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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 II. Interplay of local backbone conformational dynamics and long-range hydrophobic interactions in hairpin formation

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Abstract

Two peptides, corresponding to the turn region of the C-terminal β -hairpin of the B3 domain of the immunoglobulin binding protein G from *Streptococcus*, consisting of residues 51–56 [IG(51–56)] and 50–57 [IG(50–57)], respectively, were studied by CD and NMR spectroscopy at various temperatures and by differential scanning calorimetry. Our results show that the part of the sequence corresponding to the β -turn in the native structure (DDATKT) of the B3 domain forms bent conformations similar to those observed in the native protein. The formation of a turn is observed for both peptides in a broad range of temperatures ($T = 283$ – 323 K), which confirms the conclusion drawn from our previous studies of longer sequences from the C-terminal β -hairpin of the B3 domain of the immunoglobulin binding protein G (16, 14 and 12 residues), that the DDATKT sequence forms a nucleation site for formation of the β -hairpin structure of peptides corresponding to the C-terminal part of all the B domains of the immunoglobulin binding protein G. We also show and discuss the role of long-range hydrophobic interactions as well as local conformational properties of polypeptide chains in the mechanism of formation of the β -hairpin structure.

Keywords

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

INTRODUCTION

The pathway by which the polypeptide chain assembles from the unfolded, “disordered” state to the final active folded protein has been the subject of intense investigation. Local interactions are especially important in restricting the conformational space of the polypeptide chain in the search for the native state.^{1–10} Knowledge of local interactions is most important in the very early stages of protein folding. One of the methods to detect the

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importance of local interactions is a conformational study of protein fragments corresponding to regular secondary structure.^{11–13} 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. Short protein fragments have often been used as simple model systems to identify specific noncovalent interactions that are crucial for secondary-structure formation.^{14–16} These fragments can fold in aqueous solution into native-like conformations when no tertiary interactions are present and, thus, may play an important role as nucleation centers in initiating protein folding through local interactions^{1–5, 17–19} and provide knowledge about the earliest events in protein folding.

While the literature is rich in studies of α -helical peptides,^{20–26} small water-soluble, non-aggregating monomeric β -sheet peptides have been synthesized only relatively recently;^{27–30} earlier attempts were unsuccessful because of their marginal stability and the strong tendency to aggregate.³¹ Several features of peptides, such as side-chain side-chain interactions,³² aromatic clusters,^{33,34} salt-bridge formation^{35,36} and, especially, conformational preferences of turn sequences^{37–41} appear to be critical for the folding and stability of β -sheets and β -hairpins. The first β -hairpin peptides reported in the literature which adopt a regular β -hairpin structure in water solution were cyclic hexapeptides^{42,43} and a 17 amino-acid-residue fragment from the N-terminal part of ubiquitin.^{44–45} Other examples of peptides which could exist in the monomeric form without aggregating, were derived from tendamistat,⁴⁶ the B1 domain of protein G^{33,47} and ferredoxin⁴⁸ but, in most cases, they showed a very limited tendency to fold in the absence of tertiary contacts. The turning point in the search for a native-like β -hairpin peptide which can fold in water was the work of Blanco et al.⁴⁷ Their peptide, the C-terminal 16 amino acid residue β -hairpin fragment from the B1 domain of protein G (PGB1), remained monomeric at high concentration⁴⁷, and it was estimated that it adopts a population containing up to 40% native-like β -hairpin structure. Based on these data, Blanco et al speculated that it would be the first folding initiation site of the whole protein that could provide a nucleation center on which the rest of the polypeptide chain would find a favorable environment to fold.⁴⁷ On the other hand, Blanco et al performed their experiments at a very low temperature (278 K)⁴⁷ at which structure is usually stabilized.⁴⁹ It should be noted, however, that proteins usually fold at higher temperatures (the folding temperature of the B domains of protein G is around 353 K)^{50,51} and, consequently, the stability of the β -hairpin studied by Blanco et al could be exaggerated and their results not necessarily relevant to the mechanism of folding of the B domains of protein G.

In our previous work,^{52,53} we showed that peptide fragments of different length corresponding to the C-terminal β -hairpin of the B3 domain of the immunoglobulin binding protein G from *Streptococcus* (Fig. 1), are able to form a β -hairpin-like structure over a wide range of temperatures. We also provided evidence that β -hairpin-like structures are stabilized by cross-strand interactions between hydrophobic residues present in the investigated sequences rather, than by the hydrogen bonds suggested in earlier studies of similar sequences.^{47,54,55} In the present work, we continue our effort to understand the mechanism of β -hairpin formation, and our study is focused on the role of the turn region in this process. Muñoz et al.^{54,55} proposed that β -hairpin formation is initiated by the turn sequence which results in bending a polypeptide chain and facilitates the formation of cross-strand interactions, such as hydrogen bonds, salt bridges, and hydrophobic interactions; in other words, that the turn sequences is the “nucleation center” in β -hairpin formation. In our previous studies of peptides of different lengths (16, 14 and 12 amino acid residues),^{52,53} we showed that the sequence corresponding to the turn region in the native protein⁵⁶ possesses the ability to form turn-like structures regardless of the length of the investigated peptide.^{52,53} We also showed that formation of the turn structure is not affected by

temperature (up to 313 K). To understand the role of the turn region in the β -hairpin-formation mechanism, we have investigated two peptides of length 8 and 6 residues, respectively. These short peptides enabled us to investigate the conformational propensities of the *isolated* turn region, thereby removing the possible influence of long-range interactions on turn formation.

MATERIALS AND METHODS

Peptide synthesis

The peptides Ac-YDDATKTF-NH₂ [IG(50–57); 8 amino acid residues] and Ac-DDATKT-NH₂ [IG(51–56); 6 amino acid residues] were synthesized by standard solid-phase Fmoc-amino acid chemistry with a Milipore synthesizer. Both resins Tentagel R RAM (1g, capacity 0.19 mmol/g) were treated with piperidine (20%) in DMF, and all amino acids were coupled using 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, each of the resins was washed with methanol and dried *in vacuo*. Then the resins were 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 resins were 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. Each 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 from diethyl ether. The crude peptides were 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 0.5% CH₃CN and of 14% CH₃CN for IG(51–56) and IG(50–57), respectively, in TFA to adjust the pH approximately to 2.0. The fractions containing pure peptides were lyophilized and their purity was confirmed by analytical HPLC and MALDI-TOF analysis (*M* = 691.2 g/mol, theoretical value of molecular mass is 690.7 g/mol, and *M* = 1001.5 g/mol, theoretical value of molecular mass is 1001.06 g/mol for IG(51–56) and IG(50–57), respectively).

Circular dichroism spectroscopy

Circular dichroism (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 in water [the pH of IG(51–56) and IG(50–57) was 5.26 and 6.57, respectively], and the CD was measured at 16 different temperatures: 278, 283, 288, 293, 298, 303, 308, 313, 318, 323, 328, 333, 338, 343, 348 and 353 K. The final concentration of IG(51–56) and IG(50–57) was 0.0097g/100 ml and 0.014g/100 ml, respectively. The secondary structure content was calculated from the CD spectra using the CONTINLL method.⁵⁷

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 0.099 mM and 0.14 mM for IG(51–56) and IG(50–57), respectively. The cell volume was 0.5 ml. All scans were run at pH = 5.26 and 6.57 for IG(51–56) and IG(50–57), respectively, in water in the range of temperature from 5°C to 80°C. A given sample was cooled and reheated to check the reversibility of the transition process. The data presented for both compounds are averages from three independent measurements. The results from the DSC measurements were analyzed with the Origin 7.0 software from MicroCal using the routines of the software provided with the instrument.⁵⁸

¹H-NMR spectroscopy

The NMR spectra of IG(51–56) and IG(50–57) were recorded 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,⁵⁹ TOCSY⁶⁰ (80 ms), ROESY⁶¹ (200 ms) at 283, 305 and 313 K for IG(51–56) and additionally at 323 K for IG(50–57). The samples were dissolved in H₂O/²H₂O (9:1 by vol.), the pH of the solutions was 5.26 and 6.57 for IG(51–56) and IG(50–57), respectively, and the concentrations of IG(51–56) and IG(50–57) were 7.2 mM and 7.49 mM, respectively. The spectra were processed using VARIAN 4.3 software (Varian Instruments, Palo Alto, CA, USA) and analyzed with the XEASY program.⁶² The spectra were calibrated against the DSS (sodium 4,4-dimethyl-4-silapentane-1-sulfonate) signal.⁶³ Proton signals were assigned based on the TOCSY spectra. The sequential analysis of the peptide has been confirmed by the ROESY spectra.⁶¹ The chemical shifts of each compound measured at different temperatures are collected in supplemental data. The coupling constants between the NH and the H_α protons (³J_{HNH_α}) of IG(51–56) and IG(50–57) were obtained from the two dimensional DQF-COSY and one dimensional ¹H spectra. The intensities of the ROE signals were estimated from the ROESY spectra.⁶¹ In Figure 2 and Figure 3, the TOCSY spectra of IG(51–56) and IG(50–57), respectively, together with peak assignments, are shown.

Three-dimensional structure calculations

The ROE interproton cross-peaks of IG(51–56) and IG(50–57) were determined from the 2D H-NMR ROESY spectra, and the ³J_{HNH_α} vicinal coupling constants were obtained from 2D ¹H-NMR DQF-COSY and temperature-dependent 1D ¹H-NMR spectra. In the first step, conversion from ROESY peak volumes to upper distance bounds by using the CALIBA⁶⁴ program of the DYANA package⁶⁵ was performed. In the next step, the torsion angles were generated using the HABAS algorithm⁶⁶ of the DYANA package on the basis of the Bystrov-Karplus⁶⁷ equation. The upper distance limits and torsional angles were used as restraints in molecular dynamics calculations.

Molecular dynamics simulations with the time-averaged methodology (TAV)^{68–70} were carried out with the AMBER force field⁷¹ using the AMBER 8.0 package.⁷⁰ The interproton distances and the dihedral angles were restrained with the force constants $k = 20$ kcal/(mol × Å²) and $k = 2$ 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, using the restraints from the NMR data collected at 283, 305 and 313 K, and at 283, 305, 313 and 323 K were run for IG(51–56) and IG(50–57), respectively. All simulations were carried out in a TIP3P⁷² periodic water box at constant volume, with the particle-mesh Ewald procedure for long-range electrostatic interactions.^{73,74} MD simulations with time-averaged restraints, at these three different temperatures, were carried out with a time step of 2 fs,⁷⁵ and the total duration of the run was 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: T = N, 400, 500, 600 K (where N is the temperature of the NMR experiment, i.e., T = 283/305/313 K and T = 283/305/313/323 for IG(51–56) and IG(50–57), respectively). Running simulations at many temperatures provides an extensive sampling of the conformational space. For every trajectory and each peptide, 300 final snapshots were collected for further 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 and four NMR restraint sets

recorded at different temperatures for IG(51–56) and IG(50–57), respectively. All three sets of conformations were clustered separately, with the use of the MOLMOL program.⁷⁶ An RMS deviation cut-off of 5.0 Å was used in the clustering procedure. The RMS deviation was calculated based on C α atoms for both compounds.

RESULTS AND DISCUSSION

Differential Scanning Calorimetry (DSC)

Fig. 4b shows the heat capacity curve for the IG(50–57) peptide. A relatively sharp peak is observed, which suggests that a conformational transition connected with a major energetic effect occurs. Assuming a two-state model for the conformational transition, a transition temperature of $T_m = 316.98 \pm 0.21$ K and an enthalpy change for this transition of $\Delta H_m = 154.7 \pm 4.25$ kcal/mol were determined. It should be noted that the heat capacity becomes negative for the IG(50–57) peptide at temperatures above 330 K with a minimum at $T = 338$ K. The appearance of this minimum (at which the heat capacity is negative) suggests that the peptide starts to aggregate above $T = 330$ K.⁷⁷ However, the putative aggregation of the peptide is completely reversible. The reversibility was demonstrated by running several heating/cooling cycles using the same sample; almost identical DSC curves were obtained in every cycle.

It should be noted that the enthalpy change is very large for IG(50–57), compared to that obtained for other peptides investigated in our earlier work,^{52,53} in which the enthalpy change never exceeded $\Delta H_m = 50$ kcal/mol. This large value of the heat capacity, obtained for the IG(50–57) peptide, should be considered with caution. The rapid drop of the baseline [seen as the negative peak (~338 K)] after the phase transition peak (probably connected with reversible aggregation) suggests that the investigated process is not a typical two-state one.^{78,79} Therefore, the observed enthalpy change can correspond to multiple structural changes which occur simultaneously. On the other hand, the transition temperature calculated for the IG(50–57) peptide ($T_m = 316.98$ K) is very similar to the melting temperatures reported in our previous studies for longer IG peptides,^{52,53} even though the folding temperature is not correlated with peptide length. In our previous studies, longer 16-, 14- and 12-residue IG peptides exhibited melting points, determined by the DSC technique, of 320.11 ± 0.14 K,⁵² 330.55 ± 0.19 K and 310.24 ± 0.03 K, respectively.⁵³ Even these three are not correlated. Systematic reduction of the length of the peptides initially increases the folding temperature and then decreases it, and the enthalpy of folding is not correlated with the folding temperature) We are not able to compare our results with any other literature data because most systematic calorimetric studies of peptides concern the effect of point mutation on the thermal stability of a peptide rather than the effect of peptide length on this property.⁸⁰

The IG(51–56) peptide does not exhibit any heat-capacity peak (see Fig. 4a). In our previous studies, we postulated that the appearance of a heat-capacity peak is associated with the rupture of long-range hydrophobic interactions.^{52,53} Because IG(51–56) does not possess any hydrophobic residues, the absence of a heat-capacity peak observed for this peptide is consistent with this postulate. On the other hand, the absence of a peak does not preclude conformational changes of the peptide (which can be detected by experimental techniques other than DSC); however such changes are not associated with large changes in enthalpy.⁸⁰ For example, the absence of a clear heat capacity peak in DSC measurements is characteristic of α -helical peptides, for which most of the (small) enthalpy change effects are associated with solvation/desolvation of polar backbone groups.⁸⁰

CD measurements

CD spectra were recorded in water at pH = 5.26 and 6.57 for IG(51–56) and IG(50–57), respectively, and at sixteen different temperatures: T = 278, 283, 288, 293, 298, 303, 308, 313, 318, 323, 328, 333, 338, 343, 348 and 353 K (Fig. 5).

Fig. 5 and Fig. 6 show the molar ellipticity changes with the temperature. The molar ellipticity of both compounds increases at around $\lambda = 200$ nm and is further followed by a bathochromic shift with increasing temperature (Fig. 5, Fig. 6). At higher wavelengths, $\lambda = 220$ and 230 nm, the molar ellipticity decreases for both compounds (Fig. 5, Fig. 6); together with the previously mentioned behavior of the molar ellipticity at around 200 nm, this suggests the formation of the more ordered hairpin/turn/helix structure at the expense of a random coil conformation.^{81,82} The percentages of secondary structure elements, calculated by using the SP22X CONTINLL method,⁵⁷ are listed in Table I. The changes of the amounts of specific secondary structure elements exhibit the same trend for both compounds. Based on the data listed in Table I, it can be concluded that the percentage of β -sheet conformation decreases slowly with increasing temperature, but the percentage of turn structure increases, especially for IG(51–56). The population of random-coil conformation increases with temperature for both peptides, but this increase is much faster for the IG(51–56) peptide than for IG(50–57) peptide.

In many studies, the change of ellipticity is used to determine the melting temperature of peptides.^{52,83,84} It is seen in Fig. 6 that, for all three wavelengths (201, 220 and 230 nm), for which we observed the largest ellipticity changes, and for both peptides, the ellipticity changes are close to linear with temperature. This situation does not enable us to determine the melting temperatures for these peptides, based on CD measurements. A similar situation was observed in our investigation of the 12- and 14-residue IG(47–60), and IG(46–61) peptides.⁵³

NMR measurements

The 2D ^1H -NMR spectra of IG(51–56) and IG(50–57) peptides were recorded in water at pH = 5.26 and 6.57, respectively, at temperatures T = 283, 305, 313 K and 283, 305, 313, and 323 K for IG(51–56) and IG(50–57), respectively, to examine the influence of temperature on the structure. The chemical shifts of the proton resonances at all temperatures listed above are collected in Tables I – III and IV – VII of the supplemental data for IG(51–56) and IG(50–57), respectively.

The changes in the chemical shifts of the amide protons of IG(51–56) and IG(50–57) peptides at all temperatures listed above are shown in Fig. 7a and 7b for IG(51–56) and IG(50–57), respectively. The temperature coefficients of the chemical shifts of the amide proton determined from the 1D ^1H -NMR temperature spectra [see section “ ^1H -NMR spectroscopy” of “Materials and Methods”] are listed in Table VIII of the supplemental data. It can be seen from Fig. 7 that the chemical shifts of each amide proton of both IG(51–56) and IG(50–57) move slightly upfield with increasing temperature. For IG(51–56), the smallest temperature coefficients in absolute value were found for Ala53 (–5.1 ppb/K), Thr54 (–5.8) and Thr56 (–6.1 ppb/K), whereas for IG(50–57), the smallest coefficients were found for Asp51 (–5 ppb/K), Ala53 (–4.9 ppb/K), Thr54 (–4.1 ppb/K) and Thr56 (–5 ppb/K) (see Table VIII of supplemental material). According to literature data,⁸⁵ the amide protons with temperature coefficients more positive than –4.5 ppb/K can be considered as involved in hydrogen-bond formation or strongly shielded from the solvent. This holds for the Thr54 amide proton of IG(50–57). In addition, the temperature coefficients of the amide protons are relatively small in absolute value for both peptides studied, the coefficients of IG(51–56) being more negative. This result is consistent with that of our earlier work on

longer IG peptides, 52:53 in which the amide protons of the turn region appeared to be shielded from the solvent; however, at least three (the amide protons of Asp51, Thr54 and Thr56) had temperature coefficients more positive than -4.5 ppb/K in our previous work for longer peptides. Therefore, the present result suggests that a three-dimensional structure stable enough to protect the amide protons efficiently from the solvent appears weaker as the sequence length decreases.

In Fig. 8 and Fig. 9, the ROE effects (from which to calculate interproton distances) and the values of the $^3J_{\text{NH}\alpha}$ coupling constants for IG(51–56) and IG(50–57), respectively, obtained from NMR measurements carried out at different temperatures, are presented.

IG(51–56)—For this peptide the $\text{H}_\alpha(i) - \text{H}_\text{N}(i+1)$, $\text{H}_\beta(i) - \text{H}_\text{N}(i+1)$ and $\text{H}_\text{N}(i) - \text{H}_\text{N}(i+1)$ sequential ROE connectivities are observed at each temperature (283, 305, and 313 K) (Fig. 8). The $\text{H}_\text{N}(i) - \text{H}_\text{N}(i+1)$ sequential ROE connectivities can indicate either a hairpin/turn or an α -helix structure.⁸⁶ In the hairpin/turn structure, long-range interactions, which clip the two strands together, should also be observed. The only long-range ROE connectivity, $\text{H}_\alpha(i) - \text{H}_{\text{sc}}(i+3)$ [where the subscript “sc” denotes side-chain proton(s)], was observed for the IG(51–56) peptide at the lowest temperature of the 2D ^1H -NMR measurements (283 K) (Fig. 8a, Fig. 10a, Table II). This observation indicates that the structure at 283 K is bent and stabilized by this particular long-range interaction. No long-range interactions are observed at higher temperatures (305 and 313 K), and the $\text{H}_\text{N}(i) - \text{H}_\text{N}(i+1)$ sequential ROEs suggest that an α -helix-like structure is formed.⁸⁶ In the longer peptides studied previously^{52:53}, the hydrophobic residues Tyr50 - Phe57 usually form a long-range hydrophobic contact and keep the chain in a β -hairpin-like conformation. Because this residue pair is not present in the IG(51–56) fragment, the sequence has a tendency to bend (strong $\text{H}_\text{N}(i) - \text{H}_\text{N}(i+1)$ sequential ROEs), but an α -helix-like structure appears as a result [see results and discussion: MD simulations section].

IG(50–57)—As for the IG(51–56) peptide, the 2D ^1H -NMR spectra of IG(51–56) were recorded at 283, 305 and 313 K. Additional experiments were performed at 323 K. For IG(51–56), the NMR measurements at this high temperature were not feasible because of fast deuterium/hydrogen exchange and conformational equilibrium. The measurements at 323 K are very important because they provide information about the structure of the peptide after the melting-transition temperature (Fig. 4). At each temperature (283, 305, 313 and 323 K), the $\text{H}_\alpha(i) - \text{H}_\text{N}(i+1)$, $\text{H}_\beta(i) - \text{H}_\text{N}(i+1)$ and $\text{H}_\text{N}(i) - \text{H}_\text{N}(i+1)$ sequential ROE connectivities are usually observed (Fig. 9). In the sequence of this fragment, there is a pair of hydrophobic residues (Tyr50 - Phe57), which clearly interact at 305 and 313 K. Moreover, at 305 and 313 K, a large number of side-chain to side-chain interactions can be observed between residues other than Tyr50 and Phe57. The side-chain to side-chain long-range ROE connectivities at 305 K and 313 K are listed in Table II and shown in Fig. 9 and Fig. 10. At temperatures higher than the melting temperature ($T_m = 316.98$ K; see Fig. 4), long-range interactions are not present (Fig. 9, Fig. 10, Table II). Also, at 283 K and 323 K, only $\text{H}_\alpha(i) - \text{H}_\text{N}(i+1)$, $\text{H}_\beta(i) - \text{H}_\text{N}(i+1)$ and sequential $\text{H}_\text{N}(i) - \text{H}_\text{N}(i+1)$ ROEs are seen; their presence suggests an α -helical structure. $T = 323$ K is a temperature just after the phase-transition temperature for this peptide (see Fig. 4); thus, information about the structure and its stabilization at 323 K is obtained after the phase transition, which means that, after the global structure reorganization, i.e., after passing the melting temperature, the turn sequence still has a propensity to bend. This, in turn, means that this part of the sequence is the most stable element of the whole protein, and is the first folding initiation site. The pair of hydrophobic amino acid residues (Tyr50 - Phe57) plays a role as a hairpin staple, but only at two temperatures (303 and 313 K). At 283 K, the conformation of the peptide becomes α -helix-like [lack of long-range ROEs but presence of a large number of sequential $\text{H}_\text{N}(i) - \text{H}_\text{N}(i+1)$ ROEs; see Fig. 9, Fig. 10], at 305 and 313 K, a β -hairpin-like conformation is

observed (a large number of long-range ROEs; see Fig. 9, Fig. 10) and, finally, at 323 K, mostly an α -helix-like conformation is observed [absence of long-range ROEs but a large number of sequential $H_N(i) - H_N(i+1)$ ROEs, see Fig. 9, Fig. 10]. At low temperature, no contacts between hydrophobic residues are observed; these contacts appear at higher temperatures where the hydrophobic interactions become stronger.^{87–93} When thermal motion overcomes the hydrophobic interactions, the structure melts, which is seen in DSC experiments as a heat capacity peak (see Fig. 4b). After the melting point, no long-range hydrophobic interactions are manifest. All these observations support the results of our previous study,^{52,53} which show the importance of hydrophobic interactions in β -hairpin stabilization, and clearly connect their existence with the melting temperature recorded by DSC measurements. Aside from the role of hydrophobic interactions in structure stabilization, large numbers of strong sequential $H_N(i) - H_N(i+1)$ contacts (see Fig. 8 and Fig. 9) are always observed at all temperatures for both peptides in the peptide sequence that corresponds to the turn region (residues Asp51-Thr56). Strong sequential $H_N(i) - H_N(i+1)$ ROEs are always connected with formation of a bend/turn in a polypeptide chain.⁸⁶ Strong sequential $H_N(i) - H_N(i+1)$ were observed for longer analogue peptides investigated in our previous work;^{52, 53} however, because of the presence of cross-strand hydrophobic interactions, we could not determine whether the turn could form by itself or was only induced by long-range interactions. The results obtained in this study, indicating a turn conformation in the IG(51–56) peptide (which does not possess hydrophobic residues), enable us to conclude that the amino acid composition of the turn sequence in the C-terminal IG peptides is responsible for formation of a bent conformation regardless of temperature, chain length, or the presence of hydrophobic residues which can form cross-strand contacts.^{52,53} The formation of a bent conformation at Asp51-Thr56 is also supported by the comparatively low (in absolute value) temperature coefficients of the corresponding amide protons (see Table VIII of the supplemental material), which suggest the formation of a stable conformation. Low values of the temperature coefficients of the amide protons of the Asp51-Thr56 sequence region are also observed for the 16-,⁵² 14- and 12-, and 20- (unpublished results) residue-long IG peptides.

MD simulations

The structural data summarized in Fig. 8 and Fig. 9 were used to carry out MD simulations of IG(51–56) and IG(50–57), respectively, with time-averaged restraints, in order to determine the structure of these peptides.

IG(51–56)—The most populated conformational families of IG(51–56) at 283, 305, and 313 K, respectively, are presented in Fig. 11–13.

After MD simulations, including the restraints derived from the NMR measurements at 283 K, we obtained 5 families of conformations (Fig. 11). In the most populated family (Fig. 11a) as well as in the other, less populated families (Fig. 11b, c, d), the middle part of the sequence is bent. The turn region is preserved as in the native protein.⁵⁶ The structures shown in Fig. 11 reproduce the data presented in Fig. 8a very well. Despite the presence of a large number of sequential $H_N(i) - H_N(i+1)$ ROEs in the sequence of IG(51–56) at 283 K, α -helical conformations are not present.⁸⁶ The tendency of the peptide to bend and to form a β -hairpin-like conformation is caused by one long-range interaction between H_α (Asp52) and H_{sc} (Lys) (Fig. 8; Fig. 10; Fig. 11; Table II); this connectivity is clearly visible in most families shown in Fig. 11. The presence of an interaction between charged residues suggests the formation of a salt-bridge between residues Asp52 and Lys55 at low temperatures. The formation of this salt-bridge is observed in the structure of the whole protein.⁵⁶ The ROE between Asp52 and Lys55 was also observed at low temperature for the 16, 14, and 12 amino-acid-residue IG peptides studied in our earlier work.^{52,53} This salt bridge usually

appears at low temperatures and is more pronounced when the hydrophobic interaction between residues Tyr50 and Phe57 is not observed. Based on this finding, we can conclude that the formation of the Asp52 and Lys55 interaction is complementary to that of the Tyr50 and Phe57 interaction. A tentative conclusion can thus be drawn that these two different types of interactions facilitate turn formation under different conditions. Further studies carried out in different environments such as, e.g., solvents with increasing ionic strength to screen all long-range electrostatic interactions, are certainly required to validate this conclusion. Based on their studies of the 16-amino-acid-residues peptide corresponding to the C-terminal β -hairpin fragment of the B1 domain of protein G, Blanco et al as well as Muñoz et al.^{47,54} have shown that two pairs of hydrophobic residues (Tyr50 – Phe57 and Trp48 – Val59) are the most important in stabilizing the secondary structure of this fragment. Indeed, our previous studies confirm this statement.^{52,53} However, our present study indicates that the first stage of the folding of this C-terminal β -hairpin fragment is the creation of the turn region which seems to be the most stable element in the whole set of sequences of different length. The native-like turn position is preserved even without the Tyr50 and Phe57 residues present in studied sequence (see Fig. 8, Fig. 10, Fig. 11, Fig. 12, Fig. 13). This means that these hydrophobic residues start being important later, in the next stage of the folding process.

As temperature increases, the long-range interactions gradually disappear (Fig. 8 and Fig. 10). On the other hand, the $H_N(i) - H_N(i+1)$ connectivities, responsible for the helix/hairpin formation⁸⁶ still appear. At 305 K, these connectivities are even stronger than those observed at 283 K (see Fig. 8a, b). At 305 K, after MD simulations including NMR restraints, we obtained 5 families of conformations (Fig. 12), as in the simulations carried out at 283 K. Because of the lack of long-range ROEs, the peptide forms an α -helix-like structure (see Fig. 12).

At 313 K, the α -helix-like conformation is much more pronounced than at lower temperatures (Fig. 13). As can be seen from Figure 13a and 13 b, the first two families contain almost a regular α -helical structure, whereas the third family contains structures (Fig. 13c) bent in the middle of the sequence to the hairpin-like conformation. It should be noted that not only are the structures similar to those obtained at 305, but also the same ROEs indicate that an α -helical structure is formed in the IG(51–56) peptide (Fig. 8).

Based on the data presented above, we can conclude that the turn sequence of the IG(51–56) fragment of the C-terminal β -hairpin of the B3 domain of protein G has an intrinsic tendency to bend in aqueous solution, regardless of the temperature. The absence of the hydrophobic residues (Tyr50 – Phe57), present in the whole sequence of the C-terminal β -hairpin, whose appearance could enhance the β -hairpin conformation, results in formation of an α -helix-like structure for this peptide.

IG(50–57)—The most populated conformational families of IG(50–57) at 283, 305, 313 and 323 K, respectively, are shown in Fig. 14, Fig. 15, Fig. 16 and Fig. 17.

The sequence of the IG(50–57) peptide differs from that of the IG(51–56) peptide by the presence of the pair of hydrophobic amino acid residues (Tyr50 and Phe57). After MD simulations including NMR restraints recorded at 283 K, we obtained 4 families of conformations (Fig. 14). The long-range interactions, which could indicate an α -helix conformation at this temperature⁸⁶, are the strong sequential $H_N(i) - H_N(i+1)$ ROEs (Fig. 9). Indeed, all conformations of each family show a propensity to form the α -helix-like structure (Fig. 14). These data clearly show that the hydrophobic interactions are too weak at low temperature to stabilize β -hairpin structure even when a bend is created in the turn region.

The picture is significantly changed at 305 K (Fig. 15). At this temperature the conformations of all 4 families are bent and form β -hairpin-like structures. This is caused, first of all, by a large number of long-range interactions between the side-chains, the most important being those between the pair of hydrophobic residues (Tyr50 - Phe57) which finally act to facilitate formation of a hairpin (Fig. 9, Fig. 10, Table II) and by local interactions of medium strength manifested as sequential $H_N(i) - H_N(i+1)$ connectivities, which involve each of the amide protons of the IG(50–57) sequence (Fig. 9).

The higher the temperature, the more side-chain to side-chain ROEs are observed (Fig. 9, Fig. 10, Table II). The temperature of 313 K is very close to the melting temperature of IG(50–57) ($T_m = 316.98$ K) (Fig. 4). Thus, although a large number of long-range ROEs (Fig. 9, Fig. 10, Table II) and sequential $H_N(i) - H_N(i+1)$ connectivities (Fig. 9) are observed, the structures obtained at 313 K in each of 4 families (Fig. 16) are not as ordered as they were at 305 K. However, they still possess a tendency to form a turn/hairpin structure.

At 323 K, which is higher than the melting temperature of $T_m = 316.98$ K (Fig. 4), the structure of IG(50–57) changes dramatically one more time. The five families of conformation obtained at this temperature are shown in Fig. 17. The most populated family (33.25%) (Fig. 17a) contains bent conformations, as occurred at lower temperatures, but almost all of the other families (Fig. 17b, d, e) consist of nearly α -helical conformations. Such conformations satisfy the NMR restraints (Fig. 9d) best, because there are no long-range ROEs at 323 K. However, even at this high temperature, sequential $H_N(i) - H_N(i+1)$ connectivities, responsible for helix/hairpin formation,⁸⁶ are observed, as at 283 K where long-range ROEs were also not observed. This finding demonstrates that the turn-formation propensity in the IG peptides is independent of temperature and is an intrinsic property of their sequence. The tendency of the chain to form turn structure remains even above the melting temperature where long-range interactions between the side chains, including the hydrophobic interactions between Tyr50 and Phe57 are disrupted because of increasing entropy.

CONCLUSIONS

In this work, we present extensive conformational studies of two peptides (6 and 8 residue long) corresponding to the sequence of the turn region of the C-terminal β -hairpin of the B domains of immunoglobulin-binding bacterial proteins.⁵¹ The data presented in this paper, as well as in our previous studies^{52–53} on longer (16, 14 and 12 amino acid residues) fragments of the C-terminal β -hairpin of the B3 immunoglobulin binding domain of protein G, show that the turn region (residues Asp51 - Thr56) possesses the propensity for formation of a bent conformation at temperatures from 283 to 323K (the range of temperatures at which the NMR experiments are conducted). We also confirmed our previous finding^{52–53} that the DSC measurements combined with NMR data recorded at various temperatures, show that long-range hydrophobic interactions are responsible for structure stabilization and that breaking of the contacts between hydrophobic side chains coincides with the heat-capacity peak. We have shown that, when the investigated peptides possess at least one pair of hydrophobic residues which can form a long-range contact, as occurs for IG(50–57), a clear peak appears in the heat capacity curve which indicates a melting transition (Fig. 4b). No heat-capacity peak is observed for the shorter peptide IG(51–56), which does not possess hydrophobic residues (Fig. 4a).

Our previous^{52–53} and current studies suggest that, for the investigated peptides, the mechanism of β -hairpin formation consists of the following two consecutive steps: (i) formation of a bent conformation in the turn region, and (ii) formation of long-range

contacts between hydrophobic side chains. Step (i) prepares the ground for step (ii) to occur. Such a mechanism suggests that the part of the sequence which can form a β -hairpin should possess two essential components: a region with high propensity to form a turn and a number of hydrophobic residues located in the sequence so that they could form the hydrophobic contacts stabilizing the hairpin structure. The formation of a β -hairpin appears to be a result of the interplay between two factors: the local conformational propensity of a turn sequence and the possibility of the formation of long-range hydrophobic interactions. For the peptides investigated in our previous^{52,53} and current work, the propensity for the formation of a bent conformation by the turn sequence is independent of temperature (at least in the range of temperatures 283–323K). In contrast to this, the interactions between hydrophobic residues depend strongly on temperature.^{87–93}

In addition, our data suggest that there is another important factor influencing β -hairpin formation. To identify this factor, in Fig. 18 we again show the heat capacity curve of IG(50–57) obtained from DSC measurements, but together with the most probable structures obtained from MD simulations using NMR-derived restraints recorded at different temperatures.

As can be seen from Fig. 18, at the lowest temperature (283 K), the IG(50–57) peptide is almost α -helical and its structure changes into a β -hairpin-like shape with increasing temperature to again become an α -helix after the transition temperature. For longer peptides investigated in our previous work^{52,53} we never observed such a β -hairpin/ α -helix transition, and the peptides remained in a nearly β -hairpin conformation at all temperatures.^{52,53} The observation that the β -hairpin/ α -helix transition occurs only for very short peptides could suggest that, apart from the presence of a turn sequence and hydrophobic residues, sequence length is important in β -hairpin formation. This conclusion contradicts the suggestion of Stanger et al.,⁹⁴ that the stability of β -hairpin peptides should decrease with increasing chain length. In addition to chain length, the propensity of the chain outside the turn region to form strands is certainly important in β -hairpin formation. In our previous studies on longer IG peptides (12, 14, and 16 residues),^{52,53} we observed that parts of the sequences which form strands in a β -hairpin are in a rather extended conformation at all investigated temperatures, even at those above the melting point when all long-range interactions are disrupted.^{52,53} Computing the maximum length of a single strand as $(n-4)/2$, where n is the length of the sequence, and 4 residues are considered to form a turn, we obtain 1 and 2 for IG(51–56) and IG(50–57), respectively. These lengths are apparently too short for the respective portion of the sequence to remain in a strand conformation out of the context of long-range interactions. For IG(48–59), IG(47–60), and IG(46–61) studied in our previous work,^{52,53} the strand lengths are 4, 5, and 6, respectively; these lengths, together with local conformational propensity, are sufficient for a chain to remain in an extended conformation even after the long-range hydrophobic contacts are disrupted.^{52,53} Consequently, the peptide fragment should be at least 4 residue long for the chain to remain in an extended conformation in the absence of long-range interactions. However, to determine the minimum strand length, a 10-residue IG fragment [IG(49–58)] should be studied. This peptide was not investigated in our present study because we focused on the turn sequence and hydrophobic interactions; however, we plan to include it in our further studies.

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Fig. 1.

(a) X-ray structure of the B3 domain of protein G (1IGD). (b) Amino acid sequence of 1IGD, where the boxed fragment, IG(51–56), was synthesized and examined. (c) Amino acid sequence of 1IGD, where the boxed fragment, IG(50–57), was synthesized and examined. In (b) and (c), β denotes strand and H helix.

Fig. 2. Amino acid spin systems in the TOCSY spectra of IG(51–56) in H₂O ($t_m = 80$ ms; the diagnostic region) at (a) 283 K, (b) 305 K and (c) 313 K.

Fig. 3. Amino acid spin systems in the TOCSY spectra of IG(50–57) in H₂O ($t_m = 80$ ms; the diagnostic region) at (a) 283 K, (b) 305 K, (c) 313 K and (d) 323 K.

Fig. 4. Heat capacity curves for (a) IG(51–56) and (b) IG(50–57), recorded in water at pH = 5.26 and 6.57, respectively.

Fig. 5. CD spectra of (a) IG(51–56) and (b) IG(50–57) in water at 16 different temperatures at pH = 5.26 and 6.57, respectively.

Fig. 6. Variation of the molar ellipticity of (a) IG(51-56?) and (b) IG(50-57), with temperature at 201 nm (squares), 220 nm (circles), and 230 nm (triangles), respectively.

Fig. 7. Chemical shifts of the amide protons of consecutive amino acid residues of (a) IG(51–56) and (b) IG(50–57) at pH = 5.26 and 6.57, and at three (283, 305 and 313 K) and four (283, 305, 313, and 323 K) different temperatures, respectively.

Fig. 8. ROE effects corresponding to the interproton distances and the $^3J_{\text{HNH}\alpha}$ coupling constants of IG(51–56) measured in H₂O at (a) 283 K, (b) 305 K and (c) 313 K. The height of the bars reflects the strength of the ROE correlation as strong, medium or weak.

Fig. 9.

ROE effects corresponding to the interproton distances and the $^3J_{NH\alpha}$ coupling constants of IG(50–57) measured in H₂O at (a) 283 K, (b) 305 K, (c) 313 K and (d) 323 K. The height of the bars reflects the strength of the ROE correlation as strong, medium or weak.

Fig. 10.

Outline of the structure of (a) IG(51–56) and (b) IG(50–57) with ROE connectivities at 283 K (short-dashed lines), 305 K (long-dashed lines) and 313 K (short-and-long dashed lines) marked. In (b) at 323 K long-range ROEs are absent. [the type of long-range interactions is listed in Table II]

Fig. 11.

Five families of conformations of IG(51–56) 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) 665 (55.4%), (b) 171 (14.3%), (c) 163 (13.6%), (d) 121 (10.1%), (e) 80 (6.7%).

Fig. 12. Same as Fig. 11, but for 305 K, with the following results: (a) 757 (63.1%), (b) 184 (15.3%), (c) 175 (14.6%), (d) 59 (4.9%), (e) 25 (2.1%).

Fig. 13.

Three most populated families of conformations of IG(51–56) 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) 826 (68.8%), (b) 163 (13.6%), (c) 148 (12.3%).

Fig. 14.

Four most populated families of conformations of IG(50-57) 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) 599 (49.9%), (b) 270 (22.5%), (c) 211 (17.6%), (d) 108 (9%).

Fig. 15.

Four families of conformations of IG(50–57) 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) 577 (48.1%), (b) 307 (25.6%), (c) 234 (19.5%), (d) 82 (6.8%).

Fig. 16. Same as Fig. 15, but for 313 K, with the following results: (a) 663 (55.3%), (b) 367 (30.6%), (c) 114 (9.5%), (d) 56 (4.7%).

Fig. 17.

Five families of conformations of IG(50–57) obtained by using time-averaged MD methodology with restraints from NMR measurements at 323 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) 399 (33.25%), (b) 247 (20.58%), (c) 216 (18%), (d) 179 (14.92%), (e) 159 (13.25%).

Fig. 18. Heat capacity curves for IG(50–57) recorded in water at pH = 6.57, together with the structures that reflect the best NMR restraints recorded at 283, 305, 313, and 323 K.

Table I

Percentages of secondary structure elements of IG(51–56) and IG(50–57) at different temperatures, obtained from CD spectra using the SP22X CONTINLL method.⁵⁷

T [K]	IG(51–56)					IG(50–57)				
	α -helix	β -sheet	turn	Random coil	pp2	α -helix	β -sheet	turn	random coil	pp2
278	12.3	57.6	9.5	16	4.7	6.6	35.4	17.8	32.6	7.7
283	12.5	51.8	12	18.3	5.4	6.1	34.6	17.3	33.9	8.2
293	11.9	47.5	13.8	20.9	5.9	5.3	34.1	17.5	34.6	8.4
303	11.9	47.4	14.2	20.7	5.8	5.2	33.3	17.5	35.3	8.6
313	10.4	43.5	16	23.8	6.3	4.9	32.9	17.4	36	8.9
323	11	32	20.8	28	8.1	5	32.3	18	36.2	8.5
333	11.3	31.5	21	28.2	8	5.6	31.8	18.2	36.3	8.1
343	11	30.6	21.4	28.2	8.9	5.5	31.3	18.5	36.4	8.2
353	12	31.1	21.4	27.7	7.7	5.7	31.1	18.3	36.6	8.3

^aThe method of Reference 57 was used to de-convolute the CD spectra.

Table II

Atoms of residues $|i-j| > 1$ between which the ROE peaks were found at 283, 305 and 313 K in IG(51–56) and IG(50–57), respectively.

ROE peaks between residues $ i-j > 1$					
IG(51–56)			IG(50–57)		
283 K	305 K	313 K	283 K	305 K	313 K
α D52 – δ K55					
			$\alpha_{ac} - \delta$ F57	$\alpha_{ac} - \delta$ F57	
			δ Y50 – β A53	δ Y50 – β A53	
			δ Y50 – γ T56	δ Y50 – γ T54	
			β A53 – δ F57	δ Y50 – γ T56	
			β A53 – ϵ F57	β A53 – δ F57	
			γ T54 – δ F57	β A53 – ϵ F57	
				β A53 – N _a	
				γ T54 – δ F57	
				γ T54 – ϵ F57	
N – amide protons					
N _a – C-terminal amide protons					
α_{ac} – N-terminal alpha acetyl protons					