NMR Evidence of a Short Linear Peptide That Folds into a β-Hairpin in Aqueous Solution†

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For understanding the folding of native[1] and designed[2] proteins, it is very important to know whether short segments of an unconstrained peptide chain can autonomously fold into secondary structures other than α-helices.[2a,3] Although short linear[4] peptides can form β-turns in aqueous solution,[5,6] no experimental evidence is available[3a,9] supporting the idea that they are able to form more complex structures such as intramolecular β-hairpins.[7]

A fragment of tendamistat **1**, spanning a native β-hairpin region of the protein,[10] has been shown to form transient β-turn-like structures in the same region where the native β-turn exists, although no β-hairpin structure was detected.[6d] With the aim

1, Y-Q-S-W-R-Y-S-Q-A tendamistat 15-23

2, Y-Q-N-P-D-G-S-Q-A model peptide

3, G-H-N-P-D-G-H-G-NH_2 control peptide

of stabilizing this particular β-hairpin in order to see the effects on stability of residues flanking the β-turn, we have investigated the conformational properties of two designed peptides. In peptide **2** the four central residues were replaced by others which maximize turn probability, while in peptide **3** flanking residues different from the native ones were additionally introduced.[11] We report here strong NOE evidence in favor of the idea that peptide **2** indeed forms a significant population of isolated β-hairpins in aqueous solution, while peptide **3** does not.

NMR was essential for characterizing in detail the conformational preferences of the designed peptides in solution.[14] Although several NMR parameters may be used,[17] it is generally recognized that the pattern of diagnostic NOEs, in particular long-range NOEs, is the most conclusive evidence of structure. Our model peptide **2** showed many diagnostic NOE crosspeaks, including nonsequential ones ranging from ($i,i+2$) to ($i,i+8$) (Figures 1 and 2a). The αN ($i,i+2$) NOEs connecting residues 3–5, 4–6 (particularly intense for a nonsequential NOE), and 5–7 and an intense NN ($i,i+1$) NOE crosspeak between residues 5 and 6, together with the very small shift temperature coefficient (–2.0 ppb/K) of the amide proton of residue 5, strongly support[3a,16] the presence of a chain bend in the vicinity of residues 3–6. More striking is the presence of medium- and long-range NOEs connecting residues far apart in the sequence (as far away as $i,i+8$) that can not be explained unless, in addition to unfolded states, some of the peptide chains are in β-hairpin-like conformations. The intensities of the αN ($i,i+1$) sequential NOEs, strong for residues 1–3 and 7–9, became smaller for residues located at the central region of the peptide chain, also in agreement with the presence of β-hairpin conformations. Many linear peptides have been conformationally examined using NOEs,[1-6] but to our knowledge this is the first time that a set of cross-sheet long-range NOEs has been detected in the NOESY spectrum of a short linear peptide under nonaggregating conditions.[18] The control peptide **3** shows a similar, although less intense (Figure 2b and supplementary material), pattern of αN ($i,i+2$) NOEs, indicating that a set of β-turn structures are being formed around their central residues. Only one ($i,i+3$) NOE is visible, no long-range cross-sheet NOEs were detected, and solvent protection of the amide proton of residue 5 has been lost (temperature coefficient was –6.8 ppb/K), thus suggesting that not only the β-sheet but also the β-turn has been destabilized with respect to peptide **2**.

Although NOEs cannot be rigorously interpreted in terms of a unique structure because of the existence of conformational averaging,[20] it is not uncommon[21] to look for a model structure

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† This paper is dedicated to the memory of Prof. José Herranz.

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(7) Intramolecular sheet formation in aqueous solution has been reported but only in large protein fragments.[3a,8] Nonaggregating short β-sheets have been stabilized in dimethyl sulfoxide using rigid unnatural (diacylamino)epindolidinones as templates.[9]

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(11) The NPDG sequence at the β-turn of peptide **2** was selected because this combination had maximum individual probability at each position of the turn according to protein statistics.[12] Any sequence at the flanking residues different from the native one is valid, in principle, as a control. We selected His at both ends (aromatic residues seem to stabilize the turn[13]) that may also favor possible electrostatic interaction with the Asp, capped with two conformationally neutral Gly residues. The amidated C-terminus and the His side chains provide very convenient NMR signals, allowing us to detect possible long-range NOEs across the sheet.

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(14) The peptides were synthesized by Neosystem Laboratoire, Strasbourg, France, and found to be pure (>98%) by reverse-phase HPLC. TOCSY, ROESY, and NOESY NMR spectra, recorded in a Bruker AMX-600 by standard techniques,[15] were used to sequentially[16] assign the NMR spectrum.

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(18) Aggregation in peptides gives rise to marked concentration-dependent line widths.[19] The NMR signals of the model peptide were narrow under the conditions used (15 mM, 5 °C), and dilution by a factor of 500 (from 15 mM to 0.03 mM) did not significantly change the chemical shifts or line widths (see supplementary material), which was in agreement with the absence of aggregate species. The minor set of resonances corresponding to the *cis*-Pro species (~7% intensity), clearly identified in the TOCSY spectrum (supplementary material), was also assigned to avoid erroneous interpretation of their NOE crosspeaks as weak NOEs arising from the major *trans*-Pro peptide. Any accidental overlapping of signals from major and minor species was resolved by running spectra at several temperatures and pHs.

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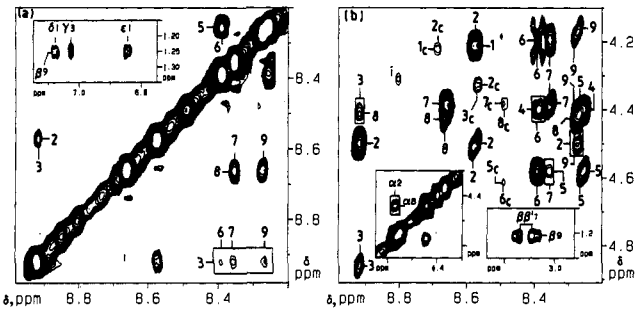

Figure 1. Regions of 600-MHz NOESY spectrum of the YQNPDGSQA model peptide. Conditions: 15 mM peptide in H_2O/D_2O 9/1 by volume, pH 4.3, 5 °C, 250 ms mixing time; insets in (b) shown at pH 5.3, 0 °C for clarity. The long-range NOE crosspeaks are shown boxed. These NOEs also appear in ROESY experiments (supplementary material), which rules out their possible spin-diffusion origin.

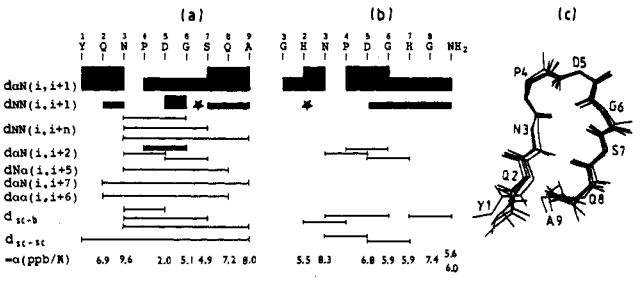

Figure 2. Summary of NOEs observed in the NOESY spectra of peptides **2** (a) and **3** (b) (sc, side chain; b, backbone; *, too close to diagonal peak). The relative intensities shown were evaluated from volume integrals of the crosspeaks. Amide shift temperature coefficients (α) are also shown. (c) View of a model structure of peptide **2** compatible with the pattern of NOEs observed. It was computed by distance geometry calculations using the NOEs shown in a and restrained energy minimization[22] (all backbone atoms shown except the H_α ones).

compatible with the experimental NOEs using distance geometry and energy minimization.[22] The tentative model structure of peptide **2** shown in Figure 2c qualitatively accounts for the pattern of NOEs observed, in particular to the structurally important αN ($i,i+2$) and NN ($i,i+1$) NOEs involving residues 4–6 and 5–6, respectively, plus the interstrand NOEs. Obviously, that particular conformation should not be considered as the only structure adopted by the peptide. In fact, the proposed model predicts intense interstrand NOEs connecting protons of residues located at the ends of the hairpin, while only weak intensities were experimentally detected, thus indicating a reduced β-sheet population in that region of the chain, i.e., multiple conformers exist. Most probably for the same reason, the hydrogen-bonded amide protons from residues located at the β-sheet (expected to be solvent protected) were found accessible as judged from their large amide temperature coefficients; also, sequential NN ($i,i+1$) NOEs were detected in addition to the αN ($i,i+1$) ones typical of sheets.

The location of the β-turn in the proposed model, with Pro located in the $i+2$ position of the turn, is in agreement with the high statistical preference shown by Pro for that position. However, the amide proton of Asp 5 was found to be protected from the solvent instead of that of Gly 6 as would be expected for the model proposed, where a typical $i+3 \rightarrow i$ hydrogen bond exists. It should be mentioned that solvent protection in β-turns cannot be explained solely on the basis of hydrogen-bonded turn conformations.[13,21d] In addition, hydrogen bonds are extremely sensitive to minor structural changes. The presence of $i+2 \rightarrow i$ hydrogen bonds, which can be formed also in β-turns,[5a,30] is a possible explanation for the protection of the amide proton of Asp 5.[25]

Peptide **1**, having the native sequence of tendamistat, did not show detectable β-hairpin formation,[6d] presumably due to the very low turn probability of the central residue. Control peptide **3**, despite having a strong preference for β-turn formation, was also unable to drive the formation of the β-hairpins.[26] Only peptide **2**, which combines high turn probability and native flanking residues, was able to form the β-hairpin. It seems that the identity of both types of residues, i.e., central and flanking ones, have a marked influence on the stability of the whole hairpin. These results are in agreement with the fact that statistical preferences have been found in proteins for the amino acids flanking other types of secondary structures (i.e., α-helices[28] and parallel β-sheets[29]). Our design strategy to stabilize β-hairpin conformations may appear simplistic, but it has yielded encouraging results and merits further experimentation. It seems feasible that isolated β-hairpin units, having enough experimentally checked stability, may be used as building blocks in protein design.

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Supplementary Material Available: Two tables and four figures showing NMR chemical shifts, the ROESY spectra of peptides **2** and **3**, a TOCSY spectrum, and the concentration independence of the 1-D NMR spectrum of peptide **2** (6 pages). Ordering information is given on any current masthead page.

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(22) Distance geometry calculations were performed with the DIANA[23] program. All the NOEs of Figure 2a were used with the observed intensity and found to be consistent with an unique conformation. To better define the folded conformer, only the nonsequential NOE intensities were modified from weak ($d < 5$ Å) to medium ($d < 4$ Å) or from medium to strong ($d < 3$ Å), and 19 structures were obtained with a root mean square deviation of 1.4 Å for the backbone atoms. Restrained energy minimization was then applied to all the structures using the GROMOS package.[24] The five structures of lowest energy are shown in Figure 2c, the sum of violations of the 37 experimental NOE restrictions being small in all of them (~1 Å).

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