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FOR THE RECORD

Solution structure and dynamics of bovine β-lactoglobulin A

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Abstract: Using heteronuclear NMR spectroscopy, we studied the solution structure and dynamics of bovine β-lactoglobulin A at pH 2.0 and 45 °C, where the protein exists as a monomeric native state. The monomeric NMR structure, comprising an eight-stranded continuous antiparallel β-barrel and one major α-helix, is similar to the X-ray dimeric structure obtained at pH 6.2, including β_I-strand that forms the dimer interface and loop EF that serves as a lid of the interior hydrophobic hole. $\{^1H\}$-^{15}N NOE revealed that β_F, β_G, and β_H strands buried under the major α-helix are rigid on a pico- to nanosecond time scale and also emphasized rapid fluctuations of loops and the N- and C-terminal regions.

Keywords: α-helix to β-sheet transition; β-lactoglobulin; dynamics; heteronuclear NMR; protein folding

Bovine β-lactoglobulin, a major component of cow's milk, has been the target of numerous structural and theoretical studies (Hambling et al., 1992; Cho et al., 1994). Recently, it has been shown that β-lactoglobulin is an intriguing model for clarifying the mechanism of the α-helix to β-sheet (α-β) transition of proteins (Hamada et al., 1995, 1996; Shiraki et al., 1995; Hamada & Goto, 1997), a key issue for understanding the folding and biological function of a number of protein molecules such as prion protein or Alzheimer's Aβ peptide (Mottonen et al., 1992; Booth et al., 1997; Dalal et al., 1997; Prusiner, 1997; Jackson et al., 1999). β-Lactoglobulin is a predominantly β-sheet protein consisting of a β-barrel of eight continuous antiparallel β-strands ($\beta_A - \beta_H$) shaped into a flattened cone or calyx, an additional β-strand, β_I, and one major and four short helices (Brownlow et al., 1997; Qin et al., 1998a, 1998b). However, the folding of bovine β-lactoglobulin is accompanied by an intramolecular α-β transition due to the inconsistency of local and nonlocal interactions (Hamada et al., 1995, 1996; Shiraki et al., 1995; Hamada & Goto, 1997).

To understand the α-β transition of β-lactoglobulin, it is essential to characterize its solution structure in detail. β-Lactoglobulin exists as a dimer at neutral pH, but dissociates into a monomer below pH 3, while it retains a native conformation even at a pH as low as 2 (Futage & Song, 1980). We have prepared recombinant β-lactoglobulin uniformly labeled with ^{15}N and ^{13}C (Kim et al., 1997; Kuwata et al., 1998). Using ^1H, ^{15}N, and ^{13}C heteronuclear NMR spectroscopy, we have assigned the resonance frequencies of the backbone nuclei in the native state and in the highly helical state induced by 2,2,2-trifluoroethanol (TFE) at pH 2.0 and 45 °C (Kuwata et al., 1998; Uhrínova et al., 1998). Secondary chemical shifts of backbone resonances and nuclear Overhauser effects (NOEs) indicated that secondary structures in the native state are similar to those of the crystal structure and that those in TFE contains many α-helical segments. The presence of the persistent α-helices in the helical state and the core β-sheet in the native state suggested that during folding native-like core β-sheet and several nonnative helices are formed first and the remaining β-sheet is subsequently induced through interaction with the pre-existing β-sheet. Thus, NMR is beginning to clarify the mechanism of the α-β transition of β-lactoglobulin.

In this paper, we report the solution structure and dynamics of β-lactoglobulin A at pH 2.0 and 45 °C based on heteronuclear NMR techniques. One of the most interesting topics of β-lactoglobulin is the Tanford transition, the pH-dependent conformational transition occurring near to pH 7 (Tanford et al., 1959; Hambling et al., 1992; Qin et al., 1998a). The present NMR structure indicates that the monomeric structure at pH 2.0 is similar to the closed conformation before the Tanford transition. Analysis of protein dynamics with the $\{^1H\}$-^{15}N NOE adds that β-strands (i.e., β_F, β_G, and β_H strands) forming the hydrophobic core are more rigid on a pico- to nanosecond time scale than the rest of the molecule.

Results and discussion: *Characteristics of the solution structure:* NMR data for structure determination were obtained for aqueous

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solutions of bovine β-lactoglobulin A at pH 2.0, without added salt, at a temperature of 45 °C, where the protein assumes the monomeric native state (Futage & Song, 1980; Kuwata et al., 1998). Of the 200 NMR structures calculated, a subset of 17 lowest energy structures was selected for analysis (Table 1; Fig. 1A; see also Materials and methods).

Comparison of the 17 structures showed that, while β-strands were defined well, the N- and C-terminals and some of loop regions were less defined. This is due to both the scarcity of NOE constraints in these regions and the conformational flexibility of the regions, consistent with results for other globular proteins (Powers et al., 1993). The overall NMR structure consists of a β-barrel of eight continuous antiparallel β-strands ($\beta_A - \beta_H$), an additional β-strand, β_I, and one major helix. Four short α-helix-like structures are seen at the N-terminal side of β_A, loop AB, loop GH, and in the C-terminal side of β_I. The β_I sheet, which forms the dimer interface, is also conserved even in the monomeric state. We can also detect the β_0 sheet, which is proposed in the recent X-ray structure (Qin et al., 1998a). The secondary structures including β_0 are consistent with those estimated from the secondary chemical shifts of backbone resonances (Kuwata et al., 1998), except the short helix-like structures that were not clear from the secondary chemical shifts.

The NMR structures were compared with the X-ray structures. The X-ray structures of variant A at pH 6.2, 7.1, and 8.2 (resolution of 2.56, 2.28, and 2.49 Å, respectively) have been reported (Qin et al., 1998a). Independent of the crystals, the global foldings are very similar, consisting of a β-barrel of eight continuous antiparallel β-sheets. However, there is a significant difference between the structures before and after the Tanford transition, which is centered at pH 7.5. The Tanford transition is primarily associated with a major conformational change in loop EF (residues 85–90), which serves as a lid to the interior of the protein. The lid is closed at low pH but which opens at high pH (Qin et al., 1998a, 1998b). Obtained NMR structure at pH 2.0 clearly showed the closure of loop EF. The global root-mean-square deviation (RMSD) values calculated for the backbone atoms between the NMR structure of the lowest energy and the X-ray crystal structures (residues 5–160) at pH 6.2, 7.2, and 8.2 are 2.70, 2.85, and 2.84 Å, respectively. If we carried out the same calculation with only β_E, loop EF, and β_F (residues 81–97), the RMSD values for the X-ray structures at pH 6.2, 7.2, and 8.2 are 1.38, 2.38, and 2.41 Å, respectively. This further confirms that the obtained NMR structure at pH 2.0 is close to the X-ray structure at pH 6.2.

However, some evident differences were also observed between the NMR and X-ray structures in the N-terminal residues, loop FG, and notably the major helix. The differences in the loop regions may arise from the low resolution of the NMR structure due to the scarcity of NOE constraint and further refinement will be required. On the other hand, orientation of the major helix and conformation of the loop residues before and after the major helix are evidently different from those of the X-ray structures, suggesting these differences represent the conformational change accompanied by the dimer–monomer transition.

Thus, we may conclude that the overall structure, except the major helix, of monomeric β-lactoglobulin at pH 2.0 and 45 °C in aqueous solution is similar to the X-ray closed conformation obtained at pH 6.2. These results are in general consistent with those deduced from the roughly determined structure by the ^{1}H-NMR spectra (Ragona et al., 1997; Fogolari et al., 1998; Uhrínova et al., 1998).

Table 1. *NMR structure determination statistics*

A. NMR constraints	
Distance constraints	
Intraresidue	2,243
Sequential ($\|i - j\| = 1$)	1,184
Medium range ($\|i - j\| <= 4$)	238
Long-range ($\|i - j\| > 4$)	434
Torsion angle constraints	
ϕ	115
χ_1	69
B. Structure statistics (17 structures)	
Violation statistics	
Number of NOE violations >0.1Å	6
Maximum NOE violation (Å)	0.41
Number of torsion angle constraint violations greater than 5°	2
Energies	
Mean constraint violation energy (kcal mol^{-1})	9.07
Mean AMBER energy (kcal mol^{-1})	−2,799
PROCHECK statistics	
Residues in most favored regions (%)	78
Residues in allowed regions (%)	18
C. RMSDs from the average structure	
Backbone atoms (N, C$^\alpha$, C′, O)	
Total (Å)	0.89
Sheets (Å)	0.29
All heavy atoms	
Total (Å)	1.53
Sheets (Å)	0.93

Pico- to nanosecond dynamics: To estimate pico- to nanosecond-order fluctuations of the β-lactoglobulin structure, we carried out ^{15}N relaxation measurement, one of the most widely used NMR methods for dynamic analysis of protein (Farrow et al., 1994). {^{1}H}-^{15}N NOE values decreased in many loops, and the ends of α-helices, and N- and C-terminal regions (Fig. 2A), indicating considerable flexibility on a pico- to nanosecond time scale. On the other hand, in the same plot on an expanded scale (Fig. 2B), {^{1}H}-^{15}N NOE values were distinctly higher in the regions corresponding to β_F, β_G, and β_H strands, and probably β_A and β_E strands. The high NOE values of loop FG were also evident. The high NOE values indicate the rigidity of the corresponding regions on a pico- to nanosecond time scale. These results suggest that one side of the β-barrel, including the β_A and C-terminal half of the molecule, is more rigid within this time scale as compared to the other side. The regions including β_F, β_G, and β_H can be considered to form a rigid core. The rigidity of the major C-terminal helix estimated by {^{1}H}-^{15}N NOE is close to those of the fluctuating β-strands.

The dynamic properties measured by {^{1}H}-^{15}N NOE were compared with the RMSD of backbone atoms of the 17 native structures determined by NMR (Fig. 2D). The backbone RMSD may represent the heterogeneity of the static structure. Significant backbone differences are evident at the N- and C-termini. However, the

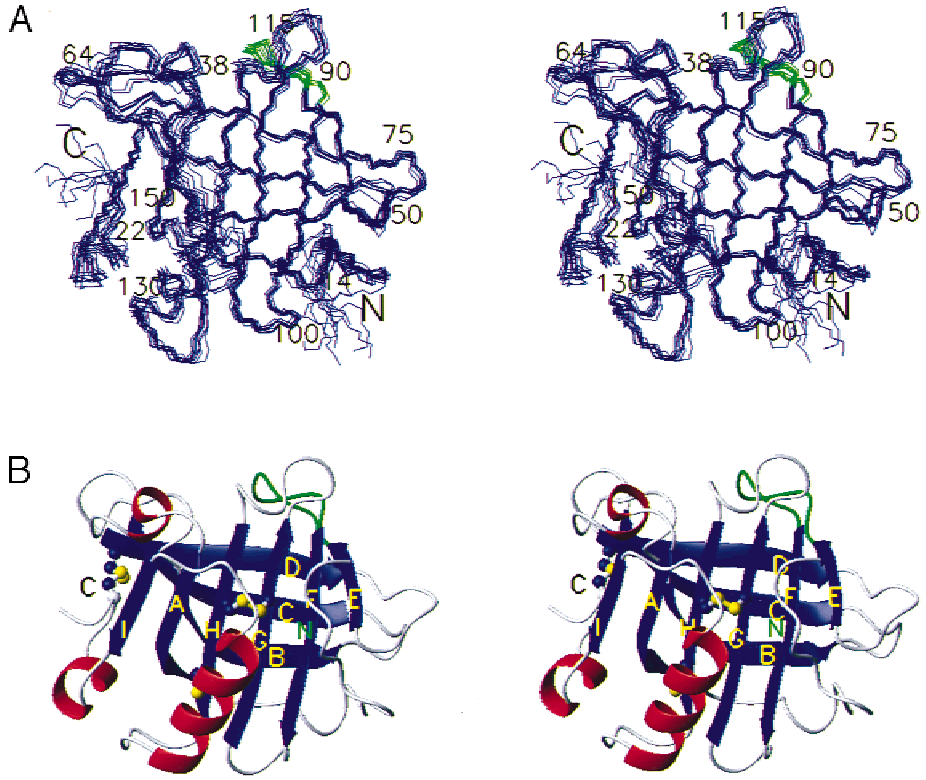

Fig. 1. (**A**) NMR and (**B**) X-ray structures of bovine β-lactoglobulin. Diagram indicating the superimposition of the 17 NMR structures was produced using MOLMOL (Koradi et al., 1996). X-ray coordinates (3blg) and the locations of secondary structures are available from the Brookhaven PDB. In **B**, the positions of disulfide bonds (Cys66–Cys160 and Cys106–Cys119) and free thiol group (Cys121) are indicated, helical regions are colored red, and loop EF is colored green.

C-terminus is less disordered than the N-terminus probably because of the presence of the disulfide linkage (Cys66–Cys160). The backbone RMSD values of all the β-strands except β_0 and β_I are similar, suggesting that the β-strands forming the β-barrel have similar stability, slightly different from the conclusion of $\{^1H\}$-^{15}N NOE. High flexibility of the loops except loop FG is also evident from the backbone RMSD profile, consistent with the $\{^1H\}$-^{15}N NOE profile.

Determinants of folding and dynamics: To address the factors responsible for the folding and dynamics of β-lactoglobulin, we plotted the hydrophobicity profile derived using the scale of Kyte and Doolittle (1982), the accessible surface area (ASA) of each residue and the residue-averaged main-chain *B*-factor values, both calculated from the X-ray coordinates at pH 6.2 (Fig. 2C). The ASA plots indicated that the β_A, β_F, β_G, and β_H strands are largely buried, and a portion of the β_C strand is also buried. Other β-strands, the major α-helix, and the loops are relatively exposed to the solvent. The side chains are usually buried in the interior of the protein molecule by hydrophobic interactions. Indeed, the hydrophobicity profile reveals that the β_A, β_E, β_F, β_G, and β_H strands are hydrophobic.

We recognize that the profile obtained from ASA or hydrophobicity plot is similar to that of *B*-factors, in which the fluctuations of the β_B, β_C, and β_D strands in addition to the loops and the N- and C-terminals are emphasized. This indicates that the regions exposed to solvent tend to form flexible structures with increased X-ray *B*-factors. Now, we can see that the profiles of the ASAs, *B*-factors, or hydrophobicities are similar to the fluctuation profiles measured by $\{^1H\}$-^{15}N NOE. The buried and rigid regions, as measured by the ASA and *B*-factor, are also rigid in $\{^1H\}$-^{15}N NOE. Such buried hydrophobic residues often form tightly packed hydrophobic cores with increased van der Waals contacts.

^1H–^2H exchange experiments conducted in the native state at pH 2 indicate the presence of core regions highly protected from the exchange, consisting of β_A, β_F, β_G, and β_H strands (Ragona et al., 1997; V. Forge, M. Hoshino, K. Kuwata, C.A. Batt, & Y. Goto, unpubl. results). The rigid regions are located roughly in the core regions described above. On the other hand, the flexible regions on a nano- to picosecond time scale correspond to areas that are less protected from ^1H–^2H exchange.

Conclusion: The solution structure and its dynamics of β-lactoglobulin A at pH 2 were studied with heteronuclear NMR techniques. The NMR structures indicated that the monomeric structure at pH 2.0 is similar to the X-ray native structure at pH 6.2, in which loop EF folded over the entrance to the β-lactoglobulin calyx. Analysis of protein dynamics with the $\{^1H\}$–^{15}N NOE indicated that β_F, β_G, and β_H strands and loop FG are more rigid on a pico- to nanosecond time than the rest of the molecule. Hydrophobicity of the amino acid side chains is one of the important factors determining the folding and dynamics of β-lactoglobulin.

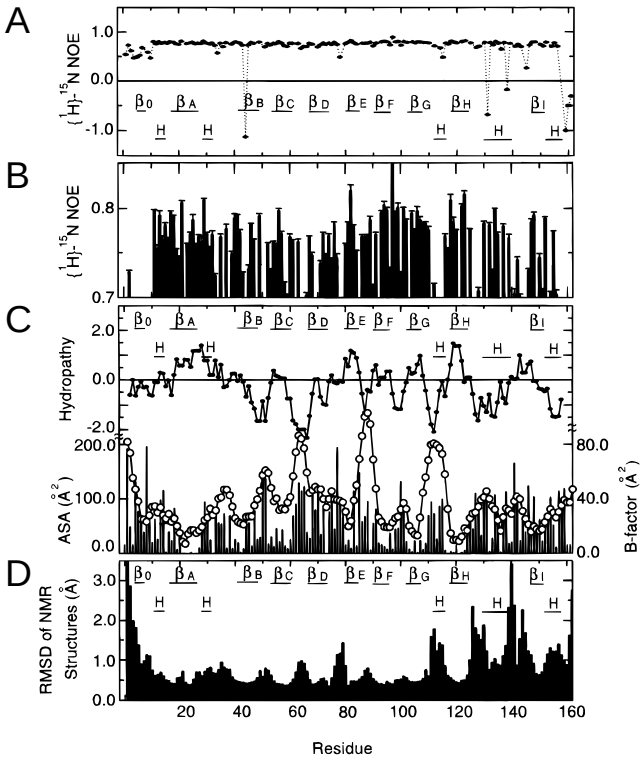

Fig. 2. Comparison of dynamics of bovine β-lactoglobulin A and possible determinants. **A:** $\{^1H\}$–^{15}N heteronuclear NOE for the backbone amide nitrogen atoms of β-lactoglobulin measured at 600 MHz on the normal scale at pH 2 and 45 °C. **B:** $\{^1H\}$–^{15}N heteronuclear NOE for the backbone amide nitrogen atoms on the expanded scale. **C:** Hydrophobicity (●), ASA (bars), and main-chain *B*-factors (○) for the X-ray structure, 3blg. Hydrophobicity was calculated using the scale of Kyte and Doolittle (1982) averaged over a sliding window of five residues. ASA was calculated assuming the water radius to be 1.4 Å, and the main-chain *B*-factor is the average value of the main-chain atoms. **D:** Main-chain RMSD in the 17 NMR structures. In **A**, **C**, and **D**, the locations of β-strands ($\beta_0 - \beta_I$) and helices (H) based on the X-ray coordinates are indicated.

Materials and methods: *Materials:* Several genetic variants of bovine β-lactoglobulin have been identified in the cow, the most common being variants A and B that differ at positions 64 (Asp/Gly) and 118 (Val/Ala). β-Lactoglobulin A was expressed in the methyltropic yeast *Pichia pastoris* by fusion of the cDNA to the α-factor prepro-leader peptide from *Saccharomyces cerevisiae* as described previously (Kim et al., 1997). Sequencing of the amino-terminal of the secreted β-lactoglobulin (Glu(-3)-Ala(-2)-Glu(-1)-Ala1-Tyr2-Val3) revealed the two residues of the α-factor prepro-leader (Glu(-3)-Ala(-2)) and the spacer repeats (Glu(-1)-Ala1), as compared to the amino terminus of intact bovine β-lactoglobulin (Leu1–Ile2–Val3). The conformation and stability of the recombinant β-lactoglobulin were indistinguishable from those of the natural protein obtained from cow's milk (Kim et al., 1997).

NMR measurements: ^{15}N relaxation measurements were conducted using a Bruker DRX-600 at the Protein Research Institute of Osaka University, Osaka, Japan. NOE spectroscopy–heteronuclear single quantum coherence (NOESY-HSQC) was measured using a Bruker DMX-750 at Kobe University. A total of 1 mg of ^{13}C, ^{15}N-labeled β-lactoglobulin was dissolved in 200 μL of 20 mM HCl (95% H_2O/5% D_2O). The pH was 2.0, and the sample was kept in a 5 mm microcell (Shigemi, Tokyo). Complete sequence-specific NMR assignments for recombinant β-lactoglobulin were obtained based on scalar coupling connectivities (Kuwata et al., 1998). The majority of distance restraints were obtained from ^1H–^{15}N NOESY-HSQC (Marion et al., 1989) and ^{13}C NOESY-HSQC (Muhandiram et al., 1993). NOESY-HSQC experiments were performed by varying the mixing time from 90 to 120 and 200 ms. The acquisition time for each three-dimensional spectrum was about four days with 2,048 points in direct dimension. Data processing and peak selection were performed using the FELIX program version 95.0 (Biosym Technologies, San Diego, California). To calculate the monomeric structure, 2,243 distance restraints were supplemented with ϕ angle restraints based on HNHA data, χ_1 angle restraints based on HNHB data, and hydrogen bond restraints based on solvent exchange and NOE data. The three-dimensional structure of β-lactoglobulin was computed using DYANA (Guentert et al., 1997) to obtain the overall structure and X-PLOR (Nilges et al., 1988) for refining the structure. The coordinates are deposited at the Protein Data Bank (PDB) under accession code 1cj5.

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