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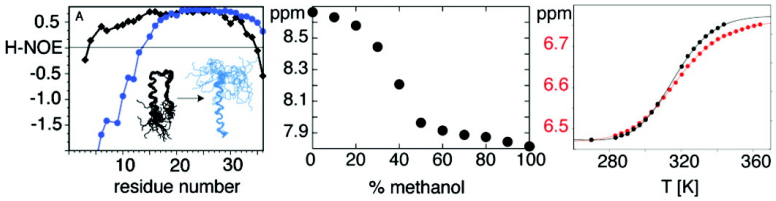

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Probing the Formation of Stable Tertiary Structure in a Model Miniprotein at Atomic Resolution: Determinants of Stability of a Helical Hairpin

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Abstract: The minimal model system to study the basic principles of protein folding is the hairpin. The formation of β-hairpins, which are the basic components of antiparallel β-sheets, has been studied extensively in the past decade, but much less is known about helical hairpins. Here, we probe hairpin formation between a polyproline type-II helix and an α-helix as present in the natural miniprotein peptide YY (PYY). Both turn sequence and interactions of aromatic side chains from the C-terminal α-helix with the pockets formed by N-terminal Pro residues are shown by site-directed mutagenesis and solution NMR spectroscopy in different solvent systems to be important determinants of backbone dynamics and hairpin stability, suggesting a close analogy with some β-hairpin structures. It is shown that multiple relatively weak contacts between the helices are necessary for the formation of the helical hairpin studied here, whereas the type-I β-turn acts like a hinge, which through certain single amino acid substitutions is destabilized such that hairpin formation is completely abolished. Denaturation and renaturation of tertiary structure by temperature or cosolvents were probed by measuring changes of chemical shifts. Folding of PYY is both reversible and cooperative as inferred from the sigmoidal denaturation curves displayed by residues at the interface of the helical hairpin. Such miniproteins thus feature an important hallmark of globular proteins and should provide a convenient system to study basic aspects of helical hairpin folding that are complementary to those derived from studies of β-hairpins.

Introduction

Despite extensive efforts in studying folding of peptides and proteins, a much better understanding of molecular determinants for particular folds and their formation is still highly desirable. Although polypeptide chains that fold into specific secondary structures are comparatively easy to design, the construction of proteins with defined tertiary structure, good side-chain packing, and cooperative folding behavior is still a major challenge. The reason for this difficulty is that tertiary contacts are often stabilized by a manifold of weaker interactions clearly underlining the necessity for a deeper understanding of how such contacts are made and stabilized.

The smallest natural domains that fold autonomously into protein-like structures have 32−40 residues,[1,2] although stable β-hairpin structures with a much smaller number of residues have successfully been isolated from a larger protein[3] or even designed de novo.[4] The availability of small and well-folded β-hairpin structures is presently advancing our understanding of factors that govern protein folding at ever-increasing pace (see, e.g., Hughes and Waters[5] for a review). Particular interest has been paid to β-hairpins that display cooperative folding behavior, since this is a hallmark of natural proteins.[6] It has been found that the stability of β-hairpins is determined by contributions from turn,[7,8] intrinsic sheet propensities,[9] and hydrophobic side-chain interactions across the strands.[4] In fact, the relative contributions of intrinsic conformational bias and interstrand side-chain−side-chain effects are suggested to be of comparable magnitude.[9]

Although the formation of individual α-helices and β-hairpins has been studied in great detail, relatively little is known about the factors contributing to helical hairpin formation. In the absence of additional covalent constraints (such as disulfide

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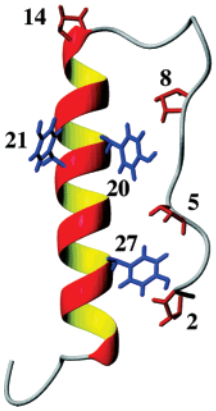


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          1          11          21          31
pPYY YPAKP EAPGE DASPE ELSRY YASLR HYLNL VTRQR Y NH2
pNPY YPSKP DNPGE DAPAE DLARY YSALR HYINL ITRQR Y NH2
bPP  APLEP EYPGD NATPE QMAQY AAELR RYINM LTRPR Y NH2
```

Figure 1. Structure of porcine PYY with side chains of Pro and Tyr residues depicted in red or blue, respectively. The sequences of porcine PYY and NPY and bovine PP are displayed on the right. Residues that are conserved among different species are underlined, and residues, which are part of the present mutational analysis, are depicted in red.

bonds[10,11]), helical hairpins have been reported to be marginally stable[12] or to oligomerize into larger assemblies such as helical bundles.[13] One of the few sequences shown to fold into a monomeric and stable hairpin in solution is α t α.[14] Although the hydrophobic interface appears to contribute significantly to the stability of the two helices in α t α, thermal unfolding was surprisingly uncooperative. This is indicative of a lack of specific tertiary interactions (by either hydrogen-bonding or interdigitating side-chains) and suggests that α t α represents a molten globule rather than a protein-like structure.

Here we present a detailed investigation on structural determinants of cooperative helical hairpin formation in 36 residue peptides, which are derived from the neuropeptide Y family of peptide hormones. Some of these peptides adopt a well-defined hairpin structure in water, which was first observed by Blundell et al.[15] for avian pancreatic peptide (aPP) using X-ray crystallography. This surprisingly stable helical hairpin is commonly referred to as PP-fold and is characterized by a C-terminal α-helix, which is back-folded via a type-I β-turn onto an N-terminal polyproline type-II helix. Tertiary contacts between the α-helix and the polyproline helix are shown in Figure 1.

The solution structure of the peptide YY (PYY) was shown to possess a highly similar helical hairpin[16,17] whereas tertiary structure is surprisingly absent in the highly homologous neuropeptide Y (NPY).[18] Despite its small size the peptide YY is stably folded in aqueous solutions, as evidenced by nonaveraged NMR parameters, a high-resolution NMR structure, and uniformly low internal backbone dynamics, as independently verified from NMR relaxation experiments.[17] To probe the energetic significance of the tertiary contacts we replaced residues at the hydrophobic interface of the hairpin-type structure of PYY by Ala and analyzed by high-resolution NMR spectroscopy the conformational and dynamical properties of the resulting peptides. By varying temperature or the methanol content of the aqueous solvent and monitoring chemical shifts we followed the residue-specific formation of tertiary contacts while changing the physical or chemical environment. The methods used by us efficiently deliver structural and dynamical information on changes in the stabilities and folding behavior of the different mutants at atomic resolution and hence provide a much more precise picture as compared to optical methods such as CD or fluorescence spectroscopy, which are usually limited to monitoring changes on molecular scales only.

The results suggest that helical hairpin formation in PP-fold peptides is both reversible and cooperative and that specific N- and C-terminal tertiary hydrophobic contacts between the polyproline and the α-helix provide the driving force for folding. In addition, structural analysis of substitutions in the turn region indicate that the loop behaves like a hinge, which may (or may not) favor, but does not constrain, the hairpin structure. To our knowledge, the present data provide the first detailed investigation on cooperative tertiary structure formation in a natural, stably folded, but otherwise unconstrained helical hairpin and show that PP-fold miniproteins can serve as a convenient system to study aspects of folding that are complementary to those using β-hairpins.

Methods

All peptides described in this paper have been produced by recombinant methods. They were expressed as insoluble fusions to ketosteroidisomerase, from which they were liberated through cyanobromide cleavage under denaturing conditions. Fusion peptides were expressed in M9 minimal media containing ^{15}N-NH_4Cl as the sole nitrogen source and verified by their MS and [^{15}N,^{1}H]-HSQC spectra. C-terminal amidation was performed by enzymatic conversion of an extra Gly residue into an amide function using the α-amidating peptidyl glycine amidase (PAM).

The structures of pPYY, Tyr7-PYY, and Ala14-PYY in solution were determined following established procedures using 2 mM peptide samples at 28 °C, pH 4.2 in 20 mM deuterated acetate buffer on a Bruker AV-700. ^{15}N relaxation data were recorded at 500 MHz proton frequency on 1 mM uniformly ^{15}N-labeled samples under similar conditions. Procedures for the spectroscopic work were previously described in more detail.[17,19] The structural characterization of the N-terminal polyproline helix proved challenging for two reasons: First,

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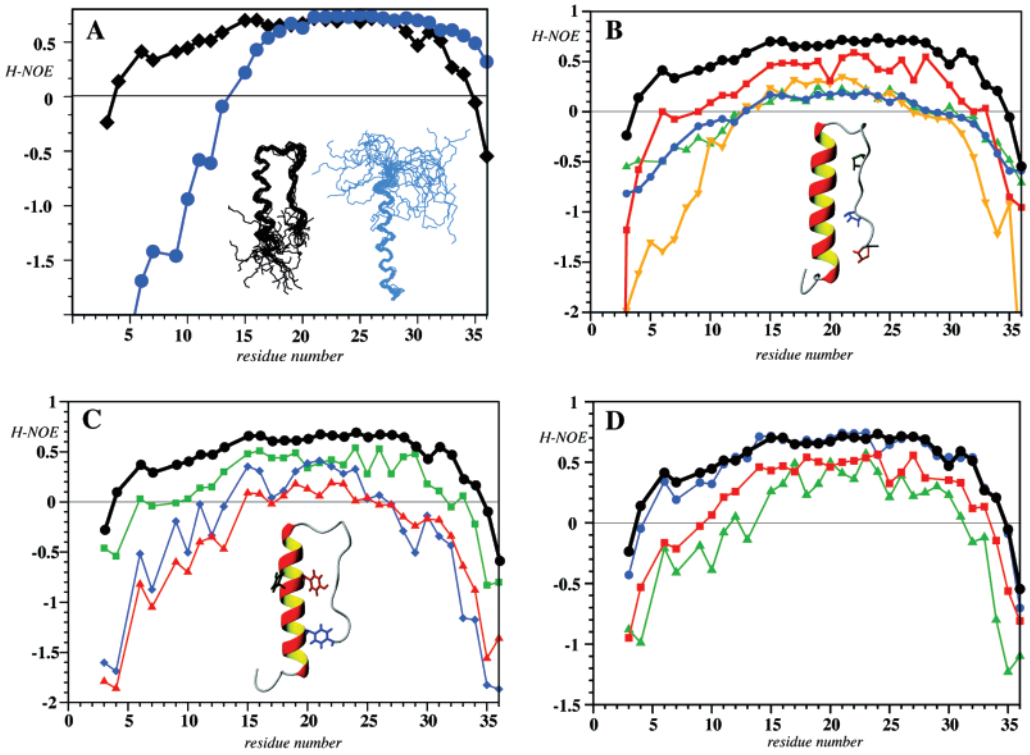

Figure 2. Values of the $^{15}N\{^1H\}$-NOE. All data were determined at 500 MHz in water using 1 mM solutions of the peptides at pH 4.2 in 20 mM acetate buffer. (A) Effect of solvent system: Back-folded PYY in aqueous solution (black diamonds) and PYY when bound to DPC micelles (blue circles), where formation of the helical hairpin is suppressed. The corresponding structures displaying backbone atoms are depicted in the inset. (B) Effect of proline mutations: PYY (black circles), Ala2-PYY (red squares), Ala5-PYY (blue circles), Ala8-PYY (green triangles), and Ala2,5,8,14-PYY (orange triangles). (C) Effect of tyrosine mutations: PYY (black circles), Ala20-PYY (red triangles), Ala21-PYY (green squares), and Ala27-PYY (blue diamonds). (D) Effect of mutations in the turn region: PYY (black circles), Ala14-PYY (blue circles), Pro13,Ala14-PYY (red squares), and Ala13-PYY (green triangles).

contributions to its stability arise both from medium-range contacts within the segment and long-range contacts to the C-terminal α-helix. Second, although a number of long-range proton−proton NOEs between protons of the C-terminal helix and the N-terminal segment clearly define the structure of the N terminus in the back-folded (native) state of PYY, the lack of both medium-range as well as long-range NOEs in the destabilized or denatured states results in great uncertainty under denaturing conditions or in destabilized mutants as to the presence of residual structure of the polyproline helix or transient back-folding to the C-terminal helix. We therefore chose to probe for residual structure by a combination of three different parameters, the $^{15}N\{^1H\}$-NOE, $^3J_{HN\alpha}$ scalar couplings, and residual dipolar couplings (RDCs) derived from samples that were partially aligned in stretched polyacrylamide gels. We have previously established a relationship between structure and backbone dynamics that allowed us to quantify the extent of back-folding as an ensemble-averaged quantity of populations of fully back-folded and fully flexible N termini solely based on $^{15}N\{^1H\}$-NOE values.[17,19] The fact that this model is consistent also with RDCs and $^3J_{HN\alpha}$ scalar couplings provides further evidence of its validity and usefulness for characterizing folding between two structurally well-characterized states by a single progress variable.

Results

Role of Residues at the PP-Fold Interface. In our attempt to understand the contributions to stability of the PP-fold, we first chose to examine contacts at the back-fold interface made between Pro and aromatic Tyr residues. Recently, π−Pro interactions were postulated to contribute substantially to the stability of long-range contacts.[20,21] Thus, we replaced Pro residues 2, 5, and 8 as well as Tyr20, 21, and 27 by Ala to produce all single mutations as well as the quadruple Ala2,5,8,-14-PYY mutant by site-directed mutagenesis. The destabilization of the helical hairpin in these mutants is probed by measuring changes in internal backbone dynamics by means of the heteronuclear NOE between the amide nitrogen and its directly attached proton. Figure 2A displays values of the $^{15}N\{^1H\}$-NOE for PYY in solution and when bound to DPC micelles. In the latter environment formation of tertiary structure is efficiently suppressed, demonstrating its utility for quantifying the stability of tertiary structure in the system under study.[19,22]

The $^{15}N\{^1H\}$-NOE data indicate that none of the Pro mutants are stably back-folded (see Figure 2B). For the quadruple Pro mutant and NPY the $^3J_{HN\alpha}$ scalar couplings are >6 Hz for all residues in the N-terminal segment, which is indicative of conformational averaging. By contrast, in the single Pro mutants the N-terminal segment is not fully flexible (see the Supporting Information). Interestingly, the rigidity of the C-terminal α-helix is highly correlated to the rigidity of the turn and the adjacent amino acids encompassing residues 6−12. Moreover, the lack of long-range contacts destabilizes in particular the last two turns of the helix.

We have additionally investigated the structural role of Tyr20, 21, and 27 again by recording the $^{15}N\{^1H\}$-NOE values of the corresponding single Tyr → Ala mutants (Figure 2C). The data confirm that these tyrosine residues and Pro2, 5, and 8 have complementary roles in the stabilization of both secondary and tertiary structure and support the data by Woll and Gellman on the Tyr20Ala and Tyr27Ala mutants.[23] In all the Tyr → Ala

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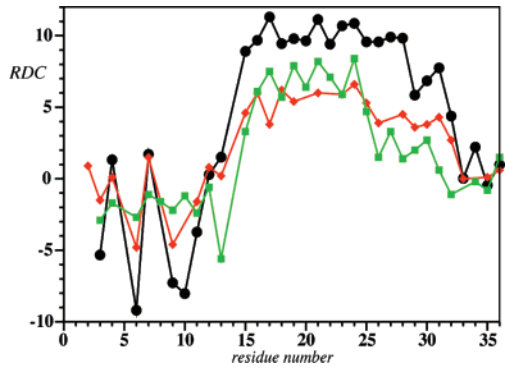

Figure 3. Residual dipolar couplings (RDCs) of PYY (black circles), Ala2-PYY (red diamonds), and Ala8-PYY (green squares) measured in stretched polyacrylamide gels at 700 MHz.

mutants, the back-fold is significantly destabilized. Mutation of Tyr20 or Tyr27, however, is far more destabilizing than mutation of Tyr21, because the π-systems of the former two residues point toward the back-folding interface and form contacts with Pro residues 5 and 8. Moreover, we have noticed in case of Ala27-PYY that the heteronuclear NOE drops in particular in the C-terminal part of the destabilized helix encompassing residues 24−30. In contrast, replacing Tyr20, which forms crucial contacts to both Pro5 and Pro8, results in a more general destabilization of the C-terminal helix. Considering that Tyr27 makes contacts with Pro2, removal of the former may result in fraying of the hairpin at its termini.

As the PYY mutants with a significantly destabilized PP-fold lack medium-range proton−proton NOEs in the N-terminal polyproline helix, we chose to probe for residual structure in the N-terminal segment by recording RDCs in samples that were partly aligned in stretched polyacrylamide gels. The values measured for the weakly and strongly destabilized PYY mutants Ala2-PYY and Ala8-PYY, respectively, are depicted in Figure 3 along with the RDCs from the stably back-folded wild-type PYY.

The comparison reveals that overall the RDCs of N-terminal residues of all three molecules follow a similar oscillatory pattern, although the amplitudes are reduced when the helical hairpin is destabilized (as inferred from the $^{15}N\{^1H\}$-NOE data). The magnitude of RDC values depends on the ensemble-averaged orientation of the NH bond vectors relative to the alignment tensor. Hence, the decreasing amplitudes indicate that the population of the helical hairpin is reduced in favor of species in which the N terminus is unstructured as seen both in Ala2-PYY and even more pronounced so in Ala8-PYY.

The differences in internal backbone dynamics of the N-terminal segment in different Pro → Ala mutants could possibly be related to (i) differences in the propensities of the various mutants to adopt the PP-fold or (ii) in changes in the intrinsic rigidity of this peptide segment due to the presence or absence of Pro residues. To resolve this ambiguity we have conducted measurements of the $^{15}N\{^1H\}$-NOE in DPC micelles, an environment in which tertiary interactions are efficiently blocked. We have previously shown in case of bPP or pPYY by using micelle-integrating spin labels that the amphipathic side of the helix becomes associated with the surface of the micelle, thereby releasing the N terminus.[17,24] In the presence of micelles, the $^{15}N\{^1H\}$-NOE data of all single as well as the quadruple mutant are virtually identical (Supporting Information Figure S3). Moreover, the C-terminal helix of all peptides is stabilized on binding to micelles, whereas the H-NOE values of residues 6−14 indicate continuously increasing flexibility toward the N terminus. A comparison of the H-NOE of the quadruple mutant in water and in DPC micelles displays highly similar values for the N-terminal segment in both environments, providing evidence that the N terminus in this particular mutant behaves more or less like a free-flight chain also in solution. The data therefore suggest that the backbone rigidity of N-terminal residues of a given mutant in solution is mainly associated with its propensity to adopt the back-fold as a result of a number of specific tertiary contacts between aromatic residues of the C-terminal helix with proline residues of the N-terminal polyproline helix rather than structural properties of the N-terminal segment alone.

Importance of Residues in the Hinge Region. The sequences of pNPY, pPYY, and bPP are displayed in Figure 1 along with the structure of pPYY. Pro residues 2, 5, and 8 and Tyr residues 20 and 27, which together form the hydrophobic core of the folded hairpin, are conserved in all three peptides. It is therefore highly unlikely that these residues account for the different propensities of NPY and PYY to adopt the helical hairpin. The sequence homology of NPY and PYY is larger than 80%, and the most prominent difference is Pro found at position 14 in PYY, whereas it occurs at position 13 in NPY. Proline 14 occupies a position in the turn region that links the C-terminal α-helix to the N-terminal polyproline helix. Due to the particular nature of the Pro side-chain we reasoned that Pro14 may direct the N-terminal segment into a favorable enough position for adopting the back-folded state. Accordingly, we have substituted Pro14 by Ala to relax this possible conformational restraint. A comparison of the structure of Ala14-pPYY with pPYY and bPP reveals that Ala14-pPYY very much resembles the structure of PYY or bPP in that the N-terminal segment is clearly back-folded (see Supporting Information Figure S1) and similar interactions between C- and N-terminal residues occur. From these data we deduce that the PP-fold of Ala14-pPYY is sufficiently supported by the hydrophobic contacts made between Pro residues from the N terminus and Tyr residues from the C-terminal α-helix and that Pro14 is not needed to constrain the turn conformationally in order to enforce back-folding.

Although Pro14 apparently does not enforce tertiary contacts in PYY, it may be that the otherwise highly homologous NPY cannot adopt the PP-fold because of a Pro in position 13, which is conserved in all NPY sequences known today. The data for the $^{15}N\{^1H\}$-NOE of Pro13,Ala14-PYY are depicted in Figure 2D and clearly prove that this mutant is not back-folded. A continuous decrease in magnitude of the $^{15}N\{^1H\}$-NOE is observed for residues preceding Ala14. Negative H-NOE values are observed for N-terminal residues, and the C-terminal helix is also significantly destabilized, but the values also indicate that the hairpin in this mutant is still more stable than in the quadruple mutant. By shifting Pro by one position toward the N terminus the naturally occurring Ser13-Pro14 is replaced by Pro13-Ala14. As a control for the effect of substituting Ser13 alone we also investigated hairpin formation by the single mutant Ala13-PYY. To our surprise we found that the PP-fold is even

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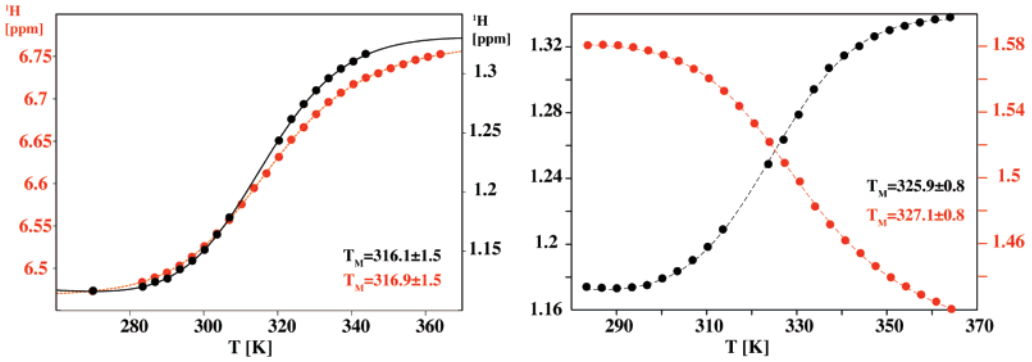

Figure 4. Proton chemical shifts of the methyl group of Ala7 (black circles) and the Hδ of the aromatic system of Tyr20 (red circles) vs temperature for PYY (left) and of methyl groups of Ala12 (black circles) and Leu17 (red circles) of Tyr7-PYY (right). The melting temperatures derived from fits to experimental data are denoted as T_M in the figure.

less stable in Ala13-PYY than in Pro13,Ala14-PYY (see Figure 2D). An energy minimization of the turn region using an implicit solvent model (see the Supporting Information) indicates the possible presence of hydrogen bonds of the hydroxyl group of Ser13 with the amide proton of Glu15 and to the side-chain carboxyl group of the latter residue. We conclude that the presence of a hydrogen bond donor at position 13 is crucial for correct formation of the β-turn and that the shift of Pro from position 14 in PYY to position 13 in NPY or the replacement of Ser13 by Ala is sufficient to disrupt the tertiary contact. It is important to note that, although the amino acid composition of the turn region determines to what extent the turn may favor the formation of tertiary structure, it is nevertheless not sufficient to constrain the helical hairpin on its own. This is evidenced both by the fact that removal of a single tertiary contact in the hydrophobic cluster can fully disrupt the back-fold and by the backbone motional dynamics of the turn region, which is significantly more rigid when the hydrophobic cluster between N- and C-termini is stabilized (see above). On the other hand it is also clear that the turn rigidity weakens gradually under increasingly more destabilizing conditions. This fact is best seen in changes of the $^3J_{HN\alpha}$ scalar couplings of residues from the turn region. For example, the coupling for residue Asp11 is always larger than 8 Hz (and often larger than 9 Hz) in the proline mutants in water but always close to 7 Hz in the DPC micelle-bound state, the latter value being indicative of conformational averaging. In addition, the value for this coupling for PYY in methanol is only moderately reduced to 8.7 Hz from the 10 Hz encountered in water. Taken together, these data indicate that the turn acts like a hinge that may support folding but does not provide sufficient stabilizing force to constrain the hairpin on its own.

Studying the Formation of the Helical Hairpin. The stability of the folded state critically depends on the delicate balance between the free energies of the folded and unfolded states. Disrupting the helical hairpin in PYY will expose hydrophobic surfaces to the solvent. Accordingly, we have studied removal of tertiary structure in PYY both by thermal and by solvent-induced denaturation.

In the thermal denaturation experiments, proton spectra of 1 mM solutions of PYY and Tyr7-PYY were measured in the temperature range from 280 to 370 K. The corresponding curves are characterized by sigmoidal shapes (see Figure 4), and the melting points of residues from different regions of the polypeptide chain are within 1° for PYY and 1.2° for Tyr7-PYY, suggesting a high degree of cooperativity. The data additionally reveal that the helical hairpin is slightly more stable in Tyr7-PYY than in PYY, as evidenced by an increase of the melting temperature from 316.5 K in PYY to 326.5 K in Tyr7-PYY.

The increased PP-fold stability seen in Tyr7-PYY as compared to wild-type PYY must result from additional intra- or intermolecular interactions. It should be noted at this point that the related avian polypeptide (aPP) exists in dimeric form in the crystal structure[15] (K_d 0.32 μM at pH 5.0[25]). Therein, the interface between the monomeric subunits is formed by a hydrophobic cluster, in which the π-systems of Tyr7, Tyr21, and Phe20 stack onto one another. By measuring chemical shift changes in a dilution series the K_d for homodimerization of PYY at pH 4.1 was determined to be 35 $\pm$ 18 mM, indicating that the monomer concentration is between 91% and 95% at 1 mM concentration. The chemical shift of the amide proton of Ala12 is very sensitive to the extent of hairpin formation, and the observed minor change of 0.06 ppm between 1 mM and 10 μM concentrations indicates that the dimer contributes only to a small extent to the stability of the helical hairpin. We therefore feel justified to treat the system as a monomer.

Apart from thermal denaturation, the PP-fold can also be destabilized by cosolvents with increased lipophilicity. It is known that coaddition of alcohols stabilizes helical secondary structures and disrupts weak hydrophobic contacts, which in turn unfolds tertiary structures. The order of effectiveness for destabilizing tertiary structure is trifluoroethanol > propanol > ethanol > methanol.[26,27] The less dramatic changes observed with methanol indicate that this solvent may be particularly useful to monitor the back-folding transition.[28] Mixtures of water and methanol with methanol contents of about 30% have often been found to result in molten-globule-type structures that possess a considerable extent of native secondary structure, while tertiary structure is largely destroyed.[28−33]

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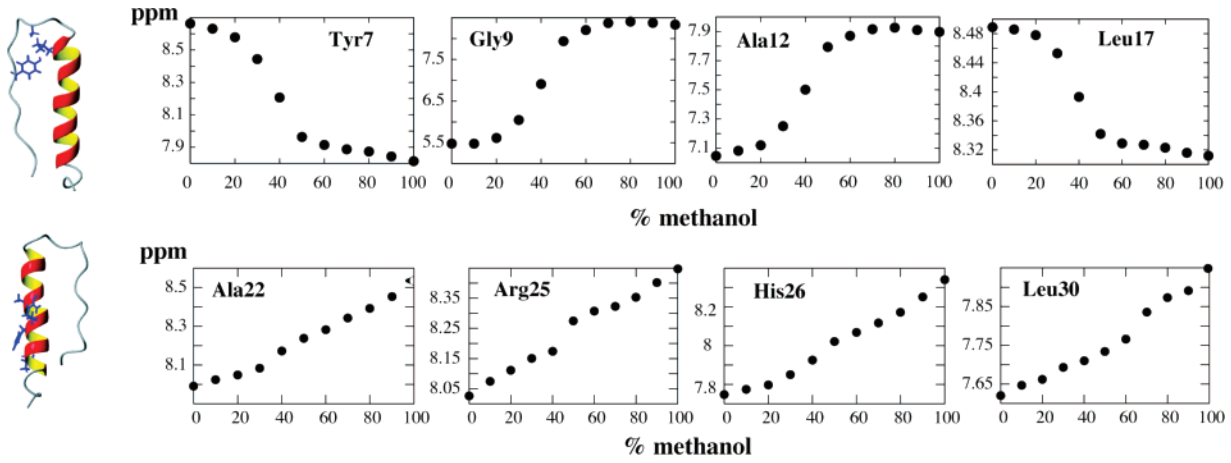

Figure 5. Proton chemical shifts of selected amide protons of Tyr7-PYY vs the methanol content for residues of the back-fold interface (top) and for those pointing away from it (bottom). On the left, side chains of these residues have been drawn in the structures to indicate their location in the molecule.

To monitor the back-folding transition we have measured the amide proton chemical shifts as a function of the methanol content. The data for selected residues of Tyr7-PYY, for which the transition between non-back-folded and back-folded species occurs at a larger methanol-to-water ratio than for PYY (and hence is better visible), are displayed in Figure 5.

The data clearly reveal that curves from residues of the back-fold interface display a characteristic sigmoidal shape. The point of inflection corresponds to a 41.8% ± 2.1% methanol/water mixture. In contrast, for residues of unstructured segments and for all residues that are pointing away from the back-fold interface the changes are limited to differences in solvent coordination and/or stability of backbone hydrogen bonds, and hence the curves appear approximately linear. The data clearly favor a model in which the PP-fold builds up *cooperatively*, because sigmoidal curves with similar inflection points are observed for the amide proton chemical shift changes for all the residues of the PP-fold interface. Similar sigmoidal curves are also seen for the values of the $^3J_{HN\alpha}$ coupling constants, which are directly dependent on the torsion angle ϕ (see the Supporting Information). The associated free energy of formation of the helical hairpin in aqueous solution, computed following the procedure introduced by Santoro and Bolen,[34] is −17.4 ± 1.3 kJ/mol, which is in agreement with the values typically observed for the free energies of folding of globular proteins ranging from −20 to −60 kJ/mol[6] and similar to the value of −12.6 kJ/mol determined for bPP.[23]

Discussion

In this study we have investigated molecular properties that lead to the cooperative formation of a helical hairpin in a natural miniprotein at atomic resolution. In particular, we have addressed the importance of the individual Pro and Tyr residues for forming tertiary structure in various mutants of PYY. Moreover, we characterized the significance of residues at specific positions in the turn region. The observation that each Pro → Ala replacement in the segment 2−8 reduces the stability of the back-fold significantly indicates that the simultaneous presence of all these contacts is required to form the hairpin. Measurements of backbone dynamics in the presence of DPC micelles, in which the formation of tertiary contacts between the helix and the N-terminal segment is efficiently prevented, revealed that differences in backbone dynamics between the various Pro → Ala mutants in the micelle-bound state are largely removed. Hence, the increased rigidity is not primarily an intrinsic property of Pro-rich peptide segments but rather due to correct positioning of residues capable of stabilizing the back-folded conformation.

In a polyproline type-II helix positions i and $i + 3$ Pro residues point in the same direction. Moreover, the aromatic residues Tyr20 and Tyr27 are located at i, $i + 7$ positions of a helix and therefore again point in the same direction. Pro residues and aromatic side-chains possess rather flat surfaces, enabling them to mutually intercalate in a zipper-type fashion resulting in a relatively large interaction surface. The importance of nonlocal Pro−aromatic interactions has been highlighted previously in different contexts, including interstrand stabilization of antiparallel β-sheets,[21] in the structure of a miniprotein called Trp cage,[35,36] and in the binding of proline-rich peptides to aromatic residues in SH3 domains.[37] It has indeed been speculated that Pro engages with an aromatic residue to form a C−H−π interaction that may provide substantial binding energy.[20,21] It is fully consistent with these observations, that the exchange of individual Tyr residues in the C-terminal α-helix by Ala destabilizes the tertiary structure considerably and that the effects of replacing Tyr20 or Tyr27, which are forming direct contacts with Pro residues from the N-terminal segment, are much larger than for Tyr21.

Our study additionally revealed that the turn region encompassing residues 10−14 is very sensitive to amino acid replacements. Whereas mutating Pro14 in PYY to Ala did not change the rigidity of the helical hairpin significantly, shifting Pro from position 14 to 13 abolished hairpin formation completely. Therefore, it can be safely excluded that Pro14 forces the backbone to adopt a PP-type fold. In contrast, a shift of a single Pro residue by one position in sequence seems to be sufficient to convert peptides that adopt the PP-fold into

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structures that more closely resemble the conformation of NPY. NPY is clearly not back-folded, although the Pro residues at positions 2, 5, and 8 and Tyr at positions 20 and 27, which together form the hydrophobic core of the hairpin in PYY, are conserved. This points toward a crucial role for the residues of the β-turn region. Interestingly, replacing Ser13 by Ala results in a similar and even stronger destabilization of the helical hairpin. Pro residues are conserved at positions 13 and 14 in NPY and PYY, respectively, and Ser is highly conserved at position 13 in PYY. Computational studies indicate that hydrogen bonds departing from the hydroxyl group of Ser may help to stabilize the β-turn. These observations indicate that residues 13 and 14 serve as a hinge region, and that conformational preferences in that segment are critical to enable stable buildup of the helical hairpin. Although the lifetimes of particular conformational states may be too short to allow characterization through meaningful proton−proton NOEs, our study suggests that backbone dynamics data can be employed for this task. The data reveal that despite significant destabilization of the helical hairpin in some mutants, transient contacts between the N-terminal segment and the helix are formed much more often than would be expected for a freely diffusing peptide chain. We therefore believe that these mutants sample conformational space similar to what would be expected for a molten globular state and that they can be considered as models of transient structures being formed during folding of wild-type PYY.

Our data indicate that formation of secondary structure in PYY occurs to some extent independently from buildup of tertiary structure. N-terminal truncation mutants of NPY possess a helix albeit at somewhat reduced stability.[38] Moreover, NPY possesses no tertiary structure, but is clearly helical, and all the non-back-folded mutants of PYY are still helical. It is therefore reasonable to assume that formation of the C-terminal α-helix in NPY occurs independently and that the tertiary contacts are initiated by hydrophobic contacts made with a preformed helix. Once the C-terminal helix is established, however, the PP-fold is formed cooperatively, because contacts of *all* Pro residues in the segment 2−8 as well as both Tyr20 and Tyr27 are required. The data suggest that the final conformation is stabilized by a manifold of weak interactions, all of which are required to drive folding to the correct state. This view is strongly supported by the thermal denaturation experiments with PYY and Tyr7-PYY and the measurements of the latter in water−methanol mixtures.

In summary, our results on the folding of an unconstrained helical hairpin suggest that both a turn-promoting sequence and specific nonlocal Pro−Tyr interactions add to the overall stability of the native fold. While a disfavorable sequence in the β-turn region suffices to destabilize the PP-fold significantly, a favorable sequence alone does not enforce it. Overall, the system should therefore prove useful to investigate the individual roles of the turn and the hydrophobic cluster in the folding dynamics of helical hairpins, for example, using ϕ-value analysis.[6] Such a study has recently been conducted for a 16-residue β-hairpin from which it was suggested that turn formation is the rate-limiting step during folding, whereas the hydrophobic cluster between the strands slows unfolding. In remarkable analogy to the helical hairpin studied by us it was proposed that main chain and side-chain residues of the turn region of this β-hairpin form a characteristic hydrogen-bond network, which might play a critical role in stabilizing the folding transition state.[39] While we have drawn a comprehensive picture of the molecular interactions underlying the thermodynamics of helical hairpin formation in PYY, studies of the folding kinetics of PYY and the mutants presented here are now needed to shed more light on the mechanistic details of helical hairpin formation.

Conclusions

By NMR spectroscopy and denaturation experiments on a series of mutants we could demonstrate that tertiary structure formation in PYY is strictly dependent on the presence of a number of specific contacts. We could also demonstrate that formation of the PP-fold from preformed secondary structures occurs cooperatively. Most of the Pro → Ala or the Tyr → Ala mutants possess increased backbone dynamics, and the differences in N-terminal mobility among them reflect various degrees to which they sample conformations close to the PP-fold. Molten globules are generally considered being structurally related to the native structure, with side-chain conformations less well defined, but many native-like tertiary contacts at least being transiently formed. In that respect many of the mutants studied in this work may actually be considered to be similar to the molten globular states of PYY.

The results may also have implications for our understanding of the binding of these peptides to their cognate membrane-embedded receptors. We have recently postulated that receptor binding is preceded by binding of the ligands to the membrane.[22] Changes in solvent properties accompany this change in environment. The solvent mixture experiments may therefore simulate the structural transition occurring when a peptide diffuses from the bulk solution toward the membrane. We have noticed that peptide structures are very similar when bound to either micelles or in methanol,[22] and hence the transition from the membrane-bound state to water may be mimicked by the buildup of tertiary structure in PYY when changing from methanol to water solvent.

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Supporting Information Available: Additional information on the structures of the described peptides, CD data, and details on the calculation of the turn region as well as chemical shift lists and relaxation data. This material is available free of charge via the Internet at http://pubs.acs.org.

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