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Conformational Studies of the C-Terminal 16-Amino-Acid-Residue Fragment of the B3 Domain of the Immunoglobulin Binding Protein G from *Streptococcus*

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Abstract

The structure and stability of the 16-amino-acid-residue fragment [IG(46–61)] corresponding to the C-terminal β -hairpin of the B3 domain of the immunoglobulin binding protein G from *Streptococcus* was investigated by means of CD and NMR spectroscopy and by differential scanning calorimetry. The CD and 2D NMR experiments were carried out (i) in water at different temperatures and (ii) at one temperature (305 K), with only CD, at different TFE concentrations. Our results show that the IG(46–61) peptide possesses organized three-dimensional structure at all investigated temperatures. The three-dimensional structure of the IG(46–61) peptide resembles the general shape of a β -hairpin that is also observed for this peptide in the experimental structure of the B3 domain in the whole G protein; the structure is stabilized by hydrophobic interactions between nonpolar side chains. Our study shows that the melting temperature of the IG(46–61) peptide is about 320 K which supports the hypothesis that the investigated peptide can serve as a folding initiation site of the B3 domain of the immunoglobulin binding protein G.

Keywords

peptide structure; β -hairpin; B3 domain of protein G; NMR; CD

Introduction

Proteins fold in different ways but a folding pathway always proceeds through sequential events, which lead to the native conformation.¹ One of the methods, which help to deduce the folding mechanism, involves a conformational study of protein fragments corresponding to regular secondary structure. Short fragments of proteins enable one to study local interactions isolated from the protein context and, therefore, indicate the importance of these interactions in determining protein secondary-structure elements. It has been shown^{2,3} that some short protein fragments can fold in aqueous solution into native-like conformations when no tertiary

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This article is dedicated to Professor Zbigniew Grzonka on the occasion of his 70th birthday.

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interactions are present. Thus, these fragments may play important roles as nucleation centers in initiating protein folding through local interactions,^{4–6} and provide knowledge about the earliest events of protein folding.

Unfortunately, most structural studies of peptides, which are related to secondary structure elements, are carried out at very low temperatures, less than 283 K.^{2,3} Low-temperature studies or structural studies performed at a single temperature, however interesting, do not provide valuable information about the forces driving protein folding. For example, it is well known that hydrophobic effects are strongly temperature dependent.⁷ Examples in which thermodynamics data are coupled with structural information are rarely found in the literature. Such data would be extremely valuable for understanding the complex process of protein folding. The main motivation of this work is to conduct structural studies at different temperatures to provide information about the relation between structure and thermodynamics of local interactions, which play a role in the very early stages of protein folding. Even if such studies are related to a particular protein/peptide, they can lead to general, useful information about the protein-folding problem. More specifically, they can provide data with which to parameterize our course-grained united-residue (UNRES) effective energy function.^{8,9}

The β -hairpin structural motif consists of two strands that are adjacent in the primary sequence, oriented in an anti-parallel arrangement, and linked by a short loop of two to five amino acids. Although the literature is rich in studies of α -helical peptides,^{10–17} water-soluble, nonaggregating monomeric β -sheets have been studied only relatively recently.^{18–21} For reasons of design and chemical synthesis, these are almost exclusively anti-parallel β -sheets, although others have used non-natural linkers to engineer parallel-strand alignments.^{22,23} The early focus on peptides excised from native protein structures provided the first insights into autonomously folding β -hairpins. Studies of peptides derived from tendamistat,²⁴ the B1 domain of protein G,^{2,25} ubiquitin,^{3,26} and ferredoxin²⁷ showed that these sequences could exist in monomeric form without aggregating, but that in most cases they showed a very limited tendency to fold in the absence of tertiary contacts. The use of organic co-solvents appeared to induce native-like conformation.^{25–27} Focusing on the C-terminal 16-amino-acid-residue β -hairpin fragment from the B1 domain of protein G (PGB1), Blanco et al.² showed the first example of native-like folding in water. This peptide remains monomeric at high concentration.² It was estimated that this peptide adopts a population containing up to 40% native-like β -hairpin structure and, therefore, it was speculated that it would be the first folding initiation site of the protein that could provide a nucleation center on which the rest of the polypeptide chain would find a favorable environment to fold.² It should, however, be mentioned that Blanco et al. performed their experiments at very low temperature, 278 K. Low temperature usually stabilizes the structure; however, such results cannot be used directly to determine the mechanism of folding of proteins for which the folding temperatures are much more higher²⁸ (the folding temperatures of the B domains of protein G are around 353 K).^{29,30}

For our investigations, we have chosen the B3 domain of protein G (PDB ID code 1IGD) from *Streptococcus* sp. (strain G148) (Figure 1)³¹ because, as well as the B1 and B2 domains, it (i) has a unique symmetrical topology, consists of two anti-parallel packed β -hairpins and an α -helix in the middle of the sequence packed to the β -sheet, (ii) possesses a compact fold, in which 95% of the residues are involved in regular secondary structure elements, (iii) has a tight packing of the hydrophobic core, and (iv) has a large number of hydrogen bonds. All of these features (small size and high thermal stability) make the B3 domain an ideal system with which to study protein folding.^{29,32–35} The sequence of the peptide investigated in this work differs by only one amino acid from that of the peptide investigated by Blanco et al.²

Results

Differential Scanning Calorimetry (DSC)

As a first step of our investigation, we performed DSC measurements of the investigated peptide from the B3 domain. DSC measurements can answer two questions: (a) whether the folding/unfolding process for the investigated peptide is well defined and (b) whether the investigated peptide has a tendency to aggregate [see “Materials and Methods: Differential Scanning Calorimetry (DSC)” section]. The heat capacity curve for the investigated peptide is presented in Figure 2. A relatively sharp peak on the heat capacity curve is observed for the IG(46–61) peptide; this peak corresponds to a phase transition. On the basis of the heat capacity curve (using the two-state model), we determined the transition temperature (320.11 ± 0.14 K) and the enthalpy change for this transition ($\Delta H = 48.01 \pm 0.60$ kcal/mol).

Honda et al.³⁶ studied the folding of the C-terminal β -hairpin of the B1 domain of the immunoglobulin binding protein G. The peptide studied by Honda et al. differs from the one studied in this work; in our peptide the N-terminus was acetylated, the C-terminus was free, and it was mutated in one position. They determined the folding temperature of their investigated peptide to be 295.3 and 293 K from DSC and NMR measurements, respectively. Streicher and Makhatadze³⁷ investigated the designed peptide called “trpzip4”. This peptide is a triple mutant (three hydrophobic residues of the peptide studied by Honda et al.³⁶ are mutated to tryptophanes), and it has an amidated C-terminus and a free N-terminal amino group. Using DSC techniques, Streicher and Makhatadze, determined the folding temperature of the investigated peptide to be 348 K. With CD measurements, they obtained a transition temperature of 347 K. It should be noted that, in earlier studies of the same peptide, Cochran et al.³⁸ used CD measurements and determined the folding temperature as 343 K, five degrees lower than the temperature determined by Streicher and Makhatadze.³⁷ A comparison of our DSC measurements with those of other studies for similar peptides shows that even a small modification in the peptide sequence (single amino acid mutation, acetylation of N-terminal group) can change the melting temperature by 15–20 degrees.³⁶

CD Measurements

The CD spectra of the B3 domain of peptide IG(46–61) of protein G were recorded under different conditions: (a) H₂O at temperatures 278, 283, 293, 303, 313, 323, 333, 343, and 353 K (pH = 4.02) (Figure 3a) and in 10, 50, and 90% TFE/H₂O mixture at 305 K (Figure 3b).

A positive band around 190 nm and one negative band around 220 nm characterize a regular β conformation.^{39,40} The CD spectra of the IG(46–61) peptide change with temperature (Figure 3). The negative band at 220 nm, characteristic of a regular β conformation^{39,40} (Figure 3a), becomes more negative for temperatures up to 343 K. These results suggest that the population of regular β conformation of the peptide increases with increasing temperature. It is interesting that the ellipticity at 201 nm becomes more positive with increasing temperature (Figure 3a). According to literature data,^{39,40} the negative band at 201 nm is characteristic of a statistical-coil conformation. Thus, the positive increase of the ellipticity at 201 nm corresponds to decreasing statistical-coil content. This finding suggests that the structure of the investigated peptide becomes more ordered with increasing temperature (up to 353 K). On the basis of the CD data, we calculated the amount of regular secondary structure for all temperatures using the SELCON3 SP37A algorithm⁴¹ (see Table I). The data presented in Table I show that the content of β structure increases slowly and the content of statistical coil conformation decreases with increasing temperature. It can be seen that the highest content of β structure and the lowest content of statistical coil structure occurs at 343 K. The calculated β structure content (Table I) lies between 36% and 48.9% for all investigated temperatures. It is interesting that Blanco

and Serrano² reported about 40% of native-like β -hairpin for a very similar peptide, from the B1 domain of protein G in water at 278 K.

In Figure 3b and in Table I, the results of CD measurements performed in solutions with three different trifluoroethanol (TFE) concentrations are shown. These results indicate that the presence of trifluoroethanol does not change the β -structure content dramatically. The β structure of the IG(46–61) peptide is stabilized up to 50% of TFE concentration but, when the amount of TFE is greater, a conformation other than a β type becomes dominant. For a similar peptide, Blanco and Serrano⁴² showed that about 64% of β structure content was present at equilibrium with the statistical coil structures in 30% TFE. Our CD measurements showed that the amount of β structure never exceeds 50%. However, it should be noted that the overall shape of the CD spectra starts to develop features characteristic of the α -helical conformation with increasing TFE concentration. A positive increase of the ellipticity at 190 nm and a rapid decrease of ellipticity around 205 nm and 222 nm, which are characteristic features of the α -helical conformation, are observed. The deconvolution procedure detects an increased amount of helical structure with increasing TFE concentration (see Table I).

In the previous section, we described the results of our DSC measurements, which showed that the melting temperature of the investigated peptide in water is about 320 K. Cochran et al.³⁸ observed the largest changes in the ellipticity at 229 nm for the peptide from the B1 domain in water and, therefore, used the CD data at this wavelength to determine the melting temperatures of the peptides that they investigated. For our peptide, we also observed large changes of CD spectra in water at 229 nm (Figure 4); however, the ellipticity becomes increasingly negative rather monotonically at $\lambda = 229$ nm with temperature without sharp changes characteristic of a phase-transition. But we observe large changes of ellipticity not only at 220 nm but also at 190 nm. At these two wavelengths, the observed ellipticity changes have a characteristic sigmoidal shape, which enabled us to estimate the transition temperatures, which we estimated to be 311 and 303 K (based on CD measurements at 190 and at 220 nm, respectively) (Figure 4). Both estimated transition temperatures are lower than those estimated from the DSC experiment.

NMR Measurements

A more detailed structural analysis was carried out using NMR spectroscopy. The 2D ^1H - ^1H NMR spectra of IG(46–61) peptide were recorded in water at pH 4.02 at three different temperatures 283, 305, and 313 K to examine the influence of temperature on the structure. The chemical shifts of the proton resonances for the IG(46–61) peptide at these three temperatures are listed in Tables I–III of supporting information. In Figure 5, the ROE effects corresponding to interproton distances and the values of the $^3J_{\text{NH}\alpha}$ coupling constants are presented for NMR measurements carried out at different temperatures.

The sequential $\text{H}_\alpha(i) - \text{H}_\text{N}(i+1)$ ROE connectivities are always observed in the recorded spectra and they are mostly very strong. The $\text{H}_\beta(i) - \text{H}_\text{N}(i+1)$ connectivities are much weaker but are also found at all temperatures. In the β -hairpin structure, strong $\text{H}_\text{N}(i) - \text{H}_\text{N}(i+1)$ connectivities and weak $\text{H}_\alpha(i) - \text{H}_\text{N}(i+1)$ sequential ROE connectivities are expected in the turn region whereas, for residues in the β -hairpin, we should expect weak $\text{H}_\text{N}(i) - \text{H}_\text{N}(i+1)$ and strong $\text{H}_\alpha(i) - \text{H}_\text{N}(i+1)$ ROEs signals.^{2,43} Figure 5 shows that, at all temperatures, the $\text{H}_\alpha(i) - \text{H}_\text{N}(i+1)$ ROEs are weak or not observed in the turn region, defined by residues Ala53 and Thr54, and strong in the other part of the peptide sequence. The $\text{H}_\text{N}(i) - \text{H}_\text{N}(i+1)$ connectivities are observed only at 305 K, and they are very strong in the middle of the sequence, as in the turn region. Additionally, the $\text{H}_\text{N}(i) - \text{H}_\text{N}(i+1)$ ROEs at 305 K, are also preserved in the N- and C-terminal parts of the IG(46–61) peptide, which suggest bends in these parts of the molecule. Long-range distance ROE connectivities are also observed: $\text{H}_{\text{sc}}(i) - \text{H}_\text{N}(i+11)$, $\text{H}_{\text{sc}}(i) - \text{H}_\text{N}(i+12)$ and $\text{H}_\text{N}(i) - \text{H}_{\text{sc}}(i+15)$ at 283 K, $\text{H}_{\text{sc}}(i) - \text{H}_\text{N}(i+3)$, $\text{H}_{\text{sc}}(i) - \text{H}_{\text{sc}}(i+7)$ and $\text{H}_{\text{sc}}(i) - \text{H}_{\text{sc}}(i$

+12) at 305 K, and $H_{sc}(i) - H_{sc}(i+10)$ [where the subscript “sc” denotes side-chain proton(s)] at 313 K, respectively (Figures 5 and 6, Table II). These connectivities actually span the whole β -hairpin structure. The long-range connectivity in the turn region, $H_{sc}(i) - H_N(i+3)$, appears only at 305 K, which supports the very large number of $H_N(i) - H_N(i+1)$ ROEs in this part of the molecule. With increasing temperature, the number of long-range ROE signals decreases, which suggests a more flexible structure, especially in the turn region. It is interesting that, at the lowest temperature (283 K), these ROEs appear between the amide protons from one β strand and the side-chain protons from the other β strand. When the temperature increases, the side-chain protons start to be closer to other side-chain protons. This interesting observation suggests that more interactions between hydrophobic side chain protons appear when the temperature increases. This finding supports the general idea of a hydrophobically-driven folding process^{7,44} of this β -hairpin peptide and that hydrophobic interactions are stronger with increasing temperature.^{7,44–49}

It can be seen from Figure 6 and Table II that the main residues which stabilize the structure of the IG(46–61) peptide are hydrophobic residues: Trp48, Tyr50, Phe57, and Val59. These data support the statement that hydrophobic interactions (not hydrogen bonds) are responsible for the stabilization of the β -hairpin structure of the B3 domain, and are opposite to the findings of Blanco et al.² for the B1 domain.

To check for the existence of hydrogen bonds, we used the data from 1D ^1H -NMR spectra recorded at 283, 289, 297, 305, 313, and 321 K, to calculate the temperature coefficients for all amide protons in the investigated peptide (Table IV of supporting information). Very low values of the temperature coefficients are observed for the amide protons of the turn region: Thr54 (3.7 ppb/K), Thr56 (4.3 ppb/K), and Asp51 (–4.2 ppb/K), which indicates that these residues could either be involved in a stable hydrogen bond or are buried inside the peptide structure causing their isolation from the solvent.⁵⁰ We did not observe any signals in the ROESY spectrum, which could enable us to identify the potential acceptors of hydrogen bonds. If these hydrogen atoms were definitely involved in hydrogen-bond formation in β -structure, the ROE signals between H_α and NH hydrogen atoms would also be observed, but they are not.

Honda et al.,³⁶ as well as others authors,^{21,51} used chemical shifts of protons recorded at various temperatures to determine folding temperatures. Typically, chemical shifts of aromatic protons of tyrosine are used^{21,51} in such studies. In Figure 7 changes in chemical shifts of the δ and ϵ protons of Tyr50 induced by temperature are shown. The chemical shifts of the tyrosine δ protons show a rather monotonic increase with increasing temperature. The overall plot is almost identical with those reported by Wei and coworkers for a similar sequence.²¹ The estimated melting temperature is around 287 K. In Figure 7, we also present the chemical shifts of the Tyr50 ϵ protons as a function of temperature. From these data, we estimated the folding temperature to be 306 K.

MD Simulations

We used the structural data summarized in Figure 5 to carry out MD simulations of IG(46–61) with time-averaged restraints, to determine the structure of this peptide. In Figures 8–10, the most populated conformational families collected at 283, 305, and 313 K, respectively, are presented.

Using NMR restraints obtained at 283 K in MD simulations, we obtained four main families of conformations, which exhibit β -hairpin-like structure (Figure 8). All conformations in these families have preserved the turn region, which additionally is observed in exactly the same sequence position as in the native β -hairpin fragment. One of the most populated (25%) family (Figure 8b) exhibits a regular tightly packed native-like β -hairpin structure. The most important

difference in the structure between all of the conformations calculated using the restraints from NMR measurements at 283 K and that of the native β -hairpin is the position of the hydrophobic cluster (Trp48, Tyr50, Phe57, and Val59). In the structure of the whole protein, all these hydrophobic residues are located on one side of the β -hairpin structure whereas, in the peptide studied, the Trp48 and Val59 pair is on one side and the Tyr50 and Phe57 pair is located on the opposite side of the plane defined by the β -hairpin. This feature is even preserved in the family, which is the most similar to the native structure (Figure 8b). The long-range interactions (side-chain to backbone) (Figures 5 and 6) define the structure.

Using the restraints from the NMR spectra recorded at 305 K, we obtained 5 families of conformations, which together with the structures presented in Figure 8, exhibit the β -like hairpin shape (Figure 9). The turn region contains the same residues as in the native fragment, and the structure is defined by the long-range connectivities from the hydrophobic cluster (Trp48, Tyr50, Phe57, and Val59), as well as from the hydrophilic turn residues (Asp52 and Lys55). The interactions between Asp52 and Lys55 keep the turn tight and strengthen the sequential amide proton interactions (Figure 6). It seems that the turn region is much more stabilized at 305 K than at 283 K. It is clearly seen that the structures based on NMR data recorded at 305 K are more diffused and less packed than those obtained using NMR data recorded at 283 K.

Using the restraints obtained from the NMR spectra recorded at the highest temperature (313 K), we obtained 12 clusters of conformations, of which the five most populated are shown in Figure 10. At this temperature, the turn region is much more flexible than at lower temperatures (283 and 305 K), but the structure still possesses the shape similar to the native β -hairpin because of the one ROE between the side-chain protons of Thr49 and Val59 residues (Figures 6 and 7 and Table II), which allows the structure to bend into the β -like hairpin. Additionally, strong sequential $H_{\alpha}(i) - H_N(i+1)$ connectivities at the N- and C-terminal part of IG(46–61) (Figure 5) force small bends at these parts, similarly as at 305 K. However, most important is the location of the four hydrophobic residues in the middle of the sequence (Trp48, Tyr50, Phe57, and Val59). It can be observed that the location and orientation of the hydrophobic residues in the β -hairpin changes systematically with increasing temperature. At 283 K, Trp48 and Val59 are on the opposite side of the β -hairpin with respect to Tyr50 and Phe57. At 305 K, Trp48, Tyr50, and Val59 are on the opposite side of the β -hairpin with respect to Phe57. Finally, at 313 K, all four hydrophobic residues are located on the same side of the backbone of IG(46–61) to form a cluster. It should be noted, though, that the distances between these four hydrophobic residues from the cluster are too long to observe Overhauser effects.

The pattern of ROE connectivities observed in our NMR experiments at all temperatures, and the results obtained are very similar to those observed by Blanco and Serrano for a similar sequence.² One difference is that we have never observed long-range interactions between Trp48 and Phe57. The presence of this contact would imply the formation of a hydrophobic cluster composed of Trp48, Tyr50, Phe57, and Val59. Recently, Wei et al.²¹ have reported an NMR study of mutants of the peptide studied by Blanco and Serrano.² Wei et al. did not observe a Trp48 and Phe57 interaction, which supports our finding that, at low temperature, the hydrophobic residues of the peptide do not form the single hydrophobic cluster characteristic of the native β -hairpin of the entire protein.³¹

On the basis of the 1D ^1H -NMR spectra recorded at different temperatures, we calculated the temperature coefficients of all amide protons in the investigated peptide (Table IV of supporting information). The changes in the chemical shift of the amide protons at 283, 305, and 313 K (temperatures at which the 2D NMR experiments were recorded) are shown in Figure 1 in supporting information. The very low values that are observed for the amide protons of Thr54 (3.7 ppb/K), Thr56 (4.3 ppb/K), and Asp51 (−4.2 ppb/K) indicate that these protons

could be involved in hydrogen bond formation. However, we did not find any $H_{\alpha}(i) - H_N(i+1)$ connectivity in the ROESY spectra. On the basis of this finding, we did not use any restraints related to hydrogen-bond formation for those amide protons in our MD simulations. To determine the possibility of hydrogen bond formation involving Asp51, Thr54, and Thr56, we have analyzed the conformations of the MD trajectories. For a given structure, we assumed that a hydrogen bond is formed when the distance between the amide proton and the potential acceptor is smaller than 2.5 Å and the N—H···X angle (where X is a proton-acceptor atom) is in the range of 150–180 degrees. The MD trajectories obtained using NMR data recorded at 283, 305, and 313 K were analyzed separately. The data presented in Table III and Figure 11 indicate that, at 283 K, the amide protons from residues Asp51, Thr54, and Thr56 can form hydrogen bonds with many acceptors (listed in Table III); however, most of these hydrogen bonds are not native ones (see Figure 11). Increasing the temperature generally reduces the number of observed hydrogen bonds, as shown in Table III and Figure 11. We found that, except for the amide protons from residues Asp51, Thr54, and Thr56, all of the other amide protons form hydrogen bonds only rarely (in less than 10% of the structures from the given MD trajectory; data not shown). This analysis shows that structures from MD trajectories can reproduce some experimental data, which are not used as restraints in MD simulations.

Discussion

In this work, we have carried out extensive conformational studies of the 16-amino-acid-residue peptide corresponding to the C-terminal β -hairpin of the B3 domain of the immunoglobulin binding protein G from *Streptococcus*. The investigated peptide has a relatively high folding temperature of 320 K at pH = 4.02, as determined by the DSC technique. In comparison, the folding temperature reported in the literature for peptides of identical length and similar in structure range from 275 to 352 K, depending on sequence and experimental technique.^{21,36–38,51} Generally, the folding temperatures determined by using different experimental techniques agree well for a given sequence, as pointed out by many authors, supporting the statement that the observed processes could be described by a simple two-state model.³⁶ From our study, we found that the melting temperature determined from DSC measurements (320 K; Figure 2) does not correspond to those estimated from CD or NMR measurements; moreover, the latter differ depending on the specific observable that is monitored. Using the CD measurements, the melting temperature estimated from ellipticity changes at $\lambda = 190$ nm is 311 K but drops down to 303 K when estimated from ellipticity changes at $\lambda = 220$ nm (see Figure 4). Using the NMR measurements, the melting temperature estimated from the temperature dependence of the chemical shifts of the aromatic δ or ϵ protons of tyrosine is 287 or 306 K, respectively (see Figure 7). At first glance, the CD and some of the NMR data suggest that the melting temperature of the investigated peptide is about 305 K, which conforms with the results of Honda et al. obtained for a similar sequence.³⁶ However, by analyzing the ROESY spectra, it can be seen that the largest number of long-range interactions is observed at $T = 305$ K. If the melting temperature were close to $T = 305$ K, a significant decrease of the number of contacts should be observed, which is not the case (Table II, Figures 5 and 6). On the other hand, some agreement can be observed between the results of DSC measurements and the temperature dependence of the ROESY spectra. At $T = 313$ K, some strand-strand interactions characteristic of a β -hairpin or, at least, a U-shaped structure are still observed (Table II, Figures 5 and 6). This leads to a conclusion that the heat capacity peak observed at 320 K in the DSC measurements is associated with the final disruption of all cross-strand long-range hydrophobic interactions or, in other words, that the major energetic effect of folding is associated with the formation of the strand-strand hydrophobic contacts.

The change in the chemical shift of aromatic protons and especially the tyrosine residue has been used to determine the folding temperature of many sequences derived from the C-terminal hairpin of the B domains of immunoglobulin binding protein G.^{21,36,51,52} It can be seen from

Figure 7 that the chemical shifts of the δ protons of tyrosine increase almost linearly with increasing temperature until they approach the chemical shift limit expected for a peptide in the statistical-coil conformation, completely exposed to the solvent.^{21,51,53} Although there is no inflection point on the $\delta_\delta(T)$ curve, we determined the respective “melting temperature” (by fitting a two-state model to the data of Figure 7) to be $T_m = 287$ K. By analysis of the data of Figure 7 and Table II, we conclude that, at $T = 283$ K, the δ protons of tyrosine are not involved in any long-range interaction. At $T = 305$ K, these protons interact with aromatic protons of the phenylalanine residue, and at $T = 313$ K no long-range interactions can be observed. Consequently, as opposed to the information provided by the ROESY spectra, the change of the chemical shift of the tyrosine δ -protons seems to be very slightly related to the change of local environment (Table II). We can conclude that the “melting transition” temperature ($T_m = 287$ K) determined from the temperature dependence of the chemical shift of the δ protons of tyrosine rather corresponds to the passing of these protons from a water environment into close proximity of the phenylalanine aromatic ring which occurred in range of temperatures 283–305 K (see Table II) but is unrelated to the melting temperature of the whole peptide as determined from DSC measurement (see Figure 2).

The temperature dependence of the chemical shifts of the ϵ protons is different from that of the δ protons (Figure 7). It can be seen from Figure 7 that these chemical shifts do not change too much between $T = 283$ K and $T = 298$ K. A rapid increase of chemical shift values is observed above $T = 298$ K. The transition temperature determined by assuming a two-state model is $T_m = 306$ K. Once again, from the data of Figure 7 and Table II, it follows that the ϵ protons of tyrosine are involved in some long-range interactions only at $T \geq 305$ K. Therefore, in this case, the rapid change in chemical shift and the “melting temperature” are related to breaking long-range interactions (observed at 305 K but not at 313 K) and the full exposure of the ϵ protons of tyrosine to the solvent.⁵³

From the analysis of the temperature dependence of the chemical shifts of the δ and ϵ protons of tyrosine, we can conclude that, although the melting temperatures based on the changes in chemical shift of individual protons are correlated with some conformational changes of the peptide, it seems that those changes have only local character and, consequently, cannot be identified with global conformational changes upon folding of the whole peptide. If we identify the folding transition of the peptide under study with the formation of long-range strand-strand contacts, we can easily explain the difference between the “folding temperatures” determined from DSC and CD measurements. The CD data reflect a statistical distribution of peptide groups among different local conformational states; however, they provide no information as to long-range contacts. Therefore, similar to the chemical shifts, CD data can provide information about only local conformation of a polypeptide chain but the formation of local structure is not necessarily related to the formation of the final folded structure. Of course, especially when studying the folding of proteins and not peptides, formation of local structure often coincides with the formation of a global fold; in such a case, the melting temperature calculated from the temperature dependence of any structure-related observable (in particular, ellipticity or chemical shifts) conforms with that obtained directly from DSC measurements^{36,37} (although there are well-documented examples where the formation of secondary and tertiary structure is not correlated⁵⁴). However, this does not seem to be the case for the peptide studied in this work.

We made an attempt at demonstrating that long-range hydrophobic interactions are responsible for the transition peak observed in DSC measurements. We tried to record the NMR spectra above the folding temperature to check whether or not long-range contacts continue to appear. Unfortunately, we were not able to record quality NMR spectra at temperatures above $T = 320$ K. On the other hand, very recently, we are now investigating related peptides, which have lower melting temperatures than 320 K, and found that the long-range contacts indeed vanish

above the melting temperature determined from DSC measurements (Skwierawska et al., unpublished).

Taking into account all the available data, we can propose that the investigated peptide possesses a structure, which can be called “native” for this sequence at about $T = 305$ K. At this temperature, we can observe the maximum number of short-range and long-range contacts in the ROESY spectra (Table II, Figures 5 and 6), compared with the spectra measured at the other two temperatures. Consequently, the restraints determined from the ROESY spectra at 305 K determine the structure of the peptide very well, except for the N- and C-terminal dipeptides (Figure 9).

It is clearly shown that the structure at $T = 305$ K is stabilized by the interactions between the hydrophobic residues, and the turn region is quite well defined, even if we are not able to observe many hydrogen bonds in this region (Table III and Figure 11). Below and above 305 K, the number of long-range and short-range interactions observed in the ROESY spectra is diminished, which indicates structural changes. At $T = 283$ K, the structure is defined by many long-range interactions but most of these interactions occur between the amide and the side-chain protons. This type of interaction is also clearly visible in our hydrogen-bond analysis; as shown in Figure 11b and Table III, most of the observed hydrogen bonds are between amide protons and polar donors from side chains. This shift in contact pattern has an influence on the stability of the turn region. The number and strength of the short-range interactions observed in the ROESY spectrum is lower at 283 than at 305 K. Increase of temperature rapidly reduced the number of long-range interactions; at 313 K only one such interaction can be seen, namely T49–V59, and this is clearly a hydrophobic interaction (see Table II).

Looking at the presented results from the point of view of the mechanism of folding of proteins, the most important is the fact that, regardless of the side-chain to side-chain contact pattern, the investigated peptide forms a structure, which generally resembles the β -hairpin-like shape in a broad range of temperatures. This hairpin structure can indeed serve as a nucleation center for this class of proteins as postulated previously.² We provide evidence that hydrophobic interactions are responsible for structure stabilization. In the investigated peptide, we have five hydrophobic residues (two valines at position 47 and 59, respectively, a tryptophan at position 48, a tyrosine at position 50, and a phenylalanine at position 57). Our investigation shows that all of these hydrophobic residues contribute to the formation and stabilization of the β -hairpin structure. However, it will be interesting to know which hydrophobic residue/residues are absolutely essential for hairpin formation. Muñoz et al.⁵⁵ suggested that formation of the β -hairpin is initiated from the turn region and, when the turn region is formed, all other interactions zip up and stabilize the structure. Such mechanism would suggest that the pair of hydrophobic residues in positions 50 and 57, which are the closest to the turn region, has immediate impact on structure formation and stabilization.

To investigate the mechanism of the formation of the β -hairpin structure, we are currently carrying out systematic structural studies of peptides, which are based on the sequence of the investigated peptide but are systematically shortened by one residue at a time from both the N- and the C-terminus. Such systematic studies will eventually answer the question as to whether the turn region forms first and then the hairpin structure is stabilized by hydrophobic interactions or whether the hydrophobic interactions force the main chain in the turn region to bend and, consequently, will have considerable impact on our understanding of the very early steps in protein folding when only relatively short-range interactions are present.

Materials and Methods

Peptide Synthesis

The peptide: Ac-GVWTYDDATKTFTVTE-OH was synthesized using standard solid-phase Fmoc-amino acid chemistry with a Mili-pore synthesizer. The resin for the free C-terminal group, Tentagel R PHB (1 g, capacity 0.2 mmol/g) was treated with piperidine (20%) in DMF, and all amino acids were coupled using the DIPCI/HOBt methodology. The coupling reaction time was 2 h. Piperidine (20%) in DMF was used to remove the Fmoc group at all steps. After deprotection of the last Fmoc N-terminal group, the resin was washed with methanol and dried in vacuo. Then the resin was treated with 1M 1-acetylimidazole in DMF at room temperature for 2 h to attach the acetyl group to the N-terminal part of the peptide. In the final step, the resin was treated with a TFA/water/phenol/triisopropylsilane (8.8/0.5/0.5/0.2) mixture (10 ml per 1 g of the resin) at room temperature for 2 h. The resin was separated from the mother liquid, and the excess of solvent was evaporated to a volume of 2 ml and the residue was precipitated with diethyl ether. The crude peptide was purified by reverse-phase HPLC using a Supelcosil™ SPLC-ABZ C₁₈ semi-preparative column (10 × 250 mm, 5 μm) with 4 ml/min elution and a 120 min isocratic mixture of 36% CH₃CN in TFA to adjust the pH to approximately 2.0. The fractions containing pure peptide were lyophilized and the purity was confirmed by analytical HPLC and MALDI-TOF analysis (*M* = 1876.9 g/mol; the theoretical value of the molecular mass is 1875.98 g/mol).

Circular Dichroism (CD) Spectroscopy

CD spectra were recorded on a Jasco J-815 spectropolarimeter with 100 nm/min scan speed, and data were collected from 260 to 190 nm with a 1 mm path length quartz cell. The samples were dissolved (a) in water (pH = 4.02) and measured at 9 different temperatures: 278, 283, 293, 303, 313, 323, 333, 343, 353 K and (b) in water and water solution of CF₃CH₂OH [H₂O/CF₃CH₂OH was 9:1, 1:1 and 1:9 by vol.] and measured at 305 K. The final concentration of IG(46–61) was 0.01 g/100 ml. The secondary structure content was calculated from CD spectra using the self-consistent method.⁴¹

Differential Scanning Calorimetry (DSC)

Calorimetric measurements were made with a VP-DSC microcalorimeter (MicroCal) at a scanning rate of 90°C/1h. Scans were obtained at a peptide concentration of 0.047 mM. The cell volume was 0.5 ml. All scans were run at pH 4.02 in water in a range of temperatures from 278 to 253 K. By cooling and reheating the same sample three times, we checked the reversibility of the transition. During repeated heating/cooling cycles we did not observe any signs of such processes as aggregation or hydrolysis (the scans were always reversible, and we did not observe any negative values of heat capacity). The presented data are averages over three independent measurements. Results from the DSC measurements were analyzed with the Origin 7.0 software using the routines provided with the instrument.⁵⁶

¹H-NMR Spectroscopy

NMR spectra of IG(46–61) were measured on VARIAN 500 and 600 MHz spectrometers. The following spectra were recorded: 1D ¹H-NMR (at 283, 289, 297, 305, 313, and 321 K) and 2D ¹H-NMR: DQF-COSY,⁵⁷ TOCSY⁵⁸ (80 ms), ROESY⁵⁹ (200 ms) at 283, 305, and 313 K. The sample was dissolved in H₂O/²H₂O (9:1 by vol.) (pH = 4.02) and the concentration of the sample was 3 mM. The spectra were processed using VARIAN 4.3 software (Varian Instruments, Palo Alto, CA) and analyzed with the XEASY program.⁶⁰ The spectra were calibrated against the DSS (sodium 4,4-dimethyl-4-silapentane-1-sulfonate) signal.⁶¹ Proton signals were assigned based on the TOCSY spectra, where the spin system of each amino acid in this peptide was easy to recognize. The sequential analysis of the peptide was confirmed by

the ROESY spectra.⁵⁹ In Figure 12, the fragments of the ROESY spectra recorded at 283, 305 and 313 K are shown; these indicate the presence of the long-range interactions listed in Table II. The chemical shifts are collected in supporting information. The coupling constants between NH and H_α protons (³J_{HNHα}) of IG(46–61) were obtained from the two-dimensional DQF-COSY and one-dimensional ¹H spectra. The intensities of ROE signals were estimated from the ROESY spectra.⁵⁹ In Figure 2 in supporting information, the assigned TOCSY spectra and the amino acid spin systems are shown.

Determination of the Midpoint from the Spectroscopic Data

To determine the phase transition temperature, spectroscopic data were fitted by

$$\gamma = \frac{\gamma_U + \gamma_F \exp \left[\frac{\Delta H_m}{R} (1/T - 1/T_m) \right]}{1 + \exp \left[\frac{\Delta H_m}{R} (1/T - 1/T_m) \right]} \quad (1)$$

where γ_U is the unfolded baseline, γ_F is the native baseline, T_m is the midpoint of the thermal unfolding curve, and ΔH_m is the enthalpy change at the T_m .⁵¹

Three-Dimensional Structure Calculations

The ROE inter-proton cross-peaks of IG(46–61) were derived from 2D ¹H-NMR ROESY spectra, and vicinal coupling constants ³J_{HNHα} were obtained from 2D ¹H-NMR DQF-COSY and temperature-dependent 1D ¹H-NMR spectra. The number of ROE inter-proton cross-peaks and vicinal coupling constants are listed in Table IV. In the first step, the ROESY peak volumes were converted to upper distance bounds by using CALIBA⁶² of the DYANA package.⁶³ In the next step, torsion angles were generated using the HABAS algorithm of the DYANA package⁶⁴ on the basis of the Bystrov-Karplus⁶⁵ equation. The upper distance limits and torsional angles were used as restraints in molecular dynamics calculations.

Molecular dynamics simulations with the time-averaged-restraint methodology (TAV)^{66–68} were carried out with the AMBER force field⁶⁹ using the AMBER 8.0 package.⁶⁸ The interproton distances were restrained with the force constant $k = 20$ kcal/(mol × Å²), and the dihedral angles with $k = 2$ kcal/(mol × rad²), respectively. The dihedral angles ω were restrained with a center at 180° and $k = 10$ kcal/(mol × rad²). The improper dihedral angles centered at the C^α atoms (defining the chirality of amino acid residues) were restrained with $k = 50$ kcal/(mol × rad²). Three sets of separate simulations were run, using the restraints from the NMR data collected at 283, 305 and 313 K. All simulations were carried out in a TIP3P⁷⁰ periodic water box at constant volume, with the particle-mesh Ewald procedure for long-range electrostatic interactions.^{71,72} MD simulations, with time-averaged restraints from these three different temperatures, were carried out with a time step of 2 fs,⁷³ and the total duration of the run was 2 ns. Coordinates were saved every 2000 steps of MD simulations. All TAV MD simulations here started with the fully extended peptide chain.

For every NMR restraint set, four independent TAV MD simulations were run at the following temperatures: N , 400, 500, 600 K (where N is the temperature of the NMR experiment, i.e., runs at 283 K (respectively, 305 and 313 K) for three independent sets of calculations). By carrying out simulations at many temperatures, as good a sampling of conformational space as possible is ensured. From every trajectory, a final set of 300 last conformations was collected for analysis. The structures from four trajectories, obtained from simulations performed using the same NMR restraint set, were combined together. After TAV MD simulations, we obtained three sets of 1200 conformation each (four runs, with 300 conformations from every run) corresponding to three NMR restraint sets recorded at different temperatures. All three sets of

conformations were clustered separately, with the use of the MOLMOL program.⁷⁴ An RMS deviation cut-off of 5.0 Å was used in the clustering procedure. The clustering procedure provided 4, 5, and 12 families of conformations, for simulations, which used NMR data recorded at temperatures 283, 305 and 313 K, respectively. The four most populated families at 283, five at 305 and five at 313 K were selected for presentation. The RMS deviation was calculated based on C^α atoms.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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FIGURE 1. (a) The X-ray structure of the B3 domain of protein G (1IGD).³¹ (b) The amino acid sequence of 1IGD. In (b), A denotes β -strands and H1 the α -helix. The boxed part of the sequence, IG (46–61), was studied in this work.

FIGURE 2.
Heat capacity curve for IG(46-61) recorded in water at pH = 4.02.

FIGURE 3.

CD spectra of IG(46–61) in (a) water at different temperatures (278, 283, 293, 303, 313, 323, 333, 343, and 353 K) (pH = 4.02) and (b) water solutions of trifluoroethanol (10, 50, and 90% of TFE).

FIGURE 4.

Molar ellipticity of IG(46–61) at different wavelengths at pH = 4.02 plotted against temperature. Estimated folding temperature using two-state model (Eq. 1, see “Materials and Methods: Determination of the midpoint temperature from spectroscopic data” section) are 311 and 303 K using ellipticity measured at 190 and 220 nm, respectively.

FIGURE 5.

ROE effects corresponding to the interproton distances and the $^3J_{\text{NH}\alpha}$ coupling constants of IG(46–61) measured in H₂O at (a) 283 K, (b) 305 K, and (c) 313 K, at pH = 4.02. The height (vertical thickness) of the bars reflects the strength of the ROE correlation as strong, medium or weak.

FIGURE 6. Outline of the structure of IG(46–61) with marked ROE connectivities at 283 K (blue-dotted lines), 305 K (green-dotted lines), and 313 K (red-dotted lines).

FIGURE 7.

Chemical shift changes for aromatics protons of the Tyr50 residue plotted against temperature. Estimated folding temperatures using a two-state model (Eq. 1, see “Materials and Methods: Determination of the midpoint temperature from spectroscopic data” section) are 287 and 306 K using the chemical shifts of the δ and ϵ protons, respectively.

FIGURE 8.

Four families of conformations of IG(46–61) obtained by using time-averaged MD methodology with restraints from NMR measurements at 283 K. Left columns show all conformations from a family (only backbones are shown for clarity), right columns show the lowest-energy conformation from the corresponding family (all heavy atoms are shown). 1200 conformations were subjected to a cluster analysis, leading to the following numbers and percentages of each clustered family: (a) 576 (48%), (b) 300 (25%), (c) 185 (15.4%), (d) 139 (11.6%).

FIGURE 9.

Five families of conformations of IG(46–61) obtained by using time-averaged MD methodology with restraints from NMR measurements at 305 K. Left columns show all conformations from a family (only backbones are shown for clarity), right columns show the lowest-energy conformation from the corresponding family (all heavy atoms are shown). 1200 conformations were subjected to a cluster analysis, leading to the following numbers and percentages of each clustered family: (a) 505 (42%), (b) 279 (23.3%), (c) 170 (14.2%), (d) 130 (10.8%), (e) 116 (9.7%).

FIGURE 10.

Five most populated families of conformations of IG(46–61) obtained by using time-averaged MD methodology with restraints from NMR measurements at 313 K. Left columns show all conformations from a family (only backbones are shown for clarity), right columns show the lowest-energy conformation from the corresponding family (all heavy atoms are shown). 1200 conformations were subjected to a cluster analysis, leading to the following numbers and percentages of each clustered family: (a) 301 (25.1%), (b) 301 (25.1%), (c) 131 (10.9%), (d) 88 (7.3%), (e) 79 (6.6%).

FIGURE 11.

The native C-terminal β -hairpin structure fragment of IGD with marked (a) native hydrogen bonds,³¹ (b) possible hydrogen bonds in IG(46–61) fragment at 283 K, (c) possible hydrogen bonds in IG(46–61) fragment at 305 K, and (d) possible hydrogen bonds in IG(46–61) fragment at 313 K.

FIGURE 12.

Fragments of the ROESY spectra recorded at pH = 4.02 at 313 K (a), 305 K (b), and (c), and 283 K (d) which indicate the presence of the key long-range interactions (boxed signals) listed in Table II.

Table I
Percentage of Secondary Structure Elements of IG(46-61) Under Different Temperature and Thrifluoroethanol Conditions
Obtained From CD Spectra Using the SELCON3 SP37A Method⁴¹ at pH = 4.02

Conditions	α -helix/ β -sheet	Turn	Statistical Coil	ppII
278 K	036	13.4	21	15.6
283 K	037.5	14.6	15.6	16.8
293 K	034.8	13.8	23.2	15
303 K	043.4	21.7	11.8	20.2
313 K	045.6	21.2	7.2	23
323 K	044.3	22.4	9.2	21.6
333 K	042.6	21.3	5.6	20.9
343 K	048.9	23.7	1.5	23.7
353 K	040.7	21	15.9	18.9
10% TFE	047.0	22.7	5.1	22.7
50% TFE	0.847.5	17.3	17.9	10.6
90% TFE	13.030.4	17.4	29.4	7.0

Table II
Atoms of Residues $|i - j| > 1$ Between Which ROE Peaks Were Found in ROESY Spectra Recorded at 283, 305, and 313 K at pH = 4.02

ROE Peaks Between Residues $ i - j > 1$		
283 K	305 K	313 K
HNG46 – γ E61	ζ_3 W48 – γ_1 V59	γ T49 – γ_1 V59
γ V47 – HNV59	δ Y50 – δ F57	
β W48 – HNV59	δ Y50 2 ϵ F47	
β W48 – HNT60	ϵ Y50 – δ F47	
α Y50 – HNF57	ϵ Y50 – ϵ F47	
	β D52 – HNK55	
	δ F57 – γ_1 V59	
	ϵ F57 – γ_1 V59	
	ϵ F57 – γ_2 V59	
	ζ F57 – γ_2 V59	

Table III
Percentage of Occupancy of Possible Hydrogen Bond From MD Trajectories Obtained with Geometrical Restraints Based on NMR Measurements Performed at Temperatures: 283, 305, and 313 K at pH = 4.02

Donor	Acceptor	% Occupancy		
		283 K	305 K	313 K
HNT56	OD52	51.85	67.2	
HNT54	Oδ2D52	49.75		12
HNT54	Oδ1D52	46.85		23.27
HNT56	Oδ1D52	33.35		
HND51	OF57	26.35		
HND51	OT56	20.1		
HNT56	Oδ2D52	18.25		
HND51	Oγ1T49	11.6		
HNT54	OD52		9.2	
HNT54	OY50			31.13
HNT54	OD51			30.33

Table IV
Number of Restraints Used to Obtain the Upper Distance Limits and Torsional-Angle Ranges Which Were Used in Molecular Dynamics Calculations

	Number of ROE Peaks ^a		
	283 K	305 K	313 K
All	102	151	83
Between residues $ i - j > 1$	5	10	1
	Number of Vicinal Coupling Constants ^b		
$^3J_{\text{HNH}\alpha}$	10	15	8

^a Obtained from 2D ^1H -NMR ROESY spectra.

^b Obtained from 2D ^1H -NMR DQF-COSY and temperature-dependent 1D ^1H -NMR spectra.