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Mechanism of formation of the C-terminal β -hairpin of the B3 domain of the immunoglobulin binding protein G from *Streptococcus*. Part III. Dynamics of long-range hydrophobic interactions

Agnieszka Lewandowska^{1,2,#}, Stanisław Oldziej^{1,2}, Adam Liwo^{2,3}, and Harold A. Scheraga^{2,*}

¹ Laboratory of Biopolymer Structure, Intercollegiate Faculty of Biotechnology, University of Gdańsk, Medical University of Gdańsk, Kładki 24, 80-822 Gdańsk, Poland ² Baker Laboratory of Chemistry and Chemical Biology, Cornell University, Ithaca, NY 14853-1301, USA ³ Faculty of Chemistry, University of Gdańsk, Sobieskiego 18, 80-952 Gdańsk, Poland

Abstract

A 20-residue peptide, IG(42–61), derived from the C-terminal β -hairpin of the B3 domain of the immunoglobulin binding protein G from *Streptococcus* was studied using CD, NMR spectroscopy at various temperatures and by differential scanning calorimetry. Unlike other related peptides studied so far, this peptide displays two heat capacity peaks in DSC measurements (at a scanning rate of 1.5 deg/min at a peptide concentration of 0.07mM) which suggests a three-state folding/unfolding process. The results from DSC and NMR measurements suggest the formation of a dynamic network of hydrophobic interactions stabilizing the structure, which resembles a β -hairpin shape over a wide range of temperatures (283 – 313 K). Our results show that IG(42–61) possesses a well-organized three-dimensional structure stabilized by long-range hydrophobic interactions (Tyr50 ... Phe57 and Trp48 ... Val59) at T = 283 K and (Trp48 ... Val59) at 305 and 313 K. The mechanism of β -hairpin folding and unfolding, as well as the influence of peptide length on its conformational properties, are also discussed.

Keywords

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

INTRODUCTION

Conformational studies of peptides related to protein fragments have been recognized as an important method to understand early stages of protein folding.^{1–3} Most of the conformational studies of peptides are focused on peptide fragments that correspond to regular secondary structure elements (α -helices^{4–11} or β -hairpins)^{12–19} in the native protein structure. Most of the conformational studies of protein fragments are carried out by using the NMR technique, and they are strongly coupled to, and influenced by, the native structure of the protein from which the peptide sequence was derived. In such NMR studies, there was

*Corresponding author. Address: Baker Laboratory of Chemistry and Chemical Biology, Cornell University, Ithaca, NY 14853-1301, USA, Tel.: (607) 255-4034, Fax: (607) 254-4700, has5@cornell.edu.

#Formerly Skwierawska

no determination of inter-proton distances from NOESY/ROESY spectra for use as the main source of structural information, but proton chemical shifts were analyzed instead.^{13–16,20–23} The chemical shifts are measured for the investigated peptide and then compared to those of related protons observed in the parent protein and in the reference unfolded system (short model peptides are usually used as chemical shift reference for the unfolded state).²⁴ Assuming that proton chemical shifts change linearly with the amount of native conformation in the peptide under investigation, information about the degree of folding can be inferred from chemical-shift data without a sometime-complicated structural analysis of inter-proton distances obtained from the NOESY/ROESY spectra.

There are a few reports in which NOESY/ROESY methods were used to investigate peptides derived from sequences of proteins to determine peptide structure and dynamics.^{17,18,25–27} Because sometimes there are only a few long-range structural restraints, this method of peptide structure determination can lead to a diffuse ensemble of conformations. On the other hand, the use of NOESY/ROESY spectra enables us to obtain information about conformational dynamics of the investigated peptide. In our previous studies, we investigated the structure and conformational dynamics of peptide fragments of different lengths (from 16- to 6-residues) which are based on the amino-acid sequence of the C-terminal β -hairpin of the B3 domain of the immunoglobulin binding protein G.^{17,18,27} These studies showed that the investigated peptides form structures that resemble the general shape of a β -hairpin structure observed in the respective part of the sequence of the parent protein not only at low temperatures (below 283 K), as earlier studies of other authors suggested,^{12–16} but also at temperatures as high as 313 K.^{17,18} We also showed that long-range interactions between hydrophobic residues are responsible for structure formation and stabilization, and that rupture of these long-range interactions is associated with the appearance of a heat capacity peak (phase transition) in Difference Scanning Calorimetry (DSC) measurements. We also showed that the turn sequence is very important for β -hairpin formation.²⁷

Our research is focused on understanding the mechanism of β -hairpin formation as well as the role of the investigated sequence fragment in the folding of the full protein. In this paper, we report an investigation of the role of some possible tertiary contacts in the stability and mechanism of formation of the β -hairpin derived from the C-terminal part of the B3 domain of the immunoglobulin binding protein G. As the object of our study, we chose a 20-residue C-terminal fragment of this protein²⁸ (see Fig. 1). In our previous studies,^{17,18,27} we have already demonstrated the importance of long-range interactions between hydrophobic residues Trp48, Tyr50, Phe57 and Val59 in the formation and stabilization of the β -hairpin structure. In the native structure of the B3 domain, the hydrophobic cluster formed within the C-terminal β -hairpin sequence interacts with other hydrophobic residues located mainly in the central α -helix as well as in the N-terminal β -hairpin. All of these hydrophobic interactions between secondary structure elements are long-range (more than 12 residues separate interacting amino acids).²⁸ However, in the structure of the protein, the Val44 residue forms contacts with the Trp48 and Val59 residues. The Val44 residue is located in the loop region connecting the C-terminal β -hairpin and the central α -helix (i.e., outside the C-terminal β -hairpin); thus, conformational studies of this peptide, which can incorporate such interactions, can provide some insight into the interactions of the fast-formed secondary structure elements with another part of the protein in the very early stage of the folding of the protein.

In the present study, we use the 20-residue fragment IG(42–61) which corresponds to the 16-residue C-terminal β -hairpin plus the four-residue loop which, in the parent protein, connects the C-terminal β -hairpin with the central α -helix (see Fig. 1b). In our previous work,¹⁷ we showed that four nonpolar residues of the C-terminal β -hairpin (Tyr50, Phe57,

Trp48, and Val59) are able to form a hydrophobic “micro-core” within the β -hairpin sequence. In the present study, we try to determine how this hydrophobic “micro-core” increases in size. We speculate that folding can progress in such a manner that the “micro-core” forms within one secondary structure element and expands in some continuous manner (e.g., when other nonpolar residues, which are close in sequence, join the hydrophobic cluster one by one), and this possible mechanism will be considered in this work. Another possible scenario is that, in another part of the protein sequence, other hydrophobic “micro-core/micro-cores” are formed, and then folding progresses by accidentally meeting such nucleation parts.^{29–35}

MATERIALS AND METHODS

Peptide synthesis

The peptide Ac-NGVDGVWTYDDATKTFTVTE-OH [IG(42–61); 20 amino acid residues] was synthesized by the standard solid-phase Fmoc-amino acid chemistry with a Milipore synthesizer. The resin for the free C-terminal group, Tentagel R PHB (1g, 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 1 M 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 one gram 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; finally, the residue was precipitated with diethyl ether.

The crude peptide was dissolved in 16.7% CH₃CN in TEA/H₃PO₄ and purified by reverse-phase HPLC using a SupelcosilTM SPLC-ABZ C₁₈ semi-preparative column (10 × 250 mm, 5 μ m) with 4 ml/min elution and a 120 min isocratic mixture of 16.7% CH₃CN in TEA/H₃PO₄ to adjust the pH to approximately 7.0. To identify fractions containing the pure peptides, HPLC was run first with a small amount of the crude peptide and the absorbance at 222 nm was measured for each fraction. A plot of absorbance vs. retention time was constructed, and the interval of the retention time to separate the pure peptide was estimated as that corresponding to the large peak in the plot. Subsequently, a semi-preparative HPLC run was carried out and the fractions containing the pure peptide were collected and lyophilized. The purity of the peptide was confirmed by analytical HPLC and MALDI-TOF analysis ($M = 2261.37$ g/mol; the theoretical value of the molecular mass is 2261.36 g/mol).

Circular dichroism (CD) spectroscopy

CD spectra were recorded on a Jasco J-815 spectropolarimeter with a 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 in (a) phosphate buffer (C_{KH₂PO₄} = 1/15 mol/ml, C_{Na₂HPO₄} = 1/15 mol/ml; pH = 6.81), and the CD spectra were measured at 16 different temperatures, i.e., at 5 deg intervals between 278 and 353 K, and (b) in water and water solution of CF₃CH₂OH [H₂O/CF₃CH₂OH ratio was 9:1, 1:1, and 1:9 by vol.] and the CD spectra were measured at 305 K. The final concentration of IG(42–61) was 0.015g/100 ml. The secondary structure content was calculated from CD spectra using the CONTINLL method.³⁶

Differential Scanning Calorimetry (DSC)

Calorimetric measurements were carried out with a VP-DSC microcalorimeter (MicroCal) at a scanning rate of 1.5 degree/minute. Scans were obtained at peptide concentrations of 0.07

and 2.1 mM. The cell volume was 0.5 ml. All scans were run at pH = 6.81 in phosphate buffer ($C_{\text{KH}_2\text{PO}_4} = 1/15$ mol/ml, $C_{\text{Na}_2\text{HPO}_4} = 1/15$ mol/ml) in the range of temperatures from 5 °C to 80 and from 5 °C to 90 °C at a peptide concentration of 0.07 and 2.1 mM, respectively. The reversibility of the transition was checked by cooling and reheating the same sample. No hysteresis of heat capacity was found in the repeated heating and cooling cycles and, moreover, no largely negative values of heat capacity were observed. This demonstrates that no irreversible processes such as, e.g., aggregation or hydrolysis, occurred during the thermal transition. The presented data are averages over three independent measurements. Results from the DSC measurements were analyzed with the Origin 7.0 software from MicroCal using the software routines provided with the instrument.³⁷

¹H-NMR spectroscopy

The NMR spectra of IG(42–61) were measured on VARIAN 500 MHz and 600 MHz spectrometers. The following spectra were recorded: 1D ¹H-NMR (at T = 283, 289, 297, 305, 313 and 321 K) and 2D ¹H-NMR: DQF-COSY,³⁸ TOCSY³⁹ (80 ms), ROESY⁴⁰ (200 ms) at T = 283, 305 and 313 K. In our previous studies^{17,18,27} we dissolved the investigated peptides in pure water. However, IG(42–61) did not dissolve well in pure water; so we used phosphate buffer for this peptide. The samples were dissolved in phosphate buffer/²H₂O (9:1 by vol.) ($C_{\text{KH}_2\text{PO}_4} = 1/15$ mol/ml, $C_{\text{Na}_2\text{HPO}_4} = 1/15$ mol/ml; pH = 6.81), and the concentration of the samples was 2 mM. The spectra were processed using VARIAN 4.3 software (Varian Instruments, Palo Alto, CA, USA) and analyzed with the XEASY program.⁴¹ The spectra were calibrated against the DSS (sodium 4,4-dimethyl-4-silapentane-1-sulfonate) signal.⁴² Proton signals were assigned based on the TOCSY spectra. The sequential analysis of the peptide was confirmed by the ROESY spectra.⁴⁰ The chemical shifts are reported in Tables I – III in supplemental data. The coupling constants between NH and H_α protons (³J_{NH_{Hα}}) of IG(42–61) were obtained from two-dimensional DQF-COSY and one-dimensional ¹H spectra. The TOCSY spectra, with peak assignments of IG(42–61), are shown in Fig. 1 in supplemental data. The intensities of ROE signals were estimated from the ROESY spectra;⁴⁰ the relevant part of the ROESY spectra showing long-range interactions are illustrated in Fig. 2 in supplemental data.

Three-dimensional structure calculations

The ROE inter-proton cross-peaks of IG(42–61) were derived from 2D ¹H-NMR ROESY spectra, and the vicinal coupling constants ³J_{NH_{Hα}} were determined from 2D ¹H-NMR DQF-COSY and temperature-dependent 1D ¹H-NMR spectra. In the first step, the ROESY peak volumes were converted to upper distance bounds by using CALIBA⁴³ of the DYANA package.⁴⁴ In the next step, torsion angles, based on the Bystrov-Karplus⁴⁵ equation, were generated using the HABAS algorithm of the DYANA package.⁴⁶ The upper distance limits and torsional angles were used as restraints in molecular dynamics calculations.

Molecular dynamics simulations with the time-averaged methodology (TAV)^{47–49} were carried out with the AMBER force field⁵⁰ using the AMBER 8.0 package.⁴⁹ The interproton distances were restrained with the force constant $k = 20$ kcal/(mol × Å²), and the dihedral angles with $k = 2$ kcal/(mol × deg²), respectively. The dihedral angles ω were restrained with a center at 180° and $k = 10$ kcal/(mol × deg²). The improper dihedral angles centered at the C^α atoms (defining the chirality of amino acid residues) were restrained with $k = 50$ kcal/(mol × deg²). Three sets of separate simulations, using the restraints from the NMR data collected at 283, 305 and 313 K, were run for IG(42–61). All simulations were carried out in a TIP3P⁵¹ periodic water box at constant volume, with the particle-mesh Ewald procedure for long-range electrostatic interactions.^{52,53} MD simulations with time-averaged restraints at these three different temperatures, were carried out with a time step of 2 fs,⁵⁴ and the total duration of the run was 3.2 ns. Coordinates were saved every 2000 steps of MD simulations.

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, 305 K, and 313 K), respectively, for three independent sets of calculations]. The purpose of running simulations at many temperatures including elevated temperatures was to enhance sampling. From every trajectory, 300 final conformations were collected for the 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 for IG(42–61). All three sets of conformations were clustered separately, with the use of the MOLMOL program.⁵⁵ A root-mean-square deviation (RMSD) over the C α atoms, with a cut-off of 5.0 Å, was used in the clustering procedure. The clustering procedure provided 48, 43, and 43 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 and 305, and three at 313 K were selected for presentation.

RESULTS AND DISCUSSION

Differential Scanning Calorimetry (DSC)

As in our previous studies^{17,18,27} we performed DSC measurement of the investigated peptide as the first step of our investigation. As stated in “Materials and methods”, DSC experiments provide information about two very important properties, namely thermal stability¹ and possible aggregation of a protein or peptide.⁵⁶ In other studies, ultracentrifugation was used to detect possible aggregation;^{25,57} however, most such studies were limited to only one temperature while it is known that a temperature change can induce oligomerization/aggregation processes.⁵⁶ Ultracentrifugation can be conducted under a controlled temperature regime; however, it is very difficult and time/sample consuming to perform a series of experiments using different temperatures. For practical purposes, the DSC experiment can detect oligomerization/aggregation processes in a wide range of temperatures, using a very small amount of the investigated compound, and can show if the process of aggregation/oligomerization is reversible²⁷ with changes of temperature; also, a wide range of heating/cooling speed can be used to detect oligomerization/aggregation processes if the only interest is detection of such processes but not their thermodynamic/kinetic effects.⁵⁶

The heat capacity curve, with two peaks corresponding to transition temperatures of 308.05 ± 0.13 K and 341.68 ± 0.32 K for IG(42–61), is presented in Fig. 2a. Based on the heat capacity curves, and the use of a three-state model, the enthalpy changes related to these transitions are $\Delta H = 62.90 \pm 0.12$ kcal/mol and $\Delta H = 16.84 \pm 0.10$ kcal/mol, respectively. For all previously studied peptides of length from 16 to 8 residues, based on the sequence of the C-terminal β -hairpin of the B3 domain of protein G, we observed only one heat capacity peak in DSC measurements.^{17,18,27} The appearance of two heat capacity peaks during DSC measurements suggests a more complex folding/unfolding process than for all previously investigated peptides. The first transition temperature observed for the IG(42–61) peptide is 308.05 ± 0.13 K which is below the transition temperatures observed for shorter peptides derived from the C-terminal part of the B3 domain ($T = 320.11 \pm 0.14$ K, 330.55 ± 0.19 K, 310.24 ± 0.03 , 316.96 ± 0.21 K for 16- [IG(46–61)], 14- [IG(47–60)], 12- [IG(48–59)], and 8- [IG(50–57)] residue peptides, respectively).^{17,18,27} On the other hand, the second transition temperature recorded for IG(42–61) is 341.68 ± 0.32 K, which is higher than all transition temperatures recorded so far for shorter peptides.^{17,18,27} In our previous studies, we showed that the phase transition recorded in the DSC measurements is related to formation/breaking of long-range interactions between nonpolar residues.^{17,18} If we assume that only formation/breaking of long-range hydrophobic interactions is responsible for the appearance of heat

capacity peaks, then there may be a unique and detailed pattern of interactions in IG(42–61) that eventually can be described more precisely by NMR experiments [see “Results and discussion: NMR measurements” section]. On the other hand, we cannot rule out the possibility that at least one peak observed in the DSC curve may not be related to long-range interactions in the structure of IG(42–61), but may be related to other processes such as, for example, “hydrophobic solvation” of nonpolar side chains.⁵⁸ A possible more complicated scenario could be considered in which the peptide forms a dimer/oligomer at low temperatures, and the low-temperature peak in the DSC curve (308.05 ± 0.13 K) could be caused by the dissociation of the dimer/oligomer, while the high temperature peak (341.68 ± 0.32 K) could be caused by the disruption of the monomer structure. However, such a mechanism is very unlikely in view of the fact that the kinetics of dimer/oligomer formation is usually different than that of its dissociation (in this case, the reaction rates of formation and dissociation of a dimer/oligomer are different). The difference in the rates and kinetics of possible dimer/oligomer formation/dissociation should be manifested as hysteresis in the heating/cooling cycles; however, no hysteresis was observed in either of the DSC experiments performed with different sample concentration (see Fig 2).

The DSC measurement presented in Fig. 2a was performed at a peptide concentration of 0.07 mM. On the other hand, our NMR experiments were performed at peptide concentration of 2 mM. To rule out possible aggregation/oligomerisation problems related to higher concentrations of the peptide in the NMR experiments, we ran an additional DSC experiment using a peptide concentration of 2.1 mM, and the results are shown in Fig. 2b. We observed one very wide heat-capacity peak on the DSC curve presented in Fig. 2b. We did not observe two heat capacity peaks as shown in Fig. 2a because the high peptide concentration results in greater inertia and, consequently, decreased resolution of the DSC measurements, which leads to broadening of a single and overlapping of multiple heat-capacity peaks.⁵⁹ It can be seen that the maximum of the heat capacity peak in Fig. 2b is located at $T = 316$ K. This temperature appears to be close to the weighted average (315 K) over the two peaks obtained at a lower concentration (Fig. 2a) [the integral of the first peak in Fig. 2a (i.e., the enthalpy of the corresponding transition) (62.9 kcal/mol) is approximately four times bigger than that over the second peak (16.84 kcal/mol, respectively). It could be possible to obtain two peaks even at very high concentration of the sample by drastically decreasing the speed of heating/cooling of the system (we used a heating/cooling speed of 1.5 degree/minute in this experiment). However, from our experience (data not shown), increasing the DSC measurement time above 120 minutes (which corresponds to the heating/cooling speed of about 0.5–0.7 degree/minute) could lead to irreversible processes such as, e.g., aggregation.

A clear proof of the oligomerisation/aggregation processes would be the appearance of negative heat capacity peaks in the DSC curves.⁵⁶ As can be seen from Fig. 2b, a very small negative peak with a minimum at $T = 353$ K, which could be associated with oligomerisation/aggregation processes, is observed. However, if oligomerisation/aggregation occurred, a much larger signal than that observed in Fig. 2b at $T = 353$ K would appear. Moreover, the observed traces of possible oligomerisation appeared in the range of temperatures more than 30 degrees higher than $T = 313$ K which was the highest temperature of the NMR experiments. Therefore, the results obtained from the NMR experiments are not affected by oligomerisation/aggregation processes.

Stanger and coworkers observed that, with β -strand length from five to seven residues, the overall stability of a β -hairpin peptide increases and that further increase of β -strand length (above seven residues) leads to β -hairpin destabilization.⁶⁰ For peptides studied in this and in our previous papers (peptide length from 8 to 20 residues), we are not able to find any correlation between folding temperatures or enthalpies of folding with the β -hairpin peptide

length.^{17,18,27} Our data suggested that thermal stability is connected with amino acid composition of the investigated peptides rather than with peptide length as Stanger and coworkers⁶⁰ suggested. It should be noted, though, that Stanger and coworkers⁶⁰ studied a peptide composed of a core part (close to the turn region) and extensions from the N- and C-termini which was built of only threonine residues. Because only one type of amino acid was used for the extension, the thermal stability was proportional to the number of amino acids in the extension part (at least this was true for extensions of length up to five residues), which supports our hypothesis that thermal stability is primarily related to the amino acid composition of the investigated peptide.

CD measurements

The CD spectra of IG(42–61) peptide were recorded under two sets of different conditions: (a) in phosphate buffer (pH = 6.81) at 16 different temperatures, i.e., at 5 deg intervals between 278 and 353 K (Fig. 3a) and (b) in 10%, 50% and 90% TFE/H₂O mixture at 305 K (Fig. 3b).

As shown in Fig. 3a, the molar ellipticity varies with temperature. The largest changes of ellipticity appear around $\lambda = 201$, 220, and 230 nm. Changes of ellipticity around $\lambda = 201$ nm and 220 nm are responsible for the changes in the statistical coil and regular β -sheet content, respectively,^{61,62} in the peptide structure. The changes in ellipticity around $\lambda = 230$ nm are connected with the tryptophan absorbance, indicating that the indole side chain is in a defined chiral environment. In proteins, such near-UV CD bands are often taken as evidence for well organized tertiary structure.^{57,63} The ellipticity at $\lambda = 201$ nm became less negative, whereas at $\lambda = 220$ nm and $\lambda = 230$ nm more negative, with increasing temperature, with these changes usually being associated with an increase of the number of residues in well-defined secondary structure; however, all of these changes are very small (Fig. 3 in supplemental data). The percentage of secondary structure elements, calculated from CD spectra for IG(42–61), changes very little with increasing temperature (see Table IV in supplemental data). The percentage of secondary structure elements changes by less than 2% for a given secondary structure element in IG(42–61), which prevents us from drawing any conclusions from these data.

For all three wavelengths ($\lambda = 201$, 220, and 230 nm) for which we observed the largest ellipticity changes for IG(42–61), the ellipticity changes are rather linear or almost linear as in our former studies on shorter variants of this peptide.^{17,18,27} Thus, we cannot determine the melting temperature for the IG(42–61) peptide based on CD measurements, as has been done in many other studies.^{17,57}

In Fig. 3b and in Table IV in supplemental data, the results of CD measurements performed in solutions with three different trifluoroethanol (TFE) concentrations are shown. These results indicate that the β -structure is almost constant in 10% and 50% of TFE and then decreases slightly in 90% of TFE (see Table IV in supplemental data). On the other hand, increasing of the TFE concentration led to an increase in the α -helical content (see Fig. 3b) and a decrease of the statistical coil content (see Table IV in supplemental data).

NMR measurements

The 2D ¹H-NMR spectra of IG(42–61) were recorded in buffer solution at pH = 6.81 at three different temperatures T = 283, 305, and 313 K, to examine the influence of temperature on the structure. The chemical shifts of the proton resonances for this peptide at these three temperatures are listed in Tables I–III of supplemental data.

The chemical shifts of the amide protons of IG(42–61) at three different temperatures (283, 305 and 313 K) are plotted as a function of residue number in Fig. 4 in supplemental data.

The amide proton chemical shifts of all amino acid residues show a tendency to move upfield, with increasing temperature (Fig. 4 in supplemental data). As shown in Fig. 4 in supplemental data, the chemical shifts vary considerably with temperature for all amide protons of IG(42–61), which indicates that none of these amide protons can be involved in a stable hydrogen bond or buried in a hydrophobic region of the peptide. Additionally, in confirmation of this statement, Table V of supplemental data shows the temperature coefficients of all amide protons in IG(42–61). For all temperature coefficients, $\Delta\delta/\Delta T < -4.5$ ppb/K, i.e., below the threshold at which an amide proton can be considered to be screened from the solvent).⁶⁴ Our previous work on shorter β -hairpin fragments of the B3 domain of protein G showed that three amino acid residues from the turn region (Asp51, Thr54 and Thr56) have very small temperature coefficients in absolute value, which suggests that they are in a stable environment and could contribute to the stability of the turn region.^{17,18} Because none of these residues exhibits this behavior in the IG(42–61) fragment, especially in the turn region, as opposed to the shorter IG C-terminal peptides,^{17,18} it can be concluded that the turn region is somehow very loose in this peptide.

In Fig. 4, the ROE effects corresponding to interproton contacts and the values of the $^3J_{\text{NH}\alpha}$ coupling constants for IG(42–61) are presented for NMR measurements carried out at different temperatures (283, 305 and 313 K). The number of observed ROE signals recorded and identified in the NMR experiments decreases with increasing temperature which reflects the increasing mobility of the system. A similar situation occurred in our previous studies on shorter peptides.^{17,18,27} A regular β -hairpin structure is characterized by strong $H_N(i) - H_N(i+1)$ connectivities in the turn region and strong $H_\alpha(i) - H_N(i+1)$ ROEs in the strand regions.^{20,65} As seen in Fig. 4, the $H_N(i) - H_N(i+1)$ connectivities are observed mostly within the N-terminal part of IG(42–61) and only at temperatures $T = 283$ and 313 K. The N-terminal part of IG(42–61) corresponds to the loop in the structure of the whole protein, and this loop serves as a link between the C-terminal β -hairpin and an α -helix in the middle of the sequence.²⁸ This observation suggests that a turn, similar to that in the parent protein, is formed at residues 42–45 of IG(42–61).

Surprisingly, $H_N(i) - H_N(i+1)$ connectivities are not observed in the region of the sequence which corresponds to the turn of the β -hairpin (residues 51–54). In all our previous studies on shorter peptides, we observed $H_N(i) - H_N(i+1)$ ROE signals in the region which corresponds to the turn sequence.^{17,18,27} The absence of $H_N(i) - H_N(i+1)$ ROE signals in the turn sequence (residues 51–54) of IG(42–61) is consistent with the high temperature coefficients observed for the amide protons (see Table V of supplemental data). These results are not consistent with our previous studies using shorter peptides^{17,18,27} when we observed that a tight turn structure was indicated by strong $H_N(i) - H_N(i+1)$ connectivities and low temperature coefficients for the amide protons in the turn region. The results from our current research do not support our earlier claim that the turn region is always very tight, regardless of the temperature and the peptide length. We do not have an explanation for this phenomenon, but in the future we plan to study longer peptides to find a possible explanation (see Conclusion section). It should also be noted that short range $H_\alpha(i) - H_N(i+1)$ or $H_\beta(i) - H_N(i+1)$ ROE signals are observed at all temperatures. Generally, the number and strength of these signals in IG(42–61) vanish with increasing temperature (see Fig. 4). At a given temperature, the strength and appearance of the sequential $H_\alpha(i) - H_N(i+1)$ and $H_\beta(i) - H_N(i+1)$ ROE signals change along the sequence without any clearly visible pattern.

As seen in Figs. 4 and 5, and in Table I, a number of long-range connectivities can be identified at all investigated temperatures. In our previous studies, we found that a β -hairpin-like structure is stabilized mostly by hydrophobic interaction between residues Tyr50 and Phe57 (the so-called “1st pair”).^{17,18,27} We observed very weak or no interactions between residues Trp48 and Val59 (the so-called “2nd pair”).^{17,18} In the case of IG(42–61), the

situation changes dramatically. As can be seen from Figs. 4 and 5, and in Table I, the “1st pair”, which was always observed in all peptides investigated so far at the temperatures below the folding transition temperature determined by DSC measurement,^{17,18,27} is present in IG(42–61) only at T = 283 K. On the other hand, the “2nd pair”, rarely observed for shorter peptides,^{17,18} is observed at all temperatures at which NMR experiments were performed for IG(42–61).

In our previous studies, we demonstrated that breaking/formation of long-range hydrophobic contacts is associated with the appearance of the heat capacity peak during DSC measurement.^{17,18,27} The IG(42–61) peptide displays a complex heat capacity curve with two peaks at T = 308.05 K and T = 341.68 K (see Fig. 2a). A combination of DSC data (Fig. 2a) as well as NMR data (Figs. 4 and 5, and Table I) leads us to the following hypothesis. The first heat capacity peak in the DSC curve at T = 308.05 K can be associated with breaking/formation of the “1st pair” of hydrophobic interactions. The ROE connectivities between the protons of the Tyr50 and Phe57 residues are observed only at T = 283 K. At T = 305 K [which differs only by three degrees from that of the heat capacity peak in the DSC curve (Fig. 2a)] and, at T = 313 K, no interaction between the Tyr50 and Phe57 protons is observed. In this hypothesis, the second peak observed in the DSC curve (Fig. 2a) at T = 341.68 K could be associated with breaking/formation of a hydrophobic interaction between residues Trp48 and Val59 (the “2nd pair”). We observe interactions within the “2nd pair” of hydrophobic residues in the NMR spectra of IG(42–61) recorded at all investigated temperatures.

It should be noted, however, that the NH W48 and NH T60 signals at T = 305 K overlap (Table II Supplemental data). The overlapping of these two signals could lead to a different interpretation of ROESY signals than that provided in Table I. The signal interpreted as the NH W48 – βV59 long-range interaction (as stated in Table I) could, alternatively, be ascribed to a sequential NH T60 – βV59 interaction. However, with such an assignment, the NMR data would not agree with those from DSC measurements (see Fig. 2); absence of an NH W48 – βV59 long-range interaction at T = 305 K would suggest that the investigated peptide would lose its β-hairpin-like structure; however, at T = 313 K, we did observe long-range interactions (see last column of Table I) which stabilize the β-hairpin-like structure. If the peptide would have lost its structure (and, consequently, the NH W48 – βV59 long-range interaction), a positive peak in the heat capacity curve would appear at about T = 305 K. This could be the peak at T = 308 K (see Fig 2a). However, some long-range interactions are observed at T = 313 K, i.e., above the first peak (see Table I). Consequently, all long-range interactions including NH W48 – βV59 would vanish around T = 305 K and then reappear at 313 K; also a negative heat capacity peak between these two temperatures (corresponding to the formation of long-range interactions) would appear between T = 305 K and T = 313K. However, as shown on Fig. 2a, no negative heat capacity is observed within the range T = 305 K and T = 313 K. Consequently, our assignment of the peak in question as NH W48 and βV59 contact (which always maintains at least one long-range interaction) is the only one which provides consistency between the NMR and DSC data.

We noticed that long-range interactions are not observed within the “1st pair” of hydrophobic residues at 305 K, but we associated their presence with the folding temperature of 308 K (see Fig. 2a). In our earlier investigated 16-residue peptide, none of the long-range interactions in the NMR spectra at T = 313 K were observed for which the transition temperature was determined to be T_m = 320 K.¹⁷ In the case of the shorter 14-, 12- and 8-residue peptides investigated previously, the presence or absence of long-range hydrophobic interactions correlate perfectly with the transition temperatures determined from DSC measurements. It is then clear that the correlation of breaking/formation of long-range hydrophobic interaction with the phase transition is perfect when only one pair of such

interaction is observed in investigated structure.^{18,27} When the number of the possible (and observed, in NMR spectra) long-range hydrophobic interactions increases (more than one pair of hydrophobic residues was observed), we noticed that the correlation between the DSC and NMR measurements was not perfect (ref. ¹⁷, and this work). We believe that the observed differences between changes in the long-range hydrophobic pattern and the appearance of the heat capacity peak is caused by possible hydrophobic interactions within the whole hydrophobic cluster and not only within pairs of residues which we discussed above. We are not able to identify such interactions in our measurements but we also cannot rule out such a possibility if we assume that such interactions are very dynamic and cannot be observed on the NMR time-scale.

MD simulations

The NMR data summarized in Fig. 4 were used to carry out MD simulations of IG(42–61) with time-averaged restraints, in order to determine the structures of this peptide. In Figs. 6, 7 and 8, 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 (Fig. 6). These four main families either differ in the conformation of the turn region or in the position of the extended N-terminal part of IG(42–61). In general, the conformation of the β -hairpin-like structure is well-defined. The position of the turn region (residues Asp52 – Lys55) is well-developed and preserved as it is in the native protein; the extended part of this peptide (Asn42 – Asp45), which serves as a link between the C-terminal β -hairpin and the middle α -helix in the B3 domain, also has a tendency to bend in the same direction as in the native protein.²⁸ The very close position of three aromatic residues (Trp48, Tyr50 and Phe57) is also seen in the structures at 283 K. It seems that the hydrophobic cluster, which is created by the “1st pair” (Tyr50 and Phe57), becomes stronger by the additional interaction with Trp48 (Fig. 4 and Fig. 5: Table I). Additionally, the “2nd pair” also appears at this temperature. It is important to note that, among all fragments studied so far,^{17,18,27} the structure of only two, the longest fragments of the C-terminal β -hairpin, IG(42–61) (20-amino-acid-residue fragment) at 283 K, 305 K and 313 K (Fig. 5: Table I) and IG(46–61) (16-amino-acid-residue fragment) at 283 K and 305 K¹⁷ are stabilized by the “1st pair” and the “2nd pair” of hydrophobic residues. First of all, this means that the “2nd pair” (Trp48 and Val59) is important for the longer sequences, and secondly the thermal stability of the “2nd pair” is higher than that of the “1st pair” because it appears at higher temperatures (Fig. 5: Table I). The importance of these two pairs of nonpolar residues in stabilizing the secondary structure of the 16-amino-acid-residue peptide corresponding to the C-terminal β -hairpin fragment of the B1 domain of protein G was also outlined by Blanco et al.²⁰ and Muñoz et al.⁶⁶

The results of the conformational analysis using the NMR restraints recorded at T = 305 K are summarized in Fig. 7. The four most populated families of conformations show a β -hairpin-like structure of the IG(42–61) fragment. The turn position (between Asp52 – Lys55) as was also found at 283 K is also preserved in the parent protein,²⁸ but its shape reflects a rather Ω -loop-like structure caused by the lack of any cross-strand interaction close to the turn region which could stabilize this part of the peptide. At this temperature, only one long-range interaction (between the “2nd pair”) spans the hairpin structure. We should point out that the aromatic side-chains of residues Tyr50 and Phe57 (the “1st pair”) are close to each other in the first (most populated) family of conformations (see Fig. 7a), but in the other families of conformations they are far apart. The observed dynamics of the “1st pair” can explain why there are no ROE signals for this interaction in NMR spectra.

Using the NMR restraints derived from the measurements at the highest temperature ($T = 313$ K) we carried out TAV MD simulations. Three most populated families of conformation are presented in Fig. 8. The structure is stabilized mostly by the “2nd pair” of hydrophobic residues (Fig. 4 and Fig. 5, Table I). The “1st pair” at 313 K is not present and, moreover, both residues point away from each another and their side-chain aromatic rings are fully exposed to solvent. At $T = 313$ K, the turn position is preserved similar to that at $T = 305$ K but the general shape resembles an Ω -loop-like structure. The formation of the Ω -loop-like structure instead of the structure similar to a classical β -turn explains why, in the IG(42–61) peptide, none of the temperature coefficients of the amide protons around the turn region is lower than -4.5 ppb/K, indicating the absence of hydrogen bonds (Table V in supplemental data).⁶⁴

CONCLUSIONS

We performed an extensive conformational study of the 20-residue peptide IG(42–61), excised from the C-terminal part of the B3 domain of the immunoglobulin binding bacterial protein G. This study is a continuation of our effort to understand the mechanism of β -hairpin formation, and to determine the role of the C-terminal β -hairpin of the B3 domain of the immunoglobulin binding bacterial protein G in the mechanism of folding of the whole protein. In our previous studies, by using the 16-, 14-, 12-, 8- and 6-residue peptides, we showed that the turn region (residues Asp51 - Thr56) is able to form bent conformations in the temperature range from $T = 283$ to $T = 323$ K.^{17,18,27} The conformational study of the 20-residue peptide presented in this work shows that increasing the peptide length leads to a change of the conformational propensities of the turn region (residues Asp51 - Thr54). As opposed to our previous studies, we did not observe small absolute values of the temperature coefficients for the chemical shifts of the amide protons (see Table V of supplemental data) or $H_N(i) - H_N(i+1)$ connectivities in the ROESY spectra (see Fig. 4) for residues in the turn region, which indicates that the turn region is not well developed and is very dynamic. However, we observed a very interesting and complex behavior of long-range hydrophobic interactions.

In our previous studies, we demonstrated that DSC measurements combined with NMR data, recorded at various temperatures, clearly show that long-range hydrophobic interactions are responsible for structure stabilization and that breaking the side-chain contacts between the nonpolar residues coincides with the heat capacity peak recorded by using the DSC technique.^{17,18,27} The structures of the peptides which have been investigated so far are stabilized by hydrophobic interactions between two nonpolar residues, Tyr50 and Phe57 (the “1st pair”),^{17,18,27} and we very rarely observed interactions within another pair of nonpolar residues, namely Trp48 and Val59 (the “2nd pair”).¹⁷

Surprisingly, for the peptide investigated in this work, we found that the hydrophobic interactions within the “1st pair” (Tyr50 and Phe57) are not observed in NMR spectra recorded at $T = 305$ K and $T = 313$ K (see Table I) but the interactions within the “2nd pair” (Trp48 and Val59) are observed in the whole range of temperatures (Table I). Moreover, we found that the heat capacity curve registered in the DSC experiment exhibits two peaks (see Fig. 2a) suggesting a complicated thermal unfolding/folding behavior. To the best of our knowledge, this is the first example in which a three-state folding/unfolding process for a peptide is registered by using DSC experiments (typically a two-state process is observed).^{17,18,27,67}

We provide two plausible explanations for this three-state behavior of the investigated peptide, both of which are based on the assumption that the appearance of the heat capacity peaks is related to the change in the strength of hydrophobic interactions with temperature.

The first explanation is based solely on the assumption that the formation/breaking of long-range hydrophobic interactions between nonpolar residues can give rise to the formation of a peak in the heat capacity curve. The first peak in the heat capacity curve, which appears at $T = 308.05$ K, could be related to breaking the hydrophobic interaction between Tyr50 and Phe57 (the “1st pair”), which is not observed in the NMR spectra registered at $T = 305$ K (see Fig. 4). The second heat capacity peak which appears at $T = 341$ K could be associated with the breaking/formation of the hydrophobic interaction between Trp48 and Val59 (the “2nd pair”). The hydrophobic interactions within the “2nd pair” of nonpolar residues are seen in the NMR spectra even at a relatively high temperature, $T = 313$ K. We tried to register the NMR spectra at higher temperatures ($T = 323$ K) but we are unable to obtain clearly interpretable spectra because of fast chemical exchange.

The second possible explanation for the appearance of two peaks in the heat capacity curve is as follows. For the investigated peptide, the interactions between Tyr50 and Phe57 vanish at $T < 305$ K. At such low temperatures, the hydrophobic interactions are weak^{29–35} and, consequently, their breaking could be undetected in DSC measurements. The heat capacity peak observed at $T = 308.05$ K could, therefore, be associated with the breaking/formation of the Trp48 ... Val59 interaction. Interactions between the Trp48 and Val59 residues at $T = 313$ K (5 degrees above the heat capacity peak inferred from the DSC measurements) are still observed. This difference can be explained as follows. At the folding temperature, there should be equal populations of folded and unfolded states and, above the folding temperature, there could still be some fraction of folded states, that could be detected by NMR spectroscopy. The second peak in the heat capacity curve could be associated with “hydrophobic solvation” effects which would occur at high temperature.⁵⁸ However, in our previous studies^{17,18,27} on shorter peptides, we did not observe such a heat capacity peak which could be associated with “hydrophobic solvation”. Additional experiments are required to determine which of the two explanations is correct.

Our results show that, if peptide fragments are used to model the early stages of protein folding, all results should be taken with caution, because some structural properties of peptides clearly depend on peptide length. In this investigation, we showed that the observed hydrophobic interaction pattern changes with peptide length (ref. ¹⁷, and this work), even if the peptides studied share the same hydrophobic residues. The relation between structure/conformational properties and peptide length has not been studied systematically in previous studies.^{4–11,12–16}

Our previous studies^{17,18,27} have shown that the mechanism of β -hairpin formation consists of the following two consecutive steps: (1) formation of a bent conformation in the turn region, and (2) formation of long-range hydrophobic interactions. Step (1) facilitates step (2). Such a mechanism is in agreement with the so-called “zipper-mechanism” proposed by Matheson and Scheraga,⁶⁸ based on hydrophobic interactions, and by Muñoz and coworkers,⁶⁶ with the exception that, in the “zipper-mechanism” of Muñoz et al.,⁶⁶ the stabilization of a β -hairpin was assumed to be achieved by formation of a hydrogen bond network but our study shows the importance of hydrophobic interactions.⁶⁸ Muñoz and coworkers assumed that β -hairpin folding and unfolding processes are completely opposite i.e., the path of folding and unfolding are identical.⁶⁶ In this work, we have shown that the folding and unfolding processes for the investigated peptide might not be identical. We found that, at low temperature, two pairs of hydrophobic interactions (the “1st pair” and the “2nd pair”), which stabilize the β -hairpin structure, are observed. However, with increasing temperature (which corresponds to unfolding), we observe that hydrophobic interactions located closer to the turn region (the “1st pair”) vanish at lower temperature than the pair of hydrophobic interactions located further away from the turn region (the “2nd pair”). On the other hand, our previous data^{17,18,27} suggested that the folding process progresses from

formation of the turn region and by subsequent formation of the 1st and then the 2nd hydrophobic pair.⁶⁶ The proposed mechanism of folding unfolding of IG(42–61) is presented in Fig. 9.

As mentioned in the Introduction, the main goal of this work was to investigate the possibility of formation of interactions between the hydrophobic “micro-core” formed within the β -hairpin sequence and nonpolar residues located outside the β -hairpin but close in sequence. We are not able to find evidence that such interactions occur. Thus, our conclusion is that the growth of the hydrophobic core is not achieved by adding nonpolar residues, that are close in sequence to the core, one by one, but rather by collision of hydrophobic “micro-cores” formed in different parts of the sequence. This mechanism was proposed by Tanaka and Scheraga,⁶⁹ which later supporting experimental evidence by Dyson et al.⁷⁰ In our future work, we will investigate this mechanism by studying a larger peptide composed of the C-terminal β -hairpin and the α -helix sequence connected by the four residue loop (Fig. 9). We have already studied all of these fragments separately (refs. 17, 26, and this work), and it is now to be determined if these fragments, each of which has a well-defined secondary structure in the parent protein, can interact with each other in the early stages of protein folding as proposed in earlier studies.^{68–72}

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1.
 (a) X-ray structure of the B3 domain of protein G (1IGD).²⁷ (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(42–61), was investigated in this work.

Fig. 2. Heat capacity curve of IG(42–61) recorded (a) in phosphate buffer ($C_{\text{KH}_2\text{PO}_4} = 1/15$ mol/ml, $C_{\text{Na}_2\text{HPO}_4} = 1/15$ mol/ml) at pH = 6.81 and at concentration 0.07 mM and (b) in phosphate buffer ($C_{\text{KH}_2\text{PO}_4} = 1/15$ mol/ml, $C_{\text{Na}_2\text{HPO}_4} = 1/15$ mol/ml) at pH = 6.81 and at concentration 2.1 mM.

Fig. 3. CD spectra of IG(42-61) (a) in phosphate buffer (pH = 6.81) at 16 different temperatures, and (b) in TFE at three different concentrations at 305 K.

Fig. 4. ROE effects corresponding to interproton contacts, and $^3J_{\text{NHH}\alpha}$ coupling constants, of IG(42–61) measured in phosphate buffer (pH = 6.81) at (a) 283 K, (b) 305 K and (c) 313 K. The thickness of the bars reflects the strength of the ROE correlation as strong, medium or weak.

Fig. 5. Outline of the structure of IG(42–61) with marked ROE connectivities at 283 K (short-dashed lines), 305 K (long-dashed lines) and 313 K (short-and-long dashed lines). The boxed fragments of the sequence correspond to the “1st pair” of nonpolar residues (Tyr50 and Phe57) and the “2nd pair” of nonpolar residues (Trp48 and Val59).

Fig. 6.

Four most populated families of conformations of IG(42–61) 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) 223 (18.6%), (b) 81 (6.8%), (c) 74 (6.2%), (d) 72 (6%).

Fig. 7.

Same as Fig. 6, but for 305 K, with the following results: (a) 115 (9.6%), (b) 101 (8.4%), (c) 81 (6.8%), (d) 80 (6.7 %).

Fig. 8.

Three most populated families of conformations of IG(42–61) 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) 300 (25%), (b) 94 (7.8%), (c) 38 (3.2%).

Fig. 9.
A plausible mechanism for formation of the C-terminal β -hairpin fragment from the B3 domain of protein G from *Streptococcus*.

Table I

Atoms of residues separated by at least 2 residues in sequence ($|i-j| > 1$) between which ROE peaks were found at 283, 305 and 313 K at pH = 6.81.

ROE peaks between residues $ i-j > 1$		
283 K	305 K	313 K
$\gamma\text{V47} - \epsilon\text{F57}$	$\text{NW48} - \beta\text{V59}$	$\delta_1\text{W48} - \gamma\text{V59}$
$\beta_1\text{W48} - \delta\text{F57}$		$\text{NT49} - \gamma\text{V59}$
$\beta_2\text{W48} - \delta\text{F57}$		$\delta\text{F57} - \gamma\text{V59}$
$\epsilon_3\text{W48} - \beta_1\text{F57}$		
$\epsilon_3\text{W48} - \beta_2\text{F57}$		
$\zeta_3\text{W48} - \alpha\text{Y50}$		
$\eta_2\text{W48} - \gamma_1\text{V59}$		
$\eta_2\text{W48} - \gamma_2\text{V59}$		
$\alpha\text{Y50} - \delta\text{F57}$		
$\alpha\text{Y50} - \epsilon\text{F57}$		
$\beta_2\text{Y50} - \delta\text{F57}$		
$\beta_2\text{Y50} - \epsilon\text{F57}$		
$\epsilon\text{Y50} - \alpha\text{V59}$		

N – amide protons