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High helicity of peptide fragments corresponding to β-strand regions of β-lactoglobulin observed by 2D-NMR spectroscopy

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Background: Whereas protein fragments, when they are structured, adopt conformations similar to that found in the native state, the high helical propensity of β-lactoglobulin, a predominantly β-sheet protein, suggested that the fragments of β-lactoglobulin can assume the non-native helical conformation. In order to assess this possibility, we synthesized four 17–18-residue peptides corresponding to three β-strand regions and one helical region (as a control) of β-lactoglobulin and examined their conformation.

Results: We observed residual helicities of up to 17% in water, by far-UV CD, for all four peptide fragments. The helices could be significantly stabilized by the addition of TFE, and the NMR analyses in a mixture of 50% water/TFE indicated that helical structures are formed in the central region whereas both termini are frayed. Thus, the very same residues that form strands in the native β-lactoglobulin showed high helical preferences.

Conclusions: These results stand out from the current general view that peptide fragments isolated from proteins either are unfolded or adopt native-like secondary structures. The implications of the results in the mechanism of protein folding and in designing proteins and peptides are significant.

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Keywords: α-helix, β-lactoglobulin, β-strand, 2D-NMR, protein folding

Received: 26 Feb 1996
Revisions requested: 22 Mar 1996
Revisions received: 22 Apr 1996
Accepted: 26 Apr 1996

Published: 17 Jun 1996
Electronic identifier: 1359-0278-001-00255

Folding & Design 17 Jun 1996, 1:255–263

© Current Biology Ltd ISSN 1359-0278

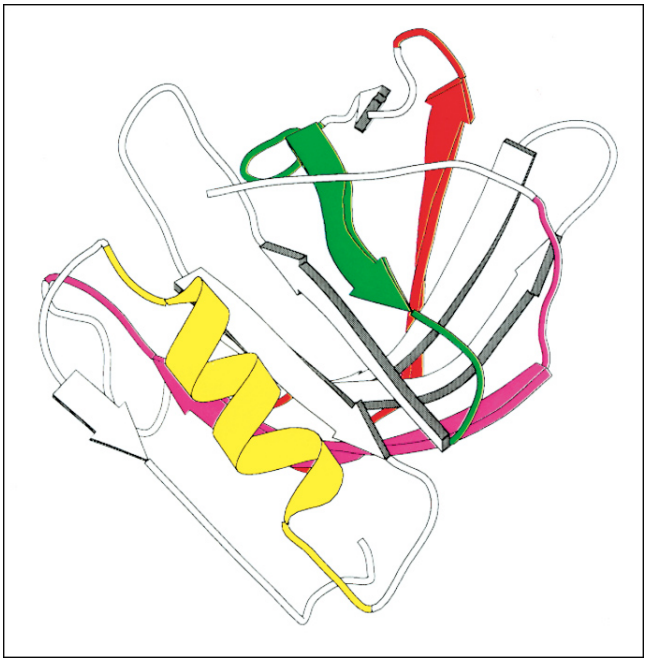
Introduction

Although the two-state protein folding model suggested a priori that no fragments should remain structured when isolated, several residual structures have now been observed in peptide fragments corresponding to regular secondary structures from native proteins [1–7]. The current consensus is that when these peptides are structured, they form secondary structures consistent with those observed in the native structure. This is known as the consistency principle [8] and supports the framework model of protein folding [9,10]. These structured fragments usually correspond to regions of the native structure that are especially stable and remain folded in the (destabilized) molten globule state [11–13]. In addition, these residual structures are observed mainly in segments that fold first [14,15], so the residual structures observed in these fragments are believed to reflect conformations that prevail in the early stage of protein folding, when local interactions predominate [2,16].

Bovine β-lactoglobulin is a predominantly β-sheet protein with nine β-strands (A–I) and one α-helix [17] (Fig. 1). Although β-lactoglobulin exists as a dimer at neutral pH and as a monomer below pH 3, it still retains a native conformation even at pH 2. Its folding process has been studied by circular dichroism (CD) stopped-flow experiments, and it has been shown that the CD signal exhibits an 'overshoot' phenomenon [18,19]. This means that the residue ellipticity is larger (in absolute value) at the burst phase intermediate of folding than in the final native state. The additional residual ellipticity is often interpreted as a far-UV CD contribution of the aromatic groups. However, this additional contribution can also be interpreted as an increase in helical structure. This alternative interpretation is compatible with more recent kinetic experiments at different wavelengths; the kinetic far-UV CD spectrum of the overshoot intermediate strongly suggests the transient accumulation of a partly α-helical intermediate [19,20]. The possibility of a helical intermediate is further reinforced by the observation of a high helical preference for the β-lactoglobulin amino acid sequence, which was clarified by the addition of *2,2,2*-trifluoroethanol (TFE) [21] and also by the reduction of the disulfide bridges [21]. This unusual preference of the β-lactoglobulin sequence for helical structure is also observed when methanol and propanol are added [22]. Thus, these reports further support the idea that the intact sequence of β-lactoglobulin has an intrinsic preference to adopt helical structure, and that the tertiary structure along with the disulfide bridges have an essential role in stabilizing the native β-strands of β-lactoglobulin.

In a previous paper [23], we reported that two peptide fragments of bovine β-lactoglobulin B which form β-strands in the native structure exhibit high helical preferences. In this paper, we further characterize the structures of the four isolated peptides of β-lactoglobulin B, including those reported previously, by two-dimensional nuclear magnetic

Figure 1



Schematic structure of bovine β-lactoglobulin drawn using Molscript [46]. The segments corresponding to the synthetic peptide fragments 1, 2, 3 and 4 are colored purple, red, green and yellow, respectively. Fragment 4 corresponds to the helix formed between residues 130 and 140 and is used as a control fragment.

resonance (2D-NMR) and show a rather high helical preference for all the peptides. The observed high helical propensities are consistent with the predicted secondary structure propensities. The results suggest a case of non-hierarchical protein folding, in which non-native β-structures may be involved in the early stage of protein folding.

Results and discussion

Selection of the peptides

The spatial positions of the segments with sequences corresponding to those of the synthetic peptides (Table 1) are shown in Figure 1. The sequences of fragments 1–3 correspond to the A, D and F strands, respectively. The sequence of fragment 4 corresponds to the helix which extends from residues 130–140 and is located between strands G and I. It is noted that fragment 3 (85–101) in the present manuscript was not included in the previous study [23] and fragment 4 (127–142) in the present manuscript corresponds to fragment 3 in the previous study [23]. These peptide segments were of particular interest because they were predicted to have a high helicity by secondary structure prediction methods [24] (see below). In determining the precise location for our fragments, we chose regions that include negatively and positively charged groups located at the N and C termini, respectively, to counter-balance the macrodipole of the helix.

Table 1

Sequences of bovine β-lactoglobulin B fragments.

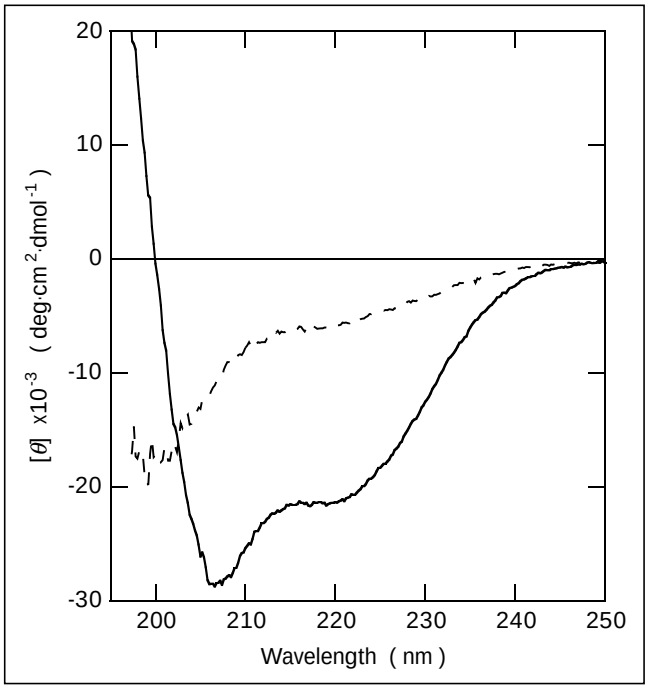
Fragment 1 (11–28)	Ac-D I Q K V^{15} A G T W Y^{20} S L A M A^{25} A S D-NH_2
Fragment 2 (61–77)	Ac-W E N G E^{65} C A Q K K^{70} I I A E K^{75} T K-NH_2
Fragment 3 (85–101)	Ac-D^{85} A L N E N^{90} K V L V L^{95} D T D Y K^{100} K-NH_2
Fragment 4 (127–142)	Ac-Y E V D D^{130} E A L E K^{135} F D K A L^{140} K A-NH_2

The sequences of the four peptides are shown with residue numbers corresponding to those of the intact β-lactoglobulin. An additional tyrosine residue has been added to the N terminus of fragment 4 for concentration determination.

Far-UV CD

The far-UV CD spectra of these fragments at pH 2 showed that the peptides retain residual helical structures even in aqueous solution (Fig. 2; Table 2; see also [23]). The peptides showed similar helical preferences at pH 7 (data not shown). However, because some of them exhibited a tendency to aggregate at pH 7 at the concentration used for the NMR measurements (1 mM), we carried out the following measurements at pH 2, where the native state is still intact. First, the helicity of fragment 4 which corresponds to the α-helix in the native

Figure 2



Far-UV CD spectrum of fragment 2 in the presence (solid line) and absence (broken line) of 80% TFE at pH 2.0 and 20°C.

Table 2

Mean residue ellipticity at 222 nm and helicity.

Peptide*	$[\theta]_{222}$ in water†	Helicity in water‡	Predicted helicity§	$[\theta]_{222}$ in 50% TFE†	Helicity in 50% TFE‡
Fragment 1	–6400	17.4 (13.4)	7.0 (6.1)	–29100	81.5 (88.3)
Fragment 2	–5500	14.8 (10.4)	3.5 (7.2)	–20100	56.1 (58.6)
Fragment 3	–3300	8.6 (3.1)	2.1 (1.4)	–27100	75.8 (81.7)
Fragment 4	–6200	16.8 (12.7)	4.8 (11.8)	–23500	65.7 (69.8)
RCM β-lactoglobulin	–6400	17.4 (13.4)	3.8 (5.3)	–27100	75.8 (81.7)

*The fragment sequences are listed in Table 1. For CD experiments, both N and C termini were protected by acetylation and amidation. RCM β-lactoglobulin is the β-lactoglobulin polypeptide with the two disulfide bridges reduced and carboxymethylated [23]. †In 20 mM HCl at pH 2.0. ‡The helicity was estimated from ellipticity values at 222 nm ($[\theta]_{222}$) according to $fH = -([\theta]_{222} + 260)/35647$ (T=20°C, and N = 17 in Scholtz *et al.* [44]). For comparison, the helicity computed using $fH = -([\theta]_{222} + 2340)/30300$ is listed in parenthesis [45]. §Averaged helicity predicted for peptides with the program AGADIR [35]. The conditions were as follows: 278K, pH 2 and both ends protected. The values in parenthesis are calculated for pH 7. As a control for this program to reproduce experimental helicities, we found a good correlation of the predicted helicity with experimentally measured values for three peptides derived from cytochrome *c* [3], for peptide III (which is an analog of the C-peptide [42]), and for a peptide derived from lysozyme that does not form helices.

β-lactoglobulin is ~17% in water (Table 2). This value is larger than those observed for the peptides corresponding to the helices of myohemerythrin [25]. It is, however, smaller than the helicity of the 15-residue peptide corresponding to the C-terminal helix of cytochrome *c* [3] or the C-peptide of RNase A [26,27], which exhibit especially high helicity even in aqueous solution. Thus, the residual helicity in fragment 4 is high but not exceptional.

On the other hand, the three other peptides corresponding to β-strands in the native protein have helicities similar to that observed for fragment 4 (Fig. 2; Table 2), although the helicity in fragment 3 (~9%) is slightly less than that of the others. This was less anticipated, as from a consistency point of view [8] one expects the same (if any) structures to be stabilized by local and non-local interactions. Inevitably, examples of residual structures adopting a β-strand are not many [28–30], because non-local interactions are absent in such fragments. For an example of a stable β-sheet peptide, Searle *et al.* [30] reported that a 16-residue peptide derived from the N-terminal sequence of ubiquitin forms a monomeric β-hairpin in aqueous solution. Instead, peptides corresponding to β-strands in the native structure are often unfolded in water [31].

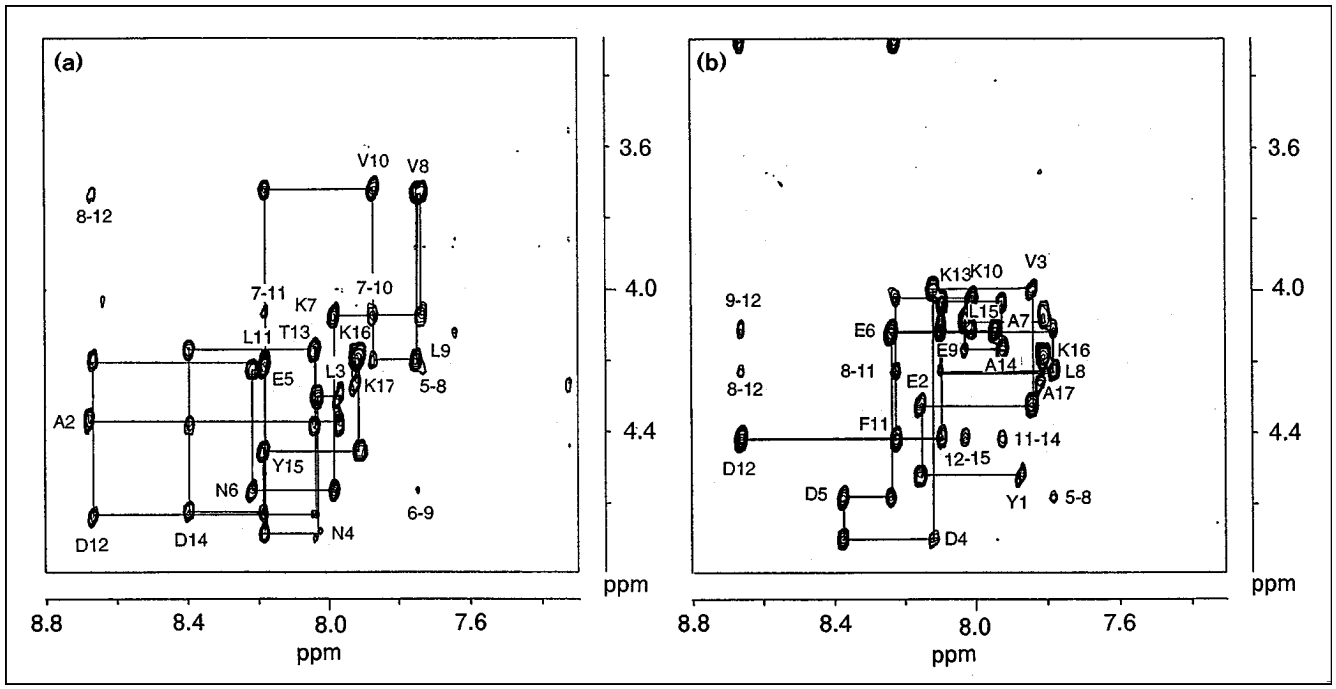
Although the far-UV CD spectra of fragments 1–3 in water are intermediate between random coil and helix, they are more characteristic of α-helix than those of peptides corresponding to helices A, B and C of myohemerythrin [25]. They are actually close to the spectra of myohemerythrin peptides in 10–15% TFE.

TFE usually increases the helicity of fragments in regions that form helices in the native structure, while leaving those derived from β-regions essentially unstructured [31–33]. Thus, the effect of TFE is generally considered to be specific. In some cases, TFE increased the helicity of peptides derived from β-strands, but the helicity is quite low, at most 50% [29,33,34], and the coil-to-helix transition is broad [23]. In contrast, the TFE dependencies of β-lactoglobulin peptides show a highly cooperative coil-to-helix transition [23]. Such an abrupt transition is clearly distinct from the gradual increase (if any) usually observed for peptide sequences corresponding to other β-proteins and emphasizes the high intrinsic helical preference of the peptides [23]. A high helical propensity in the presence of TFE is also reported for a 15-residue peptide including the last β-strand region from ubiquitin [35].

NMR analysis

The NMR analyses were performed for fragments 2, 3 and 4. To increase the solubility of fragment 2, we used a peptide with a free N terminus. The CD spectrum of the peptide with a free N terminus was the same as that with an acetylated N terminus. Some of the NMR data for fragment 2 have been reported previously [23]. NMR analyses could not be carried out for fragment 1 because it

Figure 3



Fingerprint region of the NOESY spectra of (a) fragment 3 and (b) fragment 4. The spectra were measured in 50% TFE/H_2O at 20°C, pH 2.2 and the mixing time was 250 ms. The peptide concentrations determined spectrophotometrically using molar absorption coefficients of 1400 and 5600 for tyrosine and tryptophan, respectively, were 3.3 mM for fragment 3 and 4.5 mM for fragment 4. The chemical shifts are in ppm and are referenced to 2,2-dimethyl-2-silapentane-5-sulfonate. The sequential assignments are indicated as well as *i*, *i*+3 signals.

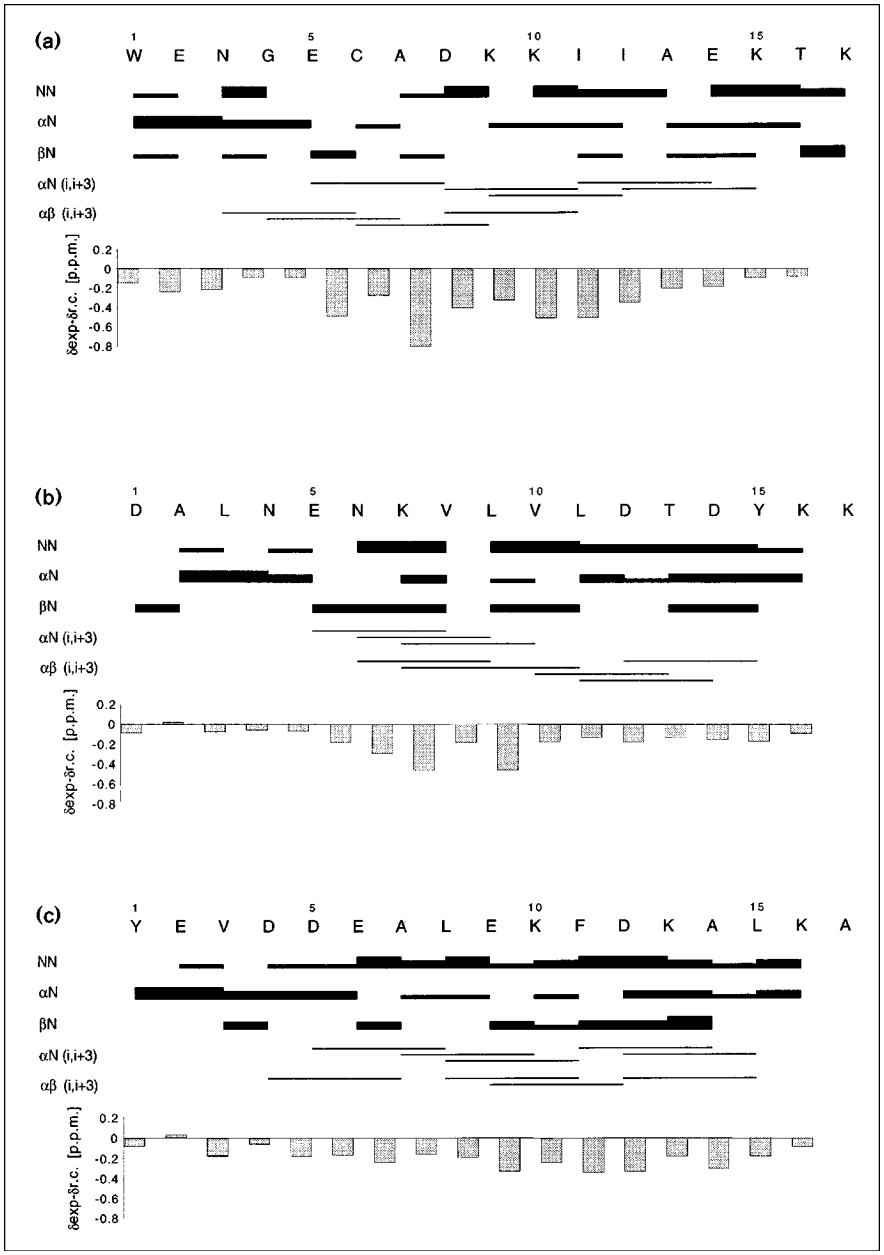
formed gels at concentrations around 1 mM, both in H_2O and in 50% TFE/H_2O. We also tried to increase the solubility of the peptide either by varying the pH or by using peptides with free termini, but without success. The assignment of the NMR resonances was obtained by standard sequential assignment method [37] using double quantum filtered correlation spectroscopy (DQF-COSY), nuclear Overhauser effect spectroscopy (NOESY; with mixing times of 250 and 350 ms) and total correlation spectroscopy (TOCSY; with mixing times of 40 and 80 ms) experiments. All peaks were assigned and tables of H-NMR resonance assignments of fragments 3 and 4 are available as Supplementary material (published with this paper on the internet; see [23] for the chemical shifts of fragment 2).

In general, the resonances were well dispersed with many H^{α} peaks shifted below 4 ppm. Panels a and b of Figure 3 show the amide-C^{α} regions of the NOESY spectra of fragments 3 and 4, respectively (see [23] for the NMR spectra of fragment 2). Many strong N(*i*,*i*+1) signals were observed. This, in conjunction with the presence of weak αN(*i*,*i*+1) signals, is characteristic of helical structures (Fig. 4). In addition, αN(*i*,*i*+3) and αβ(*i*,*i*+3) signals support the presence of helical structures in the three peptides (Figs 3,4). Although the distinction between α-helices and 3_{10}-helices on only the basis of the present NMR data is difficult, it is interesting to notice some αN(*i*,*i*+4) resonances (Fig. 3), which suggest α rather than 3_{10} structures [37].

The chemical shifts of the α protons are generally shifted upfield when the backbone adopts a helical conformation [36]. Combined with the observation of medium-range NOEs, the differences between the chemical shifts of the peptides and the values reported for random coils [37] can yield meaningful information on the extension of helical structures. As one anticipates, the NMR data confirm that the body of the peptides are helical whereas the ends are frayed (Fig. 4).

Comparison of this result with the structures observed in the native state [17] is instructive. According to the Kabsch and Sander algorithm [38], strand D starts at residue 68 and ends at 75, whereas the helix is observed in the fragment from residue 64 to residue 75 (Fig. 4). Similarly, strand F extends from residues 91–97, whereas the helix is formed in the peptide fragment by residues 90–101. This means that the helices are formed by the very same residues involved in the β-strands of the native

Figure 4



Summary of the sequential and medium-range NOE connectivities of (a) fragment 2, (b) fragment 3 and (c) fragment 4. The thickness of the lines reflects the intensity of the sequential connectivities, i.e. strong, medium, and weak, according to the number of cross-peak contours. The lower plots show the difference of chemical shifts of α-protons between the values measured in our (helical) peptides and for random coil in tripeptides [37].

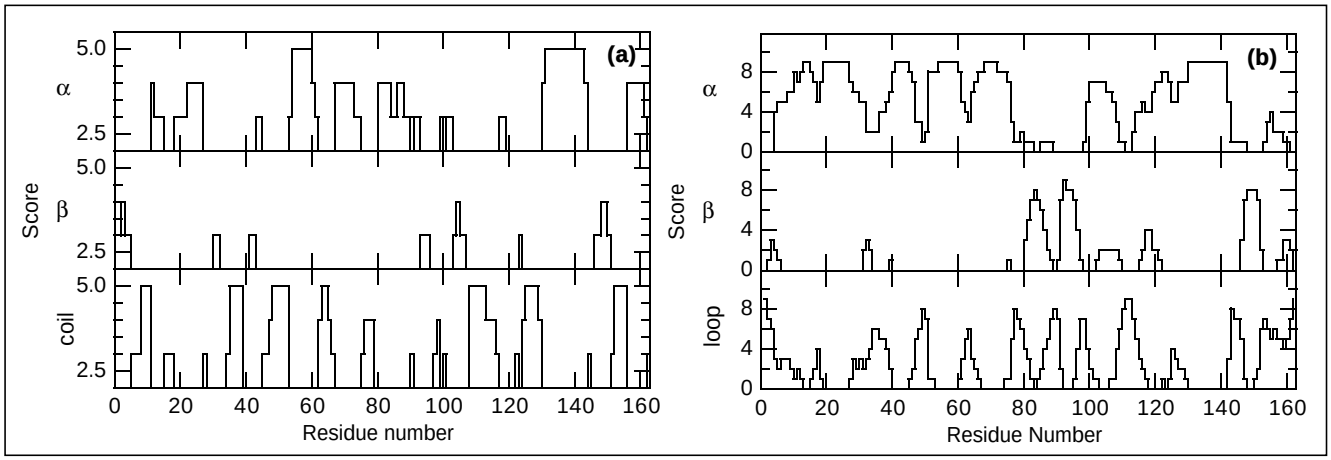
structure. Thus, the helices observed by far-UV CD are not artifacts arising from adjacent residues which form coil in the native structure.

Finally, it should be noted that no minor signals were observed and that all observed peaks were identified. As we do not expect these short peptides to form rigid helices, we interpret the absence of minor peaks in terms of a dynamic equilibrium, with the major species being in a helical conformation. The sharpness of the signals also suggests that the exchange occurs in the fast exchange limit (fast exchange with respect to chemical shift, i.e. faster than one-tenth of a millisecond).

Intrinsic preference for helical conformation

The present observation of helical conformations is supported by secondary structure prediction methods. Panels a and b of Figure 5 show the results of secondary structure prediction by a joint prediction method [24] and PHD method [39–41], respectively. Both are three-state prediction methods, predicting the probabilities of α-helix, β-sheet, and coil (or loop) on the basis of the structural

Figure 5



Predicted secondary structures of bovine β-lactoglobulin B by (a) a joint method [24] and (b) a PHD method [39–41]. See Fig. 6 for the secondary structures and their locations in the native β-lactoglobulin.

database, including various information to improve the accuracy of prediction. The PHD method, which uses multiple sequence alignment in order to find out the family's secondary structure, may be the best method available, achieving a three-state prediction accuracy better than 70%. It is argued that PHD gives a better prediction of β-strand, the most difficult of the three states to predict, than other methods. In the case of the PHD prediction of β-lactoglobulin, the 17 related proteins were aligned, although the crystallographic structure of none of them was available. It is noted that the X-ray coordinates of β-lactoglobulin, kindly provided by L Sawyer, are not yet in the Protein Data Bank.

The results of the joint method (Fig. 5a) and PHD method (Fig. 5b) are similar to each other. Both methods emphasize that the preferred backbone configuration of β-lactoglobulin is the helical one. It is remarkable that PHD, probably the best method to predict β-strand, predicted most of the strands to be helix. Both methods predicted the C-terminal helix (130–140) correctly. The parameters in these methods are calculated from different native structures without explicit consideration of protein folds, and therefore one can expect that the contribution of the non-local interaction is averaged out. This averaging, and the fact that the non-local interaction is essential for determining the secondary structures of β-lactoglobulin, may be the origin of the marked failure of secondary prediction methods to predict the native β-strands. Thus, these methods predict that the amino acid sequence of β-lactoglobulin intrinsically prefers helices compared to β-strands and coil structures. In other words, the consistency principle [8] is violated in the case of β-lactoglobulin. Still, we should be careful when claiming the violation of the consistency principle, as no natural proteins are expected to strictly follow the consistency principle.

Because the amino acid sequence of β-lactoglobulin is predicted to adopt an α-helical rather than a β-structure, we can further analyze this helical preference using a helix-coil prediction method developed for peptides (AGADIR [35]). This program considers only the coil and helix states (i.e. a two-state prediction) and, in contrast to usual secondary prediction methods, does not include long-range structure effects either explicitly or implicitly. It cannot indicate whether our peptides have a preference for β-structure, but it is well adapted to compute the helicity of our peptides in the absence of tertiary structures, as our peptides do not have an intrinsic preference for β-strands (Fig. 5).

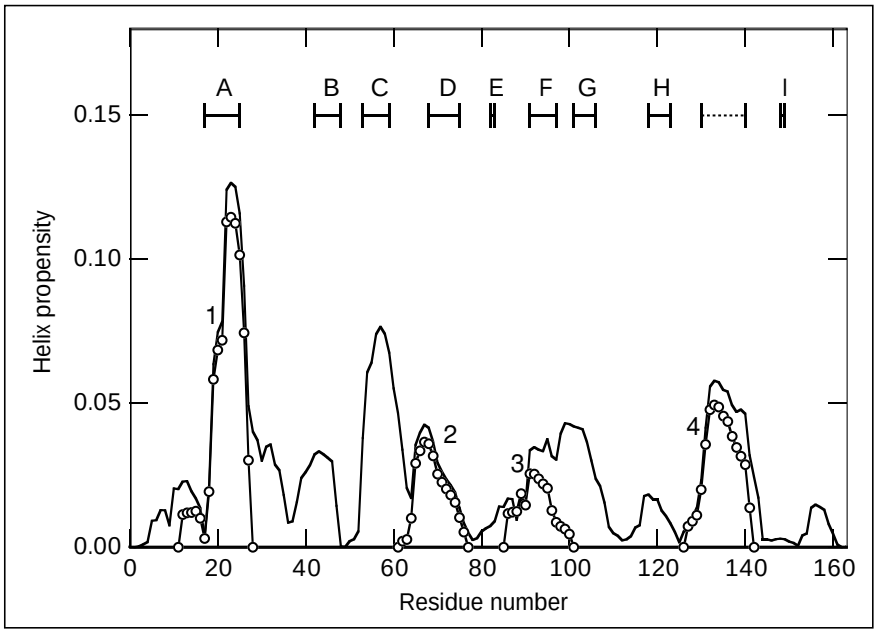
Figure 6 shows the predicted helical propensity of β-lactoglobulin and fragments 1–4. According to AGADIR, at pH 2 fragment 1 corresponds to a region that is more helical than the other regions, and some residues have predicted helicity up to 13%. The average helicities of the peptides are lowered because of end effects, and the average predicted helicities range from ~2% for fragment 3 to 7% for fragment 1 (Table 2). It is noted that the order of the calculated helical preference at pH 2 in water of the four fragments agreed with the observed order (i.e. fragment 1 > fragment 4 > fragment 2 > fragment 3).

Implications for protein folding and design

The concordance of the results obtained from peptide fragments experiments and hydrogen exchange experiments (both in native and in equilibrium intermediate states) [1,3,26,42,43] seems to support the hierarchical protein

Figure 6

Helicity of bovine β-lactoglobulin B predicted with the program AGADIR [35]. The conditions were as follows: 278K, pH 2 and both ends protected. The continuous line represents the helicity calculated for the entire β-lactoglobulin sequence. The open circles represent the helicities of the peptides synthesized in this study. The fragment numbers are indicated. The location of nine β-sheet segments (A–I) and an α-helical segment (dotted line) of the native β-lactoglobulin as determined by the DSSP algorithm [38] are indicated.



folding model, in which native-like secondary structures are formed in some regions of the unfolded protein and serve as initiation sites of protein folding [9,10]. The results are also consistent with the consistency principle [8]. Whereas the previous studies emphasized that the rate-limiting step of protein folding is located between the structured molten globule state and the native state, recent observations of rapid protein folding without detectable accumulation of the intermediate argue that folding of small globular proteins follows a nucleation-condensation mechanism [9,10]. Although the two models are different in the location of the rate-limiting step, both are similar in the sense that the elements of native structures are formed sequentially (i.e. hierarchical mechanism of protein folding). As mentioned above, our results suggest that β-lactoglobulin violates, at least partly, the consistency principle. The implications of the present results for the mechanism of protein folding and consequently for *de novo* design of proteins and peptides are significant.

The high helical preference of β-lactoglobulin and its fragments provides a reasonable interpretation for the overshoot phenomenon observed during the refolding reaction of this protein and strongly suggests the transient accumulation of a partly α-helical intermediate before formation of the native β-sheet structure [19,20]. Still, it is an important open issue whether such a non-native α-helical intermediate is a productive intermediate located on the correct folding pathway or a trapped intermediate preventing the rapid and smooth folding of this protein. Nevertheless, the refolding kinetics of β-lactoglobulin and its high helical preference imply that the non-hierarchical protein folding does occur at least for some β-sheet proteins.

Another important implication is for the design of proteins and peptides. When we design the conformation of proteins and peptides, we assume that the designed structure is similar to that in the model native structure. The present results provide an excellent example that this is not necessarily true. In the case of β-lactoglobulin, although the local interactions favor α-helix, β-sheet is stabilized in the native structure because of the participation of non-local interactions. The various methods of secondary structure prediction, which cannot consider the unique non-local interactions, concluded that β-lactoglobulin has a very high preference for α-helix. Indeed, the fragments of β-lactoglobulin as well as the disulfide-reduced β-lactoglobulin [21] exhibit markedly high helical preference, validating the secondary structure prediction.

It is expected that the extent of disagreement between the intrinsic secondary structure propensities and the native propensities varies depending on the protein species. Shiraki *et al.* [21] showed that β-lactoglobulin is not an exceptional case but is located at the edge of variation. Therefore, when we design a protein or peptide in which the native-like tertiary interaction is absent or ambiguous, we should consider the possibility that the conformation is not necessarily similar to that in the native state. In such a case, we should pay attention to the secondary structure prediction, which might be more reliable than that anticipated from the reference native structure.

Materials and methods

Peptide synthesis

The peptides were synthesized according to the standard protocol of solid phase method (F_{moc} chemistry) with an Applied Biosystems peptide synthesizer (model 430A). The N terminus was acetylated before cleavage from the resin (4-(-2′,4′ dimethoxyphenyl-F_{moc}aminomethyl) phenoxy resin). The crude peptides were purified with a reversed phase HPLC (Senshu Pak, C18, 5μ, 300 Å). The purity of the peptides were checked by analytical reversed phase HPLC, amino acid analysis and electrospray ionization mass spectroscopy.

CD

CD measurements were carried out with a Jasco spectropolarimeter, model J-500A, at 20°C at pH 2. Mean residue ellipticity [θ] was expressed in deg cm^2 $dmol^{-1}$. The peptide concentrations were 0.1–0.2 mg ml^{-1}, and a cell of 1 mm light path was used. Typically, 20 μl of peptide fragments at a protein concentration of 1 mg ml^{-1}, dissolved in 20 mM HCl, was mixed in 180 μl of 20 mM HCl (pH 1.8). The final pH of the solution containing peptides is 2.0. The protein concentrations were determined spectrophotometrically using the molar absorption coefficients at 280 nm.

NMR

DQF-COSY, NOESY with mixing times of 150, 250 and 350 ms, and TOCSY with a mixing time of 40 and 80 ms experiments were recorded with 512 t_1 increments and 2K data points. The experimental data were zero filled to give a 4K × 2K data matrix. The spectra were recorded at 20°C and pH 2.2 in 50% (v/v) H_2O/TFE with an AM500 Bruker spectrometer.

Supplementary material

Tables of H-NMR resonance assignments of fragments 3 and 4 are published with this paper on the internet.

Acknowledgements

We thank L Serrano (EMBL, Heidelberg) for providing us with the program AGADIR, C Sander and B Rost (EMBL, Heidelberg) for advice about their PHD program, and L Sawyer (University of Edinburgh, UK) for the β-lactoglobulin coordinates. We thank H Nakamura for his interest in this work. This work was supported in part by Grants-in-Aid for Scientific Research from the Ministry of Education, Science and Culture of Japan, by fellowships from the Japan Society for Promotion of Science for Japanese Junior Scientists, and by the Ciba-Geigy Foundation (Japan) for the Promotion of Science.

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