluctuating side chains, like the molten globule state observed for several proteins (Hagihara et al., 1994; Nishii et al., 1994; Ptitsyn, 1995). TNB-blg interacted with GroEL (K. Sakai & Y. Goto, unpubl. data), as well as blg modified with TMR (Yamasaki et al., 1999), whereas no interaction was observed between intact blg and GroEL. This also suggests that TNB-blg assumes a molten globule-like conformation with hydrophobic surfaces exposed. If this is a case, it is not surprising that the C_m value of TNB-blg is higher than that for intact blg. The collapsed states stabilized by the marginal hydrophobic interactions are occasionally observed to be more resistant to unfolding than the rigid native structure (Hagihara et al., 1994; Nishii et al., 1994).

Conformational stability at pH 2

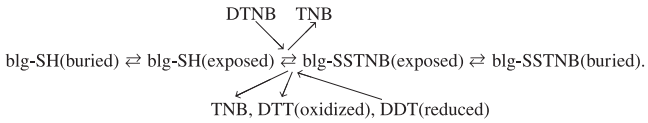
One of the most intriguing observations in the present study was that blg at pH 2.0, either in the intact or modified forms, was more stable against Gdn-HCl than blg at pH 7.5 (Fig. 7). Higher stability of the intact blg at pH 2.0 than at neutral pH was also suggested for the thermal unfolding (Griko & Kutyshenko, 1994; Burova et al., 1998), but this was not examined in detail because interpretation is complicated by the thiol-disulfide exchange reaction in the neutral and alkaline pH regions. The present results confirmed that dimer formation of the intact blg does not increase the stability of blg. The pI value of blg is 4.6 and the net charges at pH 7.5 and pH 2.0 were calculated to be -9 and $+20$, respectively, on the basis of the amino acid composition. Therefore, the net charge repulsion, which is stronger at pH 2.0 than at pH 7.5, cannot explain the increased stability at pH 2 and the exact reason for the increased stability of blg at pH 2.0 is still unclear.

The C_m value for the unfolding of TNB-blg at pH 2.0 was notably higher than that at pH 7.5 (Fig. 7; Table 2). This causes the increased driving forces of folding in the absence of denaturant, producing the more ordered structure. The unique near-UV CD spectrum with a large positive peak of TNB group and sharp peaks of aromatic residues observed for TNB-blg at pH 2.0 represents the tightly fixed side chains. Most importantly, TNB-blg at pH 3.0 exhibited a native-like spectrum measured by 1D NMR, although some differences from the spectrum of the intact blg were also evident. Further NMR analysis will be promising to clarify at the atomic level the effects of thiol modification on the conformation of blg.

Oxidation/reduction-dependent conformational cycle

The advantage of using DTNB for thiol modification is that the reaction can be reversed by external addition of reducing reagent

(Riddles et al., 1983). Whereas the reaction of blg with DTNB produced a modified blg with a mixed disulfide with a TNB group, the reaction of TNB-blg with a reducing reagent such as DTT reproduced the intact blg:



(4)

The forward and backward reactions are rate-limited by the reactivity of the thiol and disulfide groups, respectively. We observed that both reactions are accelerated by addition of Gdn-HCl, indicating that the thiol and disulfide groups are buried inside the protein molecule. The reactivity of the thiol and disulfide groups can be related with the conformational flexibility of the protein molecule. Goto and Hamaguchi (1981, 1982) measured the reaction with DTNB of the reduced C_L fragment of immunoglobulin light chain and its dependence on Gdn-HCl concentration. The estimated rate of conformational change was close to the rate of global unfolding. Further analysis of the DTNB titration of blg and the reduction of TNB-blg with DTT will provide useful data for clarifying the slow conformational dynamics of blg.

Concluding remarks

Bovine blg has a buried thiol at Cys121 that can be titrated by DTNB. Modification of the thiol group with DTNB destabilizes blg, which adopts a monomeric structure with flexible side chains at pH 7.5. This state has characteristics similar to the molten globule or compact denatured state. On the other hand, TNB-blg assumes a native-like structure at pH 2.0. Further analysis of TNB-blg will be useful for clarifying the mechanism of protein folding. The thiol modification-induced monomer formation is not surprising considering the relatively weak and delicate interactions stabilizing the dimer interface. TNB-blg can be reversed to the intact blg by reduction of the mixed disulfide bond with DTT. This oxidation/reduction-dependent conformational cycle of blg provides an intriguing opportunity for studying the mechanism of substrate recognition of GroEL as well as analyzing the mechanism of blg folding.

Materials and methods

Materials

Bovine β-lactoglobulin A was purchased from Sigma (St. Louis, Missouri). TNB-blg was prepared by titrating the thiol group of blg at 0.4 mg mL^{-1} with 0.4 mM DTNB in 1.0 M Gdn-HCl and 10 mM Tris-HCl buffer at pH 7.5. The titration reaction was followed by measuring the absorption increase at 412 nm, and it was confirmed that 1 mol of thiol group reacted with DTNB. The molar absorption coefficients of DTNB and TNB were assumed to be 18,000 M^{-1} cm^{-1} at 325 nm and 13,600 M^{-1} cm^{-1} at 412 nm, respectively (Riddles et al., 1983). The excess reagents were removed by gel filtration with a PD10 column (Pharmacia Biotech, Brussels, Belgium) or dialysis. The purity of TNB-blg was checked by HPLC ion-exchange chromatography with a DEAE 3SW column (Tosoh, Tokyo, Japan). First, the intact blg or TNB-blg was adsorbed to the column equilibrated with 50 mM Tris-HCl at pH 7.5, and then the protein was eluted with a linear gradient of 0 to 0.5 M NaCl in the same buffer. TNB-blg was separated from the intact blg as shown in Figure 9. TNB-blg used in the present manuscript was more than 95% pure. DTNB, DTT, Gdn-HCl, and other reagents were purchased from Nacalai Tesque (Kyoto, Japan).

CD

CD measurements were carried out with a Jasco spectropolarimeter, Model J-720, at 20 °C. The instrument was calibrated with ammonium d-10-camphorsulfonic acid. The results are expressed as the mean residue ellipticity $[\theta]$, which is defined as $[\theta] = 100\theta_{obs}(lc)^{-1}$, where θ_{obs} is the observed ellipticity in degrees, c is the concentration in residue moles per liter, and l is the length of the light path in centimeters. Far- and near-UV CD spectra were measured using cells with light paths of 1 and 10 mm, respectively. The protein concentrations were 0.5 and 0.8 mg mL^{-1} for the far- and near-UV measurements, respectively.

Fluorescence and absorption

Fluorescence spectra were measured with a Hitachi fluorescence spectrophotometer, F4500, at 20 °C, and a cell with a 5 mm light-path was used. Tryptophan fluorescence was measured with an excitation at 295 nm at a protein concentration of 0.2 mg mL^{-1}. Absorption measurements were carried out with a Hitachi spectrometer, U3000, at 20 °C.

Analytical ultracentrifugation

Prior to the ultracentrifugation experiments, proteins were dialyzed against the desired buffer or passed through a PD10 column equilibrated with the buffer. The buffers used were 20 mM glycine-HCl

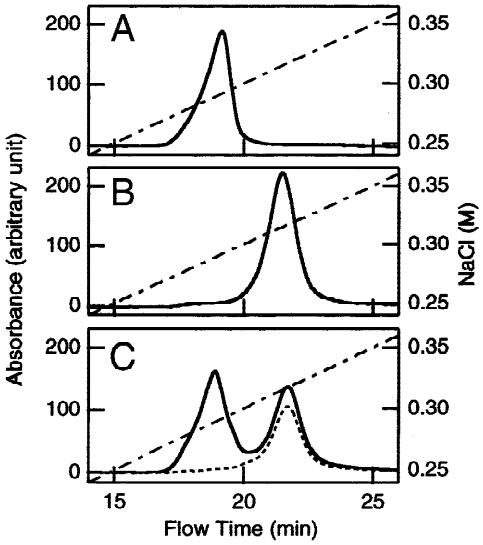


Fig. 9. Separation of intact blg and TNB-blg by HPLC ion-exchange chromatography with a DEAE 3SW column in 50 mM Tris-HCl at pH 7.5. The upper two panels show the elution patterns of (**A**) the intact blg and (**B**) TNB-blg, detected by absorption at 278 nm. (**C**) The elution pattern of a mixture of intact blg and TNB-blg detected by the absorption at 278 nm (solid line) and at 325 nm (dotted line). The linear gradient of NaCl concentration is indicated.

(pH 3.0) containing 20 mM NaCl or 10 mM Na phosphate (pH 6.5) containing 100 mM NaCl. The intact blg with a molecular weight of 18,400 and TNB-blg with a molecular weight of 18,600 were assumed to have the same partial specific volume of 0.751 mL g^{-1} at 25 °C (Joss & Ralston, 1996). A value of 0.961 cm^{-1} mL mg^{-1} at 280 nm (Townend et al., 1960) was used for the absorbance coefficient of the intact blg, and a value of 1.189 cm^{-1} mL mg^{-1} was deduced for that of TNB-blg, assuming the additivity of the absorption of blg and TNB. The densities of glycine-HCl buffer (pH 3.0) and Na phosphate buffer (pH 6.5) at 20 °C were assumed to be 0.9990 and 1.0030 g mL^{-1}, respectively.

Sedimentation equilibrium experiments were performed with a Beckman Optima XL-A analytical ultracentrifuge at 27,000 rpm at 20 °C, monitoring the absorbance at 278 nm. Concentration vs. radius data acquisition was as an average of 20 absorbance measurements over 0.001 cm intervals. All experiments used double-sector 12 mm thick charcoal-epon centerpieces and matched quartz windows. Protein concentration (C_T) was plotted against radius (r) from the arbitrary radius point (r_0). The C_T vs. r plots were analyzed to determine the association constant K, according to the theoretical equation for monomer–dimer equilibrium:

$$C_T = C_M(r_0)\exp[M\phi(r^2 - r_0^2)] + (2K/M)[C_M(r_0)]^2 \exp[2M\phi(r^2 - r_0^2)] \quad (5)$$

where $C_M(r_0)$ is the monomer concentration at r_0, M is the molecular weight of monomer, $\phi = \omega^2(1 - \nu\rho)/(2RT)$, and R, T, and ω are the gas constant, absolute temperature, and rotor speed, respectively (Teller et al., 1969). Least-squares curve fitting was done using the Igor software program (WaveMetrics, Lake Oswego, Oregon).

NMR measurements

NMR spectra were recorded on a 500 MHz spectrometer (Bruker DMX 500) equipped with a triple-axis-gradient triple-resonance probe. The protein solutions were prepared in 90% (v/v) H_2O and 10% (v/v) D_2O, and the buffers used were 10 mM Tris-HCl (pH 7.5) or 10 mM HCl (pH 3.0). For 1D measurements, the spectral width was 8,012.8 Hz, and 128 scans of 4,096 real time points were collected for 3 min. The temperature was 25 °C.

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