



## Review

 $\beta$ -hairpin-forming peptides; models of early stages of protein foldingAgnieszka Lewandowska<sup>a,b,1</sup>, Stanisław Ołdziej<sup>a,b</sup>, Adam Liwo<sup>b,c</sup>, Harold A. Scheraga<sup>b,\*</sup><sup>a</sup> Laboratory of Biopolymer Structure, Intercollegiate Faculty of Biotechnology, University of Gdańsk and Medical University of Gdańsk, Kładki 24, 80-922 Gdańsk, Poland<sup>b</sup> Baker Laboratory of Chemistry, Department of Chemistry and Chemical Biology, Cornell University, Ithaca, NY 14853-1301, United States<sup>c</sup> Faculty of Chemistry, University of Gdańsk, Sobieskiego 18, 80-952 Gdańsk, Poland

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## ABSTRACT

Formation of  $\beta$ -hairpins is considered the initial step of folding of many proteins and, consequently, peptides constituting the  $\beta$ -hairpin sequence of proteins (the  $\beta$ -hairpin-forming peptides) are considered as models of early stages of protein folding. In this article, we discuss the results of experimental studies (circular-dichroism, infrared and nuclear magnetic resonance spectroscopy, and differential scanning calorimetry) of the structure of  $\beta$ -hairpin-forming peptides excised from the B1 domain of protein G, which are known to fold on their own. We demonstrate that local interactions at the turn sequence and hydrophobic interactions between nonpolar residues are the dominant structure-determining factors, while there is no convincing evidence that stable backbone hydrogen bonds are formed in these peptides in aqueous solution. Consequently, the most plausible mechanism for folding of the  $\beta$ -hairpin sequence appears to be the broken-zipper mechanism consisting of the following three steps: (i) bending the chain at the turn sequence owing to favorable local interactions, (ii) formation of loose hydrophobic contacts between nonpolar residues, which occur close to the contacts in the native structure of the protein but not exactly in the same position and, finally, (iii) formation of backbone hydrogen bonds and locking the hydrophobic contacts in the native positions as a hydrophobic core develops, sufficient to dehydrate the backbone peptide groups. This mechanism provides sufficient uniqueness (contacts form between residues that become close together because the chain is bent at the turn position) and robustness (contacts need not occur at once in the native positions) for folding a  $\beta$ -hairpin sequence.

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\* Corresponding author. Tel.: +1 607 255 4034; fax: +1 607 254 4700.

E-mail address: [has5@cornell.edu](mailto:has5@cornell.edu) (H.A. Scheraga).<sup>1</sup> Formerly Skwierawska.

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## 1. Introduction

The  $\beta$ -hairpin-forming peptides initially received attention as model systems for investigation of the early stages of protein folding [1–4]. Over the years, studies of  $\beta$ -hairpin-forming peptides were extended to other fields such as medicine in which such peptides are studied because of their application as enzyme inhibitors or nucleic acid intercalators [5], or they are used as biomaterials in surgery and reconstruction procedures [6]. This paper, however, will focus mainly on conformational studies of  $\beta$ -hairpin-forming peptides as a model of very early events in the protein-folding process.

The marginal stability and strong tendency to aggregate [7] make  $\beta$ -hairpin-forming peptides, in general, difficult to study. The first reported  $\beta$ -hairpin peptide that was demonstrated to fold autonomously into a native-like hairpin in water was the 17-amino-acid-residue fragment from the N-terminus of ubiquitin [1,4] but, according to the authors' description, this peptide adopts only ~20% of regular  $\beta$ -hairpin structure in water solution [1,4]. Studies of other peptides, derived from tendamistat [2], from the B1 domain of protein G [3,8], and from ferredoxin [9], also showed that  $\beta$ -structured peptides could exist in the monomeric form without aggregating but that, in most cases, they showed a very limited tendency to fold in the absence of tertiary contacts.

Blanco et al. [3] studied the C-terminal 16-amino-acid-residue  $\beta$ -hairpin fragment from the B1 domain of protein G (PGB1) (residues 41–56, known as the G-hairpin), and showed the first example of native-like folding of this fragment in water. This peptide remained monomeric at high concentration [3]. It was estimated that it adopts a population containing up to 40% native-like  $\beta$ -hairpin structure, and they, therefore, speculated that it would be the first folding initiation site of a protein that could provide a nucleation center to initiate the folding of the rest of the protein [3].

Since this original discovery, the G-hairpin has been the paradigm for  $\beta$ -hairpin formation and was studied by using experimental (NMR and CD spectroscopy) [3,10–13] and computational [14–17] approaches. Many groups also tried to modify the original G-hairpin peptide to study the influence of amino acid composition on the conformational dynamics and thermodynamic stability of the structure [10,12,13,15,18–27]. All of these studies have led to suggestions that there are four main factors that could contribute to the folding and stability of the G-hairpin: (1) the intrinsic  $\beta$ -turn properties of the residues which form a turn region [19,21,23–27], (2) hydrophobic interactions between the side chains across the  $\beta$ -strands [12,20,23–27], (3) interstrand hydrogen bonds that help define and maintain the architecture of the hairpin [15], and (4) polar side-chain-to-side-chain (sc–sc) interactions, including electrostatic interactions and salt bridges [10,13,18,22].

In this review, an attempt is made to show the current state of knowledge about structure and conformational dynamics of  $\beta$ -hairpin-forming peptides derived (or based on) protein sequences. We will try to show how conformational studies of short peptides could extend our knowledge about the very early stages of the protein-folding process, stages of protein folding that are usually difficult to study in detail by currently available experimental techniques.

## 2. Experimental techniques used to determine structure and conformational dynamics of the $\beta$ -hairpin-forming peptides

### 2.1. CD and IR spectroscopies

In conformational studies of  $\beta$ -hairpin-forming peptides, various experimental techniques are used such as NMR, CD or IR spectro-

scopies. Work from the 90s [1,3,4,8] as well as more recent studies [12,23–29] showed that CD spectroscopy has very limited application in the investigation of the conformational dynamics of the  $\beta$ -hairpin-forming peptides. In the CD spectra of such investigated peptides, the signals corresponding to a turn or an extended conformation are usually very weak and cannot easily be distinguished from those corresponding to other structures such as  $\alpha$ -helix or statistical coil [3,8,23–27]. Adding structure-forcing solvents such as trifluoroethanol [3,8] or changing the temperature of the experiment [23–27] does not provide useful information. In some very special cases, CD spectroscopy could provide some valuable information [12,28,29], as discussed later in Sections 3.1 and 3.2. Reports of the use of IR spectroscopy to study conformation of  $\beta$ -hairpin-forming peptides can be found in the literature [28,29]; however, the results are very difficult to interpret because the signals, as in CD spectroscopy, are very weak and overlapping.

### 2.2. NMR spectroscopy

The method of choice for conformational studies of  $\beta$ -hairpin-forming peptides is NMR spectroscopy. Even for very dynamic systems, NMR spectroscopy can provide clean interpretable spectra which can provide valuable structural information. Generally, in the literature about  $\beta$ -hairpin-forming peptides, there are two ways to describe the structure and dynamics of the investigated peptide. One way to describe structure is based on obtaining geometrical restraints (interproton distances and torsion-angle ranges) from NMR spectra, and then to convert such restraints into a three-dimensional structure (usually an ensemble of structures). However, in the literature, there are only a few examples, such as the “tryptophan-zipper” peptide designed by Cochran et al. [12], the  $\beta$ -hairpin derived from the human YAP65 WW domain studied by Espinosa et al. [30], the structure of the  $\beta$ -hairpin derived from ubiquitin [31], and the G-peptides of different length [23–27] in which the three-dimensional structures, based on NMR restraints, are presented. Such an approach to the structure-determination of flexible peptides has some drawbacks which are related mainly to signal broadening observed very often in studies of dynamic systems.

Most of the studies use some sort of fragmentary description of the structure of the investigated peptide(s), instead of presenting the structures based on the data derived from NMR experiments. The most popular way to describe the structure of an investigated peptide is based on chemical shifts (with a method developed by Blanco et al. [3]). In this approach, the values of the  $^1\text{H}$  chemical shifts of the investigated peptide are first measured. In the next step, these chemical shifts are compared with those of the corresponding protons recorded for the structure of the full protein and with the chemical shifts of model peptides recorded in a fully denatured state [32]. Using these three sets of chemical-shift values, the fraction of folded peptide can be determined from Eq. (1).

$$CS_{\text{pept}} = U \times CS_{\text{den}} + F \times CS_{\text{Pfold}} \quad (1)$$

where  $CS_{\text{pept}}$  is the chemical-shift value of a given proton in the investigated peptide,  $CS_{\text{den}}$  is the chemical-shift value of that proton in a model denatured peptide,  $CS_{\text{Pfold}}$  is the chemical-shift value of that proton in the folded state of the parent protein, and  $U$  and  $F$  are the fractions of unfolded and folded states, respectively. Additionally, the values of  $U$  and  $F$  should satisfy the condition  $U + F = 1.0$ . This method of describing peptide structure is similar to methods that are used for the deconvolution of CD spectra into fractions of regular

structures [33,34]. With this approach, the values of  $U$  and  $F$  for all protons in the investigated peptide can be calculated and, by averaging the values of  $U$  and  $F$  of all the protons, the description of the conformational state of the investigated peptide can be obtained [3]. This approach appears very reasonable, and many researchers have used it in their studies [3,13,35].

In some studies, the  $^{13}\text{C}$  chemical shifts or volumes of NOE signals between the  $\text{H}^\alpha$  and  $\text{NH}$  protons [36] are used instead of the  $^1\text{H}$  chemical shifts. There are several problems related to this kind of structure description which, however, are rarely acknowledged in the literature. This approach is based on the assumption that the chemical-shift value of a given proton (or that of another observable), changes linearly with temperature during the folding/unfolding process. It is known that, for many protons, the values of the proton chemical shifts do not change linearly when protons are transferred from the interior of a folded molecule to a water environment [11,13,35]. Values of chemical shifts change with changing temperature [37] but, in all known studies related to peptides that are protein fragments, the temperatures at which the NMR spectra are recorded for the full protein sometimes differ by more than 20–25 degrees from those at which the NMR spectra of a model of the unfolded state and of the investigated peptide are recorded.

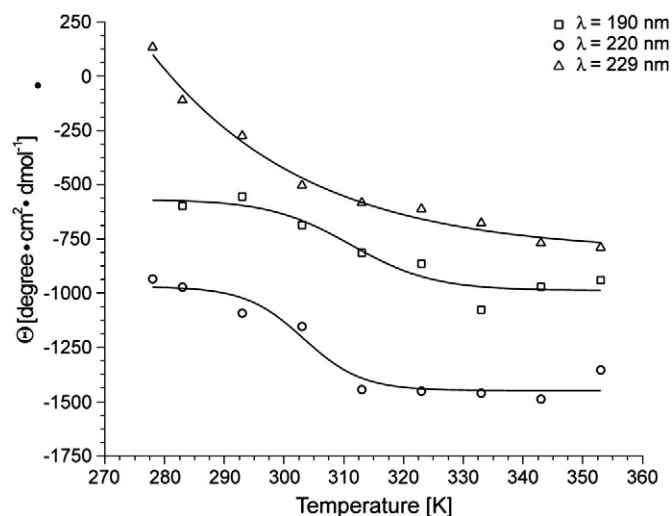
All the factors mentioned above strongly influence the results. As shown by Santiveri et al. [36], there is a weak correlation between the degree of folding obtained by using three different folding descriptors, namely  $^1\text{H}$  chemical shifts,  $^{13}\text{C}$  chemical shifts, and sequential NOE signals for sets of peptides. The description of a conformation obtained in this manner is also a very simplified one. It is assumed that the investigated peptide can exist in only two dominant conformations: folded ( $F$ ), which is essentially the conformation of this peptide identical to that of the corresponding sequence in the structure of the whole protein, and unfolded ( $U$ ), which is a conformation completely unrelated to the folded conformation. Such a two-state description of the conformational dynamics is, perhaps, elegant but cannot capture the complexity of conformational dynamics of peptides in solution.

### 3. Determination of folding-transition temperatures of $\beta$ -hairpin-forming peptides

#### 3.1. Determination of the folding-transition temperature by NMR and CD spectroscopy

CD spectroscopy is widely used for the determination of the folding-transition temperature of proteins and  $\alpha$ -helical peptides. As mentioned in Section 2.1, the usefulness of CD spectroscopy for studies of  $\beta$ -hairpin-forming peptides is very limited. There are only a few examples in the literature [12,13,23,38] in which attempts were made to determine the folding-transition temperature of  $\beta$ -hairpin-forming peptides by using CD. For the determination of the folding-transition temperature, the measurements of CD spectra at several temperatures are necessary, and then the molar ellipticities recorded at selected wavelengths are plotted against temperature and, from such plots, the folding temperature could be determined (see Fig. 1).

As shown in Fig. 1, the dependence of the molar ellipticity on temperature varies strongly with the wavelength. The curves plotted for  $\lambda = 190\text{ nm}$  and  $\lambda = 220\text{ nm}$  possess a sigmoidal shape, which suggests a conformational transition and enables us to determine the folding-transition temperature. However, the curve plotted for  $\lambda = 229\text{ nm}$  does not have any inflection point and, consequently, does not indicate any folding transition. Moreover, the folding temperatures determined from the data recorded at different wavelengths differ substantially (311 K and 303 K for the data recorded at 190 nm and 220 nm, respectively). The two problems,



**Fig. 1.** Plots of the molar ellipticities of a 16-residue peptide corresponding to a variant of the G-peptide at three different wavelengths ( $\lambda_1 = 190\text{ nm}$ ,  $\lambda_2 = 220\text{ nm}$ ,  $\lambda_3 = 229\text{ nm}$ ) vs. temperature. The folding temperatures, estimated by using the two-state model [23], are  $T_{\lambda=190\text{ nm}} = 311\text{ K}$ ,  $T_{\lambda=220\text{ nm}} = 303\text{ K}$ . Because of the absence of an inflection point in the respective curve, the folding temperature could not be determined for  $\lambda = 229\text{ nm}$ .

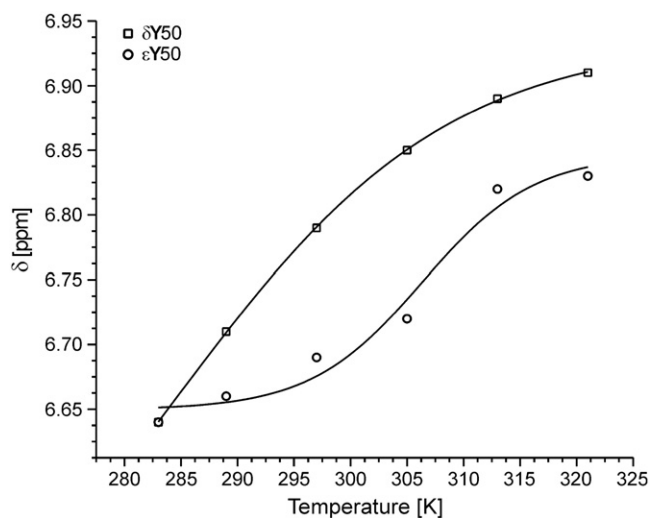
illustrated with the example shown in Fig. 1, occur rather frequently when applying CD spectroscopy to study peptide folding.

Another popular method for the determination of a folding temperature is to record NMR spectra at different temperatures and plot the changes of chemical shift of selected protons versus temperature. The main problem that arises from such an approach is that the plot of the dependence of the chemical shift on temperature often does not possess any inflection point (as encountered when using CD data for this purpose). To overcome this problem, those protons are selected for which the plot of the dependence of the chemical shift on temperature has an inflection point. For example, Kuroda [39] and Kobayashi et al. [10,11] suggested that the chemical shifts of the  $\delta$ -aromatic protons of the tyrosine residue as well as the  $\gamma$ -protons of the valine residue should be used to determine the folding temperature of the G-peptide and its variants.

As shown in Fig. 2, the selection of the protons for which the chemical shifts are observed strongly affects the value of the folding temperature. In the example shown in Fig. 2, we used the aromatic ( $\delta$  and  $\epsilon$ ) protons of the tyrosine residue as suggested by Kobayashi and coworkers [10,11]. Because the data presented in Figs. 1 and 2 pertain to the same peptide, we can compare the results. Depending on whether the chemical shifts of the  $\delta$  or  $\epsilon$  aromatic protons were used, we obtained folding temperatures of 284 K or 306 K, respectively. On the other hand, the folding temperatures determined from CD spectroscopy range from 303 K to 311 K, depending on the wavelength selected (see Fig. 1). It is clear that there is a weak agreement between the folding temperatures obtained from the CD and NMR measurements for the same compound under the same conditions.

#### 3.2. Determination of the folding-transition temperature by microcalorimetry

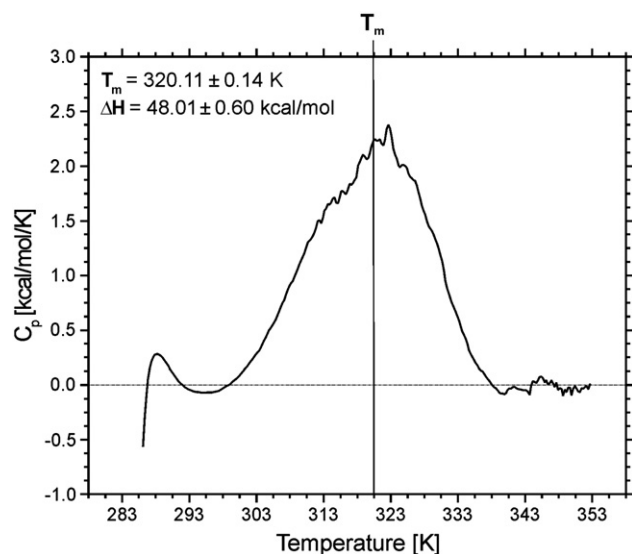
An elegant way to determination transition temperatures is to use differential scanning calorimetry (DSC). This technique enables us to determine not only the folding-transition temperature, but also the thermodynamic characteristics of the folding transition. It is also a very useful technique to detect possible aggregation of compounds under investigation [23–27]. The DSC technique was first used to determine transition temperatures of  $\beta$ -hairpin-forming peptides for the tryptophan zipper peptide, a modified variant of the G-hairpin peptide [12]. Fig. 3 shows a sample DSC



**Fig. 2.** Plot of the chemical shifts of the  $\delta$  and  $\epsilon$  protons of the tyrosine residue in the 16-residue peptide corresponding to a variant of the G-peptide versus temperature. The folding temperatures, estimated by using the two-state model [23], are  $T_\delta = 284$  K and  $T_\epsilon = 306$  K.

curve obtained for the peptide for which the folding-transition temperature was determined by using CD and NMR spectroscopy (see Figs. 1 and 2). As seen clearly in Figs. 1–3, the transition temperatures determined by these three different methods (CD, NMR and DSC) are different, which immediately raises a question as to which method gives the correct answer and which method is the best to determine the folding temperatures of  $\beta$ -hairpin-forming peptides. There are only a few examples in the literature of the use of the DSC technique to determine the folding transition [12,23–27].

Interestingly, one example shows that there is full agreement between the melting temperatures obtained from CD and DSC measurements [12,38], while other reports show disagreement between the results obtained by using various techniques as depicted in Figs. 1–3 [23]. Using the CD technique, Cochran et al. [12] determined the folding temperature of the tryptophan zipper, in which three nonpolar residues of the G-hairpin first studied by Blanco et al. [3] are mutated to tryptophans, to be 343 K. In later studies of the same



**Fig. 3.** The heat-capacity curve obtained by using the DSC technique for a 16-residue peptide corresponding to a variant of the G-peptide [23].

peptide, Streicher and Makhatadze [38] determined the folding temperatures to be 348 K and 347 K by using the DSC and CD techniques, respectively. In the study by Streicher and Makhatadze [38], the differences between the folding temperatures obtained by both techniques (CD and DSC) are minimal. Cochran and coworkers as well as Streicher and Makhatadze determined the folding-transition temperature from the temperature dependence of the molar ellipticity at  $\lambda = 229$  nm, and 228 nm, respectively, at which the ellipticity depends on the through-space interactions involving the indole moieties of the tryptophan residues [40]. On the other hand, the results shown in Figs. 1–3 are for a variant of the G-peptide possessing only one tryptophan residue, for which there are no through-space interactions between tryptophan side chains in CD spectroscopy, even if we monitor changes of the molar ellipticity at  $\lambda = 229$  nm as in the work of Streicher and Makhatadze [38]. All the results described above indicate that agreement is obtained between the melting temperatures determined from CD spectroscopy and the DSC technique when the same structure-related observables are monitored by using both methods. For the tryptophan zipper peptide, CD spectroscopy clearly shows interactions between tryptophan side chains, which means that the thermal effects observed in the DSC measurements are connected with the breaking/formation of interactions between those residues. Studies by Lewandowska (Skwierawska) and coworkers [23–27] show that there is a direct connection between the formation/breaking of the long-range hydrophobic interactions between nonpolar side chains and melting temperature determined from DSC measurements.

#### 4. Factors that determine the stability of the structure of $\beta$ -hairpin-forming peptides

##### 4.1. Turn sequence

In the G-hairpin, the  $\beta$ -turn consists of a six-residue loop (DDATKT) that forms a  $\beta$ -turn with a type IV  $\beta$ -turn in the protein structure, according to the Thornton nomenclature [41,42]. Fesinmeyer et al. [43] have shown that replacing the native turn sequence with the NPATGK sequence can substantially enhance the stability of the resulting G-hairpin. Replacement of the six-residue loop with a shorter turn-favoring sequence Val<sub>6</sub>Pro-Gly-Lys also creates a very stable G-hairpin variant [21,44]. McCallister et al. have examined the effects of replacing the turn residues with alanine in the B1 domain of protein G and found that the D47A mutation is the only one stabilizing the protein [45]. Also, replacement of one aspartic acid residue (equivalent to position 47 in the full protein) by an alanine residue in the turn-forming peptide at the turn position (with these mutation providing a six-residue loop of sequence DAATKT) is the only reported single loop-mutation that stabilizes the G-hairpin peptide [10,11,45]. The fact that the D47A mutation stabilizes the G-hairpin peptide is quite unexpected because this mutation removes not only a salt bridge with Lys50 across the turn, but also a hydrogen bond between Asp47 and Tyr45 (with residue numbers for the full sequence of the B1 domain of protein G) [45]. Most of the studies that are focused on the influence of turn sequence on stability of the peptides derived from the G-hairpin peptide agree that the increase of turn-formation propensity in the  $\beta$ -hairpin turn region increases the stability of the structure of the whole peptide [18,19,21,43,44]. On the other hand, some studies suggested that a turn sequence is not as essential as hydrophobic interactions for the stabilization of a  $\beta$ -hairpin-forming peptides structure [46].

From the analysis of the known three-dimensional structures of proteins, we know many different types of turn structures and related amino acid sequences [41,42]. However, turn sequences found in proteins can be divided into the following two groups: self-folding, which means that turn propensity is independent of



sequence context of a particular protein (i.e. the turn sequence of a short peptide excised from a protein is able to form turn structure), and induced folding which means that the formation of turn structure is context-dependent. For the G-hairpin peptides, we have proof that the turn sequence is self-folding and is able to form proper structure even in very short peptides [25,26]. From the point of view of the early stages of protein folding, it seems to be very important that short fragments of protein sequences have very well defined three-dimensional structure and this structure is well preserved over a wide range of temperature [27]. Such short (4–6 residues) sequence fragments (turn sequences) could play a role in the very first micro-nucleation centers which facilitate the formation of larger parts of the protein structure (see Section 5) and those are an important part of the protein-folding process [47]. It is interesting to note that the turn-forming sequences need not be associated only with  $\beta$ -turns; for example, Lewandowska et al. found that a turn-forming sequence is located in the loop which connects two secondary structure elements of protein G [26,27].

#### 4.2. Interactions between side chains

As mentioned above, interactions between amino acid side chains (especially between hydrophobic ones) are pointed out as the most important factor which determines structure stability of  $\beta$ -hairpin-forming peptides. In the original G-hairpin peptide, the hydrophobic cluster is formed between a valine side chain and the rings of three aromatic residues: tryptophan, tyrosine and phenylalanine [3]. Kobayashi et al. showed that single mutations of either tryptophan, tyrosine or phenylalanine to alanine in the hydrophobic core can dramatically destabilize the G-hairpin structure [10]. On the other hand, Cochran et al. mutated valine, tyrosine, and phenylalanine to tryptophan residues and obtained a peptide with very high thermal stability [12]. These two papers clearly show that nonpolar residues are essential for stabilization of G-hairpin structure; however, different kinds of nonpolar residues have different effects on stability (the importance of interaction between nonpolar residues in structure stabilization is discussed in Section 4.6.).

The pH- and salt-dependence of  $\beta$ -hairpin formation in a number of model systems suggest that long- and short-range electrostatic interactions across  $\beta$ -strands can also contribute to stability [13,46,48,49]. It has also been shown, both in designed *de novo* peptides and in the G-hairpin, that enhanced electrostatic interactions at the terminal positions of the peptide can contribute to the stability of the  $\beta$ -hairpin [13,50]. Additionally, the introduction of cross-strand Lys-Glu ion pairs near the terminal regions of designed  $\beta$ -hairpins also show increased stability [48,50]. However, these mutations might also create additional hydrophobic interactions due to the long nonpolar “neck” in the side chain of Lys, and it is difficult to separate these contributions to stability from that from ion-pair interactions. Wei et al. [35] extended this work and showed that the structure and stability of the G-hairpin can be affected greatly through only one or a few simple mutations. For example, removing an unfavorable charge near the N-terminus of the peptide (glutamic acid to glutamine or threonine) or optimization of the N-terminal charge-charge interactions (replacing glycine with lysine) both stabilize the peptide, even in water. Furthermore, a simple replacement of a charged residue in the turn (aspartic acid to alanine) changes the  $\beta$ -turn conformation [35]. Wei et al. [35] also showed that the effects of combining these single mutations are additive, suggesting that independent stabilizing interactions can be isolated and evaluated in a simple model system.

#### 4.3. Peptide length

There are a limited number of papers describing the influence of peptide length on structure stability of  $\beta$ -hairpin-forming peptides.

Stranger et al. [51] studied variants of the G-hairpin for which length was increased by adding threonine residues at both ends of the peptide. Increases of peptide length (up to five residues at both ends) led to increased thermal stability, but further increases of peptide length have no effects on structure stability or even lead to structure destabilization. On the other hand, in a series of papers, Lewandowska (Skwierawska) et al. studied variants of the G-hairpin peptide of different lengths and concluded that there is no clear connection between peptide length and structure stabilization [23–27].

#### 4.4. Hydrogen bonds

Surprisingly, it is difficult to find reports that clearly show a role for hydrogen bonds in structure stabilization of  $\beta$ -hairpin-forming peptides. Some limited reports about the role of hydrogen bonds in the stabilization of the structure of  $\beta$ -hairpin-forming peptides are related mainly to stability of the turn region (see Section 4.1) [10,23–27,45]. In all NMR structural studies of  $\beta$ -hairpin-forming peptides, there is no clear evidence such as cross-strand NOE signals for the presence of hydrogen bonds that are not located in the turn region [10,12,21,23–27].

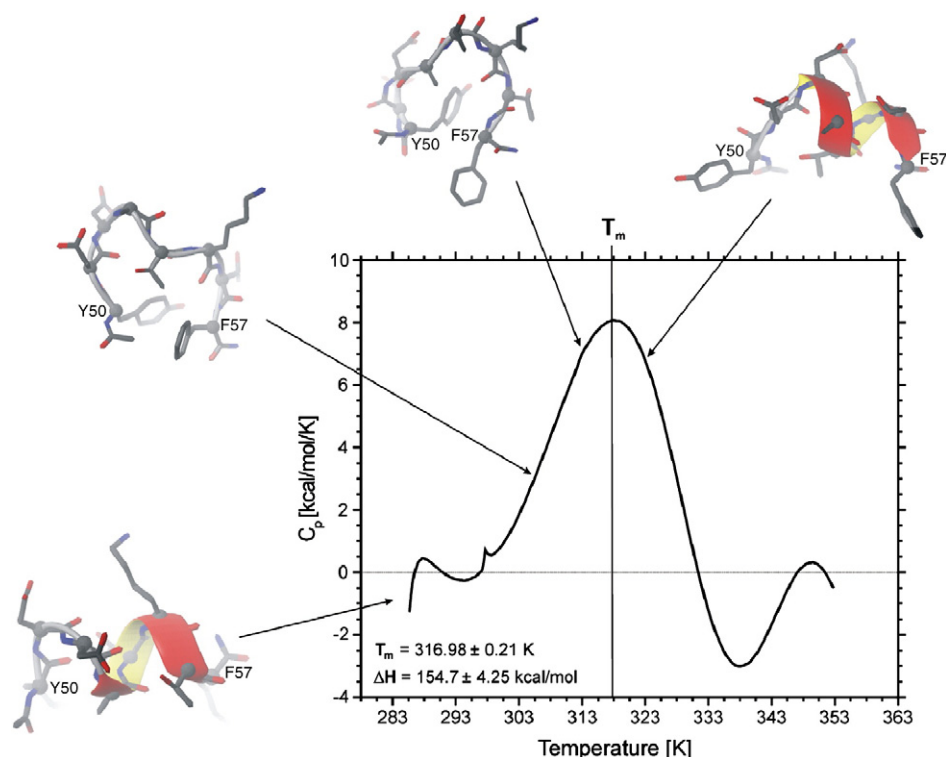
#### 4.5. Solvent

Most of the structural studies related to the  $\beta$ -hairpin-forming peptides have been performed in water/buffer solutions but there are some studies performed in water/organic solvent mixtures. For example, addition of trifluoroethanol (TFE) to water/buffer solutions increases the stability of the structure of  $\beta$ -hairpin-forming peptides substantially, but only in some sequences [36,52]. Santiveri et al. concluded that the increase of structure stabilization by addition of TFE to a solution depends strongly on the sequence under investigation [36,52]. Some structural studies of  $\beta$ -hairpin-forming peptides were performed in water/organic solvent mixtures because the measurements were conducted at temperatures lower than 273 K, which prevents use of water or buffer solution. In such studies, however, the influence of solvent on structure is not discussed or the authors assumed that solvent mixtures are a simple substitute for a water environment at temperatures below the water freezing point [35].

#### 4.6. Temperature

As mentioned in Section 3.1, many studies report spectral properties of  $\beta$ -hairpin-forming peptides at various temperatures [10,11,13,35,39]. However, only one series of papers reports structures of variants of G-peptides with various lengths [23–27] at several (3 to 4) temperatures. Such temperature-relation studies are very important for understanding the mechanism of folding of peptides/proteins. In Fig. 4, representative conformations of an 8-residue-long variant of the G-peptide, calculated with the use of restraints derived from NMR measurements performed at  $T = 283, 305, 313$ , and  $323$  K, are shown [25]. It can be seen that the peptide undergoes complex conformational changes with temperature. At low temperature ( $T = 283$  K), the peptide exhibits some  $\alpha$ -helical rather than  $\beta$ -hairpin-like structure. With temperature increase ( $T = 305$  and  $313$  K), the peptide forms a  $\beta$ -hairpin-like structure which is stabilized by strong hydrophobic interaction between the pair of nonpolar residues (marked on Fig. 4 as Y50 and F57). With further temperature increase ( $T = 323$  K), the peptide becomes  $\alpha$ -helical-like once again. In Fig. 4, the heat-capacity curve obtained from a DSC experiment is also shown.

The results of the DSC experiment show that the transition temperature for the investigated peptide is  $T_m = 316$  K. It appears that hydrophobic interactions between nonpolar side chains (tyrosine and phenylalanine) are responsible for the stabilization of the  $\beta$ -hairpin-like structure. Breaking the hydrophobic interaction between the nonpolar



**Fig. 4.** The heat-capacity curve for an 8-residue-long variant of the G-peptide recorded in water at pH=6.57 with structures calculated by using NMR restraints determined at  $T=283, 305, 313$ , and  $323$  K [25].

side chains at temperatures between  $T=313$  K and  $T=323$  K leads to a rapid conformational change that coincides with the position of the heat-capacity peak at  $T=316$  K. As in earlier studies, mentioned in Section 3.2, it was shown that there is a connection between the long-range interactions between nonpolar residues and the folding temperature determined by the DSC measurements [12,38]. On the other hand, the broad shape of the heat-capacity curve in Fig. 4 suggests a multistate character of the folding transition, consistent with the differences between the folding-transition temperature determined by DSC and those determined by CD, NMR or IR spectroscopy (Section 3). The region of the maximum of the heat-capacity curve (located at  $T=316$  K; Fig. 4) clearly shows breaking the interactions between side chains at temperatures between  $T=313$  K and  $T=323$ . We can, therefore, conclude that hydrophobic interactions are responsible for structure formation and stabilization and that the breaking/formation of such interactions is responsible for the folding transition [23–27]. It should also be noted that, at all temperatures ( $T=283, 305, 313$ , and  $323$  K), the turn sequence always forms a turn structure but, at some temperatures ( $T=283$  and  $323$  K), the observed turn is part of the helix-like structure and at other temperatures ( $T=305$  and  $T=313$  K) is part of a  $\beta$ -hairpin-like structure. The results shown in Fig. 4 demonstrate that the following two factors are essential for formation/stabilization of structure in the  $\beta$ -hairpin-forming peptides: hydrophobic interactions between nonpolar side chains and turn-formation propensities of some part of the sequence. The temperature dependence of the strength of hydrophobic interactions is also very important (the strength of such interactions increasing with increasing temperatures) but turn-formation properties for some parts of the sequence seem to be independent of temperature changes [25].

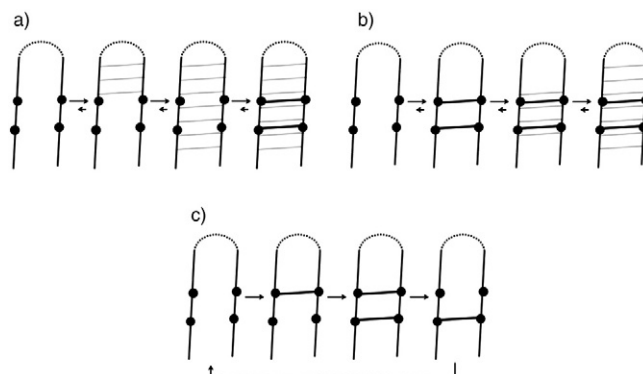
## 5. Mechanism of $\beta$ -hairpin formation

The following three mechanisms: zipper, hydrophobic-collapse, and broken-zipper mechanisms have been proposed and discussed in

the literature for the G-hairpin. These three mechanisms are depicted in Fig. 5 and summarized in the next three subsections.

### 5.1. The zipper mechanism

Kinetic analysis of the folding of variants of the G-peptide performed by Muñoz et al. [15,53,54] revealed a single exponential relaxation process with time constant  $\tau=3.7 \pm 0.3$   $\mu$ s. The data indicated a single kinetic barrier separating folded and unfolded states, consistent with a two-state model for folding. Subsequently, Muñoz et al. [15,53] developed a statistical mechanical model of the folding mechanism similar to that of Matheson and Scheraga [55] that describes the stability in terms of a minimal numbers of parameters: loss of conformational entropy, backbone stabilization by hydrogen bonding and formation of a



**Fig. 5.** Mechanisms of structure formation of the G-hairpin peptides: a) zipper mechanism [20,54,55]; b) hydrophobic-collapse mechanism [56]; c) broken-zipper mechanism [27]. Black circles represents nonpolar residues, thick horizontal bars represents hydrophobic interactions between nonpolar residues, thin horizontal lines represents hydrogen bonds, dotted line represents fragment of the sequence which has turn propensity, and arrows represents direction of folding/unfolding process.

stabilizing hydrophobic cluster. The proposed zipper mechanism of folding is shown in Fig. 5a, and it consists of the following steps. In the first step, the turn region forms a bent structure, such bend facilitating the formation of hydrogen bonds starting from the turn region and progressing to ends of the peptide (as in a zipper). After the hydrogen bond network is established, the interaction between hydrophobic residues start to form in the second step and, as they develop, they stabilize the final structure [15,53,54]. This mechanism appears reasonable to describe the folding of the G-peptide. However, the main concern is related to the role of hydrogen bonds (omitted by Matheson and Scheraga [55]) in the folding process. As was mentioned in Section 4.3, hydrogen bonds are not observed in NMR structural studies of  $\beta$ -hairpin-forming peptides (except some possible hydrogen bonds in the turn region) [10,12,21,23–27,30]. The absence of hydrogen bonds in the structure of isolated G-peptides is an argument against the zipper mechanism proposed by Muñoz [53,54]. It should be noted that a similar zipper mechanism proposed by Mattheson and Scheraga [55] is based on hydrophobic interactions.

### 5.2. The hydrophobic-collapse mechanism

Theoretical studies performed by Dinner et al. [56] suggest that the formation of  $\beta$ -hairpin structure may start with hydrophobic collapse (formation of as many as possible interactions between nonpolar residues) followed by rearrangement of un-ordered contacts between nonpolar residues to form a more organized hydrophobic cluster. After the hydrophobic core is formed, hydrogen bonds start to propagate outward from the hydrophobic cluster in both directions (see Fig. 5b) [56]. Such a model of folding does not appear to require a turn-based nucleation event. The hydrophobic-collapse mechanism also conforms to the results of the study by Fernandez and Scheraga [57], who showed that the formation of stable hydrogen bonds in secondary structure elements should be preceded by the creation of the interactions between nonpolar groups surrounding the peptide groups involved. When the number of nonpolar groups wrapping the peptide groups involved in a hydrogen bond is sufficient, the peptide groups become dehydrated and the hydrogen bond(s) between them do not compete with those to the water molecules. The hydrogen bonds between the peptide groups, therefore, become more stable, thereby, stabilizing, in turn, the entire structure [57]. The hydrophobic-collapse mechanism is an alternative to the zipper mechanism described in Section 5.1 because it emphasizes the important role of nonpolar side chains in the structure-formation process. However, this mechanism still assumes that a) the formation of hydrogen bonds is an important step of the folding process, even though hydrogen bonds are rare in the structure of the G-peptides (see Section 4.4) and, b) it ignores the importance of the turn-region structure in folding the  $\beta$ -hairpin structure, and does not agree with many studies that emphasize the role of the turn region in the initiation of the folding (see Section 4.1).

### 5.3. The broken-zipper mechanism

Recently, Lewandowska (Skwierawska) et al. [27] proposed another mechanism for folding the G-hairpin, which is referred to as the broken-zipper mechanism. This mechanism is depicted in Fig. 5c and it consists of the following steps. This mechanism recognizes the importance of local, hydrophobic, and hydrogen-bonding interactions. In the first step, the turn sequence facilitates the formation of contacts between the nonpolar residues located close to the turn sequence. The formation of these hydrophobic contacts enables the nonpolar residues farther from the turn sequence to join in and, as a result of this, the hydrophobic core grows (see Fig. 5c) [55]. As opposed to the zipper and hydrophobic-collapse mechanisms (Fig. 5a and b), it is proposed that unfolding of the

structure is not just the reverse of the folding process. Lewandowska (Skwierawska) et al. [27] observed that, during the heating of the system, the hydrophobic interaction between the residues close to the turn region (which are formed first during folding) break first, while those between the residues farther from the turn region are more resistant to the increase of temperature (see Fig. 5c) [23–27]. The broken-zipper mechanism recognizes both the importance of the turn-region sequence and the importance of the hydrophobic interactions between nonpolar side chains for the folding process as well as for structure stabilization. The broken-zipper mechanism is, to some extent, similar to the zipper mechanism proposed by Muñoz et al. [53,54] except that the main “zipping” force is the formation of hydrophobic contacts rather than backbone hydrogen bonds. It shares the recognition of the role of hydrophobic interaction with the hydrophobic-nucleation mechanism of protein folding proposed much earlier by Matheson and Scheraga [55].

## 6. Conclusions

Based on the results obtained by using different experimental techniques (CD, NMR, IR spectroscopy and DSC), the local interactions [18,19,21,43,44] and hydrophobic interactions between nonpolar residues [3,12,23–27,38,55] around the turn sequence seem to initiate the formation of  $\beta$ -hairpins. It should be noted that a mechanism for the formation of a folding nucleus by nonpolar residues close in amino-acid sequence has been proposed over 30 years ago by Matheson and Scheraga [55] (however, local interactions were ignored in this mechanism). Structural studies of the G-peptide and its analogs clearly indicate that the propensity of the turn sequence to initiate hydrophobic interactions (see Section 4.2) is responsible for structure formation and stabilization [18,19,21,43,44]. Both types of interactions enable a polypeptide chain to overcome the unfavorable conformational entropy and form a stable U-shaped folding nucleus. The contacts between nonpolar residues are near-native but not exactly native and are very dynamic in this initial stage, changing greatly with the change of temperature [23–27]; however, the U-shape of the hairpin is preserved. The turn is very loose and fluctuating. There is no experimental evidence that the formation of hydrogen bonds between backbone peptide groups plays any significant role in the initial stage of folding [10,12,22–27,55], because the hydrophobic core that a hairpin forms is insufficient to isolate the peptide groups from the solvent [57]. The lack of stable hydrogen bonds in the three-dimensional structure of  $\beta$ -hairpin-forming peptides is also in agreement with previous findings that the hydrogen bonds are not the dominant force in protein folding and are not essential in the early steps of protein folding [55,58–60]. Hydrogen-bonding interactions seem to come into play later when a cluster of nonpolar residues, sufficiently large to dehydrate backbone peptide groups, is formed. Once this happens, the backbone hydrogen bonds form and fix the structure, in particular the turn position and the native hydrophobic contacts. We term this mechanism the broken-zipper mechanism [27] (Section 5.3 and Fig. 5c).

Not only does the broken-zipper mechanism take into account all interactions important in folding initiation and is fully consistent with the experimental data on  $\beta$ -hairpin-forming peptides [27] but it also explains the uniqueness and robustness of the folding process. Let us suppose that, as in the hydrophobic-collapse mechanism (Fig. 5b) [56], only the hydrophobic interactions and not the hydrophobic interactions plus turn sequence determined folding initiation. Then multiple nucleation sites, most of them containing contacts far from native, could form in various places of the sequence where nonpolar residues are present, close enough in sequence to overcome the entropic barrier to contact formation. As Matheson and Scheraga have shown, the difference between the free energy of contact formation in various alternative sites does not appear large, even though, for most of the proteins they studied, the

approximate free energy of contact formation was the lowest for the native nucleation sites [55]. Thus, without an additional discriminative factor, the multiplicity of nucleation sites would frustrate the chain in zillions of possible conformations. The formation of protein structure by the hydrophobic-collapse mechanism can be compared to trying to assemble Lego blocks into a toy by putting together all the blocks whose shapes fit each other. There are, however, multiple such combinations, only few of them resulting in assembling a toy.

As opposed to that of hydrophobic collapse, the zipper mechanism of Muñoz et al. (Fig. 5c) [15,53,54] provides uniqueness of nucleation (even though, contrary to experimental evidence, it assumes that backbone hydrogen bonds are present in an isolated  $\beta$ -hairpin and, thereby, stabilize the structure, along with hydrophobic bonds). However, it assumes that the hydrogen bonds and the hydrophobic contacts in the  $\beta$ -hairpin, although formed gradually, are locked once they have formed. This would work to form a structure, if the interactions were as unique as modeled by Gō-like potentials, which is not the case. Because of non-uniqueness of interactions, locking interresidue contacts (both hydrogen-bond and hydrophobic) at a position close to native but yet non-native would require overcoming a significant free energy barrier to break it up and form at a correct place, once the context of the  $\beta$ -hairpin appears and interactions with other parts of the chains come into play to form greater segments of protein structure. Therefore, the zipper mechanism does not account for the robustness of folding. Structure formation according to this mechanism can be compared to assembling the Lego blocks by trying to put together and lock, e.g., the pieces of plane fuselage separately and of those of the wings separately, without knowing what the exact shape should be. It might happen that the pre-assembled wings will fit the fuselage but, more likely, the Lego blocks will have to be disassembled and assembled again into the correct (although similar to the former one) shape once the shape of the fuselage is known.

In the broken-zipper mechanism, the turn and contacts are not very strong (only hydrophobic contacts are present). They, therefore, are not locked, which provide robustness, but are in about correct places, which provide sufficient uniqueness. The contacts become locked in specific locations (by the formation of hydrogen bonds between dehydrated backbone peptide groups) only after the context of the  $\beta$ -hairpin appears. This mechanism can be compared to putting together the Lego blocks corresponding to different parts of the toy, without firmly locking them until it knows that the part of the toy will fit the other ones.

The precedence of the locking of the final structure (backbone conformation) by forming hydrophobic contacts and a crude turn shape is supported by different temperature dependence of various conformation-dependent observables and also by broad heat-capacity peaks of these peptides (Figs. 3 and 4), which suggest the multistate character of the unfolding transitions. As shown in Section 3, different experimental methods provide different folding temperatures for  $\beta$ -hairpin-forming peptides. The folding temperature determined for the same peptide under the same conditions could vary from 284 to 320 K depending on which conformation-dependent observable is being monitored (Figs. 1–3). As was shown in Section 3, the folding temperature determined by the DSC method appears to be associated with the formation of long-range interactions between nonpolar amino-acid side chains [12,23–27,38]. CD spectroscopy can also detect some long-range interactions between nonpolar amino-acid side chains and, if such CD measurements and DSC measurements are performed, the folding-transition temperatures determined from both measurements are in agreement [12,38]. As shown in Figs. 1–3, the melting temperatures determined from the DSC measurements are the highest (320 K, see Fig. 3) and those temperatures determined from CD measurements or from chemical-shift analysis are lower by ten

degrees or more. This clearly explains what is observed in the DSC measurement (breaking long-range side chain-side chain interactions); on the other hand, typical CD measurements can provide information about the average conformational state of peptide bonds (or other chromophores) in the sample, and NMR experiments can provide information about the conformational state and/or the environment surrounding a particular NMR probe (typically a proton). It can be assumed that the CD spectra and, to some extent, chemical shifts, can provide information about local conformational states of amino acids in an investigated peptide. With this assumption, we can conclude that the melting temperatures determined from CD spectroscopy [23] (or IR spectroscopy [28,29]) and, in some cases, the chemical shifts of backbone protons [11,38] show changes of the average conformational state of the backbone (secondary structure) in the investigated peptide. An analysis of the folding temperatures determined by CD spectroscopy and DSC (see Figs. 1 and 3) leads to the conclusion that rapid changes of average conformational states of the peptide backbone (disruption of secondary structure) occur at lower temperatures than the disruption of long-range hydrophobic interactions [23]. The authors of some other studies of variants of the G-peptides claim that the disruption of long-range hydrophobic interactions occur at lower temperatures than the rapid change of the average conformational state of the peptide backbone [29]. Even if the results of different studies do not agree as to whether the secondary structure melts or long-range hydrophobic interactions become disrupted first, it can be concluded that both processes (melting the secondary structure or disrupting long-range hydrophobic interactions) do not occur at the same temperature.

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