Temperature-Dependent NMR and CD Spectra of β-Peptides: On the Thermal Stability of β-Peptide Helices – Is the Folding Process of β-Peptides Non-cooperative?[1])

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Temperature-dependent NMR and CD spectra of methanol solutions of a β-hexapeptide and of a β-heptapeptide at temperatures between 298 and 393 K are reported. They establish the fact that the 3_{14}-helical secondary structures of the two β-peptides, **1** and **2**, do not 'melt' in the temperature range investigated. This is in sharp contrast to the behavior of the helices of α-peptides and proteins which undergo cooperative unfolding ('denaturing') upon heating. A non-cooperative mechanism is proposed, with a stepwise, rather than an 'unzipping' opening of H-bonded rings (*cf. Fig. 6*). The experimental results are regarded as evidence that, of the three effects which have been identified as contributing to the stability of β-peptide helices, *i.e.*, H-bonding, hydrophobic interactions, and ethane staggering, the latter one is predominant.

1. Introduction. – Since the discovery of the α-helix by *Pauling* and *Corey* almost half a century ago [2], scientists have sought to imitate and to improve the structural features of proteins, the molecules of life. In the last few years, great steps have been taken in the field of the so-called protein design, where structural motifs such as α-helices and β-sheets are formed by synthetic peptides consisting of α-amino acids. However, major shortcomings of proteins for pharmaceutical applications such as unsufficient stability and resistance to proteases are not solved by this approach.

We have recently developed a different approach to well-defined secondary structures. Short-chain β-peptides, built entirely from β-amino acids, containing only six or seven residues, form stable secondary structures in solution (review: [3]). In the beginning, we focused our interest in peptides containing only β-amino acids derived from the proteinogenic α-amino acids such as Val, Ala, and Leu [4]. It was shown by solution NMR techniques that β^3- as well as β^2-peptides form 3_{14} helices in methanol [4][5]. In contrast to this approach, *Gellman* and coworkers used constitutionally enforced and conformationally fixed cyclic β-amino acids such as aminocyclohexane- and aminocyclopentanecarboxyclic acid as building blocks. A peptide containing the aminocyclohexanecarboxylic acid also folds in a 3_{14} helix, whereas another helical structure, a 2.5_{12} helix, has been found for the cyclopentane analog [6][7]. Furthermore, an unusual helical structure which was called a 12/10/12 helix was found when a 'mixed'

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[1]) Partially presented in a conference poster, see [1].

[2]) Part of the projected Ph. D. theses of *K.G.* and *R.P.*, ETH-Zürich.

β-peptide containing both β^2- and β^3-amino acids was analyzed by 2D-NMR spectroscopy in solution [5][8].

More recently, our attention has shifted towards peptides with more polar side chains [9][10], and a β-peptide containing protected derivatives of Ser, Glu, and Asp was shown to also exhibit a helical structure in solution [9]. This helix proved to be weaker compared to that of the β-HVal, β-HAla, β-HLeu peptides, resembling a transition structure between a folded and an unfolded state. Cyclic derivatives of β-peptides have been found to be arranged in a stacked tubular manner (*nanotubes*) with sheet-like H-bonds in the solid state [11], and there has been an example of ion conductivity induced by cyclic β^3-peptides [12]. The other two common structural motifs found in proteins, namely sheets and turns, have also been found in β-peptides, both in solution and in the solid state[3]) [4][5][8][14]. β-Peptides are not degraded by a large variety of proteases, and the β-amino acids are negative in *Ames*' tests [4][16][17]. These findings led to headlines such as '*β-peptides: Nature improved*?' [18] and '*novel secondary structures take shape*' [19].

We wondered what the *thermodynamic stability* of the 3_{14} helix would be in solution. As a model system we chose β-heptapeptide **1**, since it has been extensively studied both by 2D-NMR spectroscopy [20] and by molecular-dynamics (MD) simulations [21]. This seven-residue peptide folds in a 3_{14}-helical secondary structure in methanol solution, as depicted in *Fig. 1*. The half-life values for the exchange in CD_3OD for the NH protons are quite large, ranging from 5 to 262 min (*Table 1*) [20]. From MD simulations at elevated temperatures, a 'melting' point of 340 K was estimated (folded and unfolded microstates equally populated) [21][22]. In addition, β-heptapeptide **1** was the first example where reversible peptide folding was observed in MD simulations [22][23]. This prompted us to perform temperature-dependent NMR and CD measurements to explore the thermodynamic stability of 3_{14} helices and to experimentally deduce informations regarding the thermal unfolding. A model for this process is proposed, based on the experimental evidence.

2. Results. – 2.1. *NMR Spectroscopy of the β-Heptapeptide* **1**. The NH region in a ^{1}H-NMR spectrum of a peptide is a highly sensitive probe for changes in the secondary structure [24]; in particular, three parameters are thought to reflect this. First, the observed large J values, due to the antiperiplanar arrangement of NH to C(β)–H in a helical β-peptide structure, should decrease upon loss of secondary structure [25][4]). A random coil structure of β-peptides is expected to show J values which are smaller by several Hz. Second, the dispersion of the chemical shifts, caused by the helical environment, is a qualitative evidence for secondary structure [24]. Third and last, the temperature coefficients, *i.e.*, the change in chemical shifts *vs.* temperature, indicate intramolecular H-bonded NH protons [26][27]. However, this is discussed controversely in the literature, since it is difficult to draw the line between H-bonded and not H-bonded atoms in terms of absolute temperature coefficients. Some authors state that temperature coefficients may only be used together with exchange data (indicating solvent-shielded protons) [27].

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[3]) Parallel [3][4][13] and antiparallel sheets [14][15].

[4]) β-Peptide **1** had J values ranging from 8.6 to 9.4 Hz at room temperature.

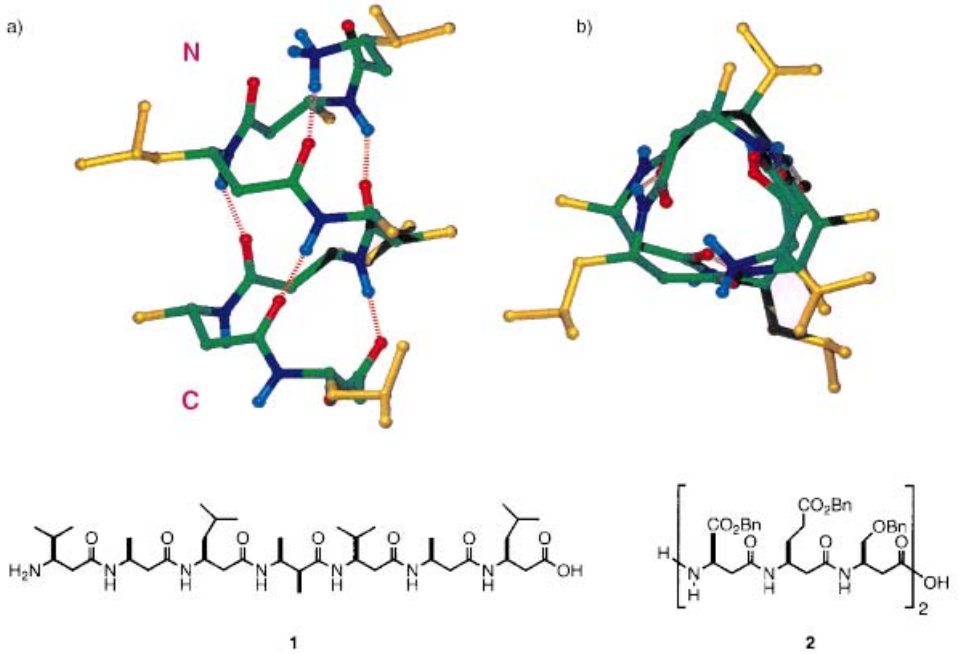

Fig. 1. *Model of a* 3_{14}*-helical structure of the β-peptide* **1** *and molecular formulae of the β-peptides* **1** *and* **2** *studied*. Backbone C-atoms colored in green, side-chain C-atoms in gold, H-atoms in cyan. N and C indicate the N and C terminus. *a*) Side view of the 3_{14} helix; this helix has 3 residues per turn and its H-bonds close 14-membered H-bonded rings. *b*) Top view of the 3_{14}-helical structure. All side chains are in close juxtaposition.

Table 1. *Half-life Values* [min] *for the H/D Exchange in* CD_3OD *of the β-Peptide* **1** [20]

NH(2)	NH(3)	NH(4)	NH(5)	NH(6)	NH(7)
5	188	230	262	37	40

We have measured NMR spectra of β-peptide **1** in CD_3OH at room temperature and in intervals of 10 K up to 393 K. The NH region of these spectra is shown in *Fig. 2*. The experiments have been carried out in a sealed NMR tube, and the solvent signal has been suppressed by presaturation. The *J* coupling constants could be easily extracted from the 1D spectra and are displayed in *Table 2*. The *J* values decrease only slowly upon increase of the temperature, and the values at 353 K indicate that the 3_{14}-helical structure is still present to a large extent. The decrease of 0.7 Hz for the residues 2 and 7 is larger than that for the central residues, where a decrease of *ca.* 0.4 Hz is observed. This may reflect the expected higher mobility of the terminal residues that would be fraying out. The second parameter examined was the dispersion of the chemical shifts. The 1D spectra (*Fig. 2*) clearly show that the dispersion remains large at all temperatures up to 393 K. The chemical-shift difference between NH of β^3-HLeu^3 and NH of β^3-HVal^5 is 0.89 ppm at 298 K and 0.84 ppm at 393 K. Again, such a large difference would not be expected for a more randomized structure. As can be seen in

Fig. 2, two resonances disappear, the first is NH of β^3-HVal[1] between 333 and 343 K and the second is NH of β^3-HAla[2] between 373 and 383 K. This may be due to exchange with the presatured CD_3OH solvent molecules, and, hence, it may be concluded that these two residues are more mobile and become more exposed to the solvent at higher temperatures.

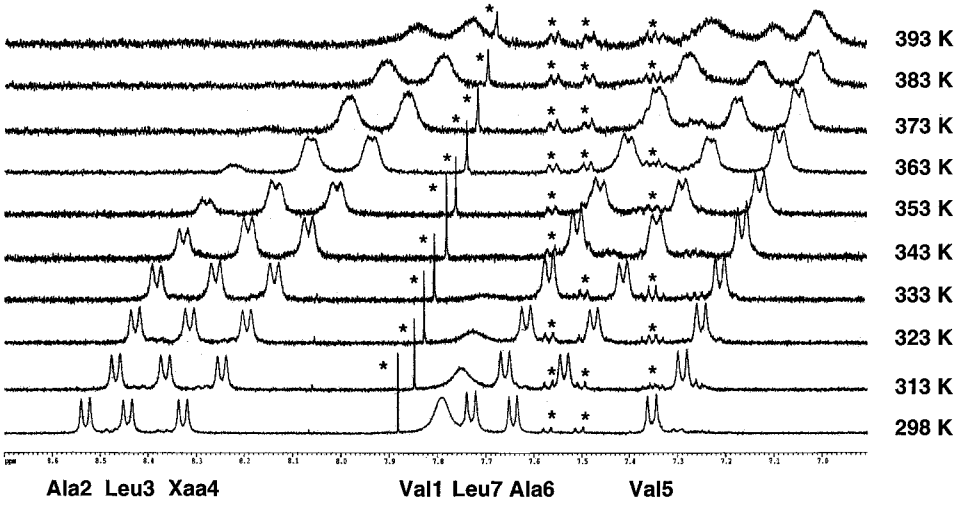

Fig. 2. *NH Region of the temperature-dependent ^{1}H-NMR spectra of the β-peptide* **1** *in CD_3OH* (500 MHz) *up to 393 K*. Signals from impurities are marked by asterisks. These spectra provide evidence that at least a partial 3_{14}-helical secondary structure is populated at all temperatures displayed. Four parameters are in full agreement with this: The *J* values remain large, the dispersion in chemical shifts is maintained, the temperature coefficients are small and linear over the whole range, and the NH signals are not disappearing by exchange with the 'presatured' solvent molecules. Only the NH resonances of β^3-HAla[2] and of β^3-HVal[1] disappear due to exchange with solvent molecules. There is no breakup of the helical structure observed.

Table 2. ^{3}J *Values* (in Hz) *for the Coupling between NH and C(β)–H Protons in β-Peptide* **1**

T/K	β-HAla[2]	β-HLeu[3]	β-HXaa[4]	β-HLeu[7]	β-HAla[6]	β-HVal[5]
298	9.1	9.4	9.0	9.4	8.6	9.4
313	9.0	9.4	8.9	9.4	8.6	9.4
323	9.0	9.2	9.0	9.4	8.4	9.5
333	9.0	8.9	8.7	8.7	8.6	9.4
343	8.9	8.9	8.6	9.1	8.2	9.1
353	8.4	9.0	8.5	8.7	7.8	9.0

The temperature coefficients, determined over a large range of 100 K[5]), are negative (*Fig. 3*), being in full agreement with those observed for the NH resonances of α-peptides and proteins. The values of the coefficients range from –2 to –6 ppb/K. It is commonly assumed in the literature that values more positive than –6 ppb/K reflect intramolecular H-bonded NH protons in H_2O [28]. The temperature coefficients

5) In the literature, there are several examples where temperature coefficients are calculated over a much smaller temperature range of 30 to 50 K.

between -4 and -6 ppb/K and the slow exchange in CD_3OD (see *Table 1*) establish that NH protons of residues 3, 4, and 5 are strongly intramolecularly H-bonded. It is not clear from the data, however, whether the NH protons of residues 6 and 7 are intramolecularly H-bonded; the temperature coefficients and the exchange data are contradictory. However, it is clear from both parameters that the NH of residue 2 is only weakly intramolecularly H-bonded. The other important region in the 1D-NMR spectra concerning secondary structure is the $C(\alpha)-H$ region displayed in *Fig. 4*. The two protons at $C(\alpha)$ have a distinct and different position with respect to the helix axis. The first proton denoted H_{ax} is in line with the helix axis, whereas the second proton denoted H_{lat} is perpendicular. This difference in the spacial surrounding generates a large chemical-shift difference as well as unequal *J* coupling constants to the adjacent $C(\beta)-H$. To illustrate this, we have indicated the $\alpha-CH_2$ groups of residues 1, 2, and 5 in green, magenta, and blue in *Fig. 4*, respectively. It is evident from this figure that the difference in chemical shifts for all three residues is maintained up to 353 K and beyond. Also, the *J* coupling constants $C(\alpha)-H_{ax}/C(\beta)-H$ remain large up to 353 K.

2.2. *CD Spectroscopy.* CD Spectroscopy is a valuable tool to analyze chiral structure, and it is complementary to NMR spectroscopy, although it does not provide structural information at atomic resolution. We have assigned a distinct CD pattern to a 3_{14}-helical structure in solution. It is characterized by an intense minimum at 215 nm and a strong maximum at 198 nm. This pattern was confirmed to reflect the 3_{14} helix for five different peptides of which the secondary structure was determined by a detailed NMR analysis [5][9][20][21].

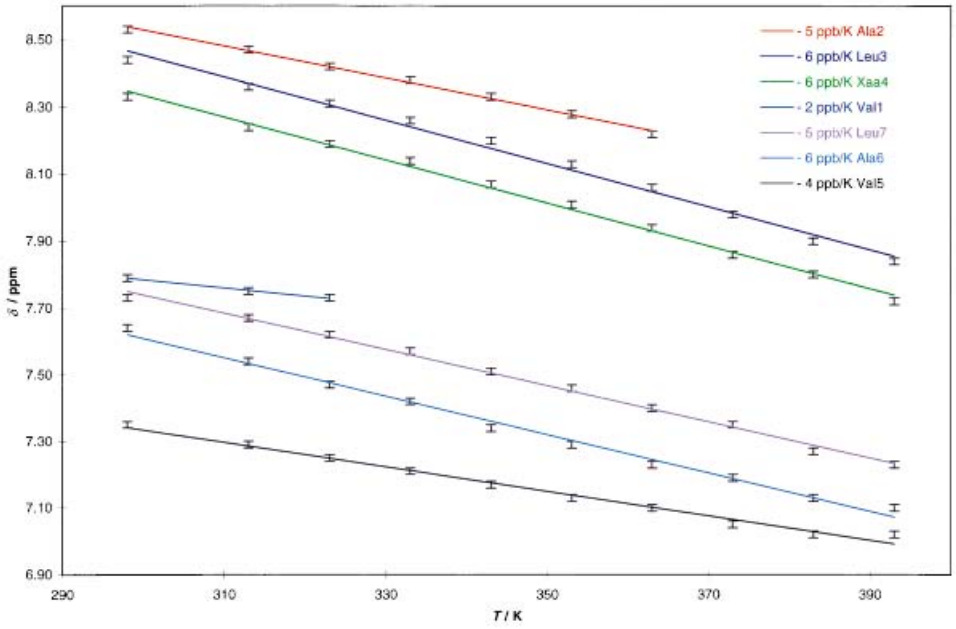

Fig. 3. *Temperature coefficients for the NH-proton chemical shifts of the β-peptide* **1** *calculated over a range of 100 K by linear regression.* The change in chemical shift *vs.* temperature is indicative of the H-bonding environment. Values more positive than -6 ppb/K may indicate intramolecular H-bonded NH protons.

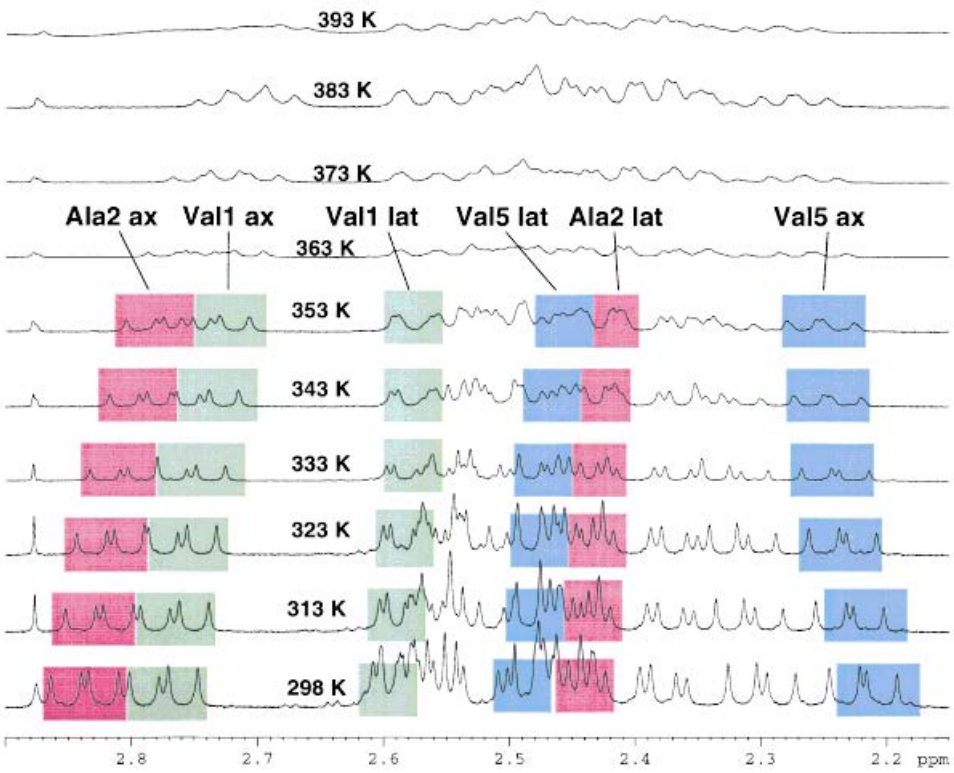

Fig. 4. *Temperature-dependent NMR spectra of the C(α) region of the β-peptide* **1** (CD_3OH, 500 MHz). The two diastereotopic protons at C(α) are separated by several 0.1-ppm units due to the helical environment. Two protons of the same residue are indicated in the same color, C(α)–H bound in line with the helix axis is denoted ax, C(α)–H bound perpendicular to the helix axis is denoted lat. The difference of chemical shifts for the two protons at C(α) in all residues is preserved at even the highest temperatures used, providing evidence for population of the secondary structure.

We have now measured temperature-dependent CD spectra of β-peptides **1** and **2** up to 333 K (*Fig. 5*). β-Peptide **1** exhibits the typical CD spectrum assigned to the 3_{14} helix at room temperature (*Fig. 5,a*); however, upon heating, the intensity of the two extrema in the CD spectrum decreases (by *ca.* 12% per 20 K). The linear decrease (*Fig. 5,b*) in signal intensity is *fully reversible* (we have performed temperature scans from 333 to 293 K backwards (data not shown)). According to NMR measurements, β-peptide **2** has a less defined 3_{14}-helical secondary structure [9]. This agrees perfectly with the CD spectra at room temperature, at 313 and at 333 K (in *Fig. 5,c*). Furthermore, the absolute decrease in intensity (*Fig. 5,d*) is much larger than for β-peptide **1**. Our initial hope to characterize fully unfolded states in the CD spectra was not fullfilled, because the decrease shown in *Fig. 5,d*, remains linear up to 333 K.

However, the correlation between CD spectrum and a random coil structure is not yet etablished for β-peptides, and, hence, it is difficult to compare the absolute decrease in terms of loss of secondary structure. The decrease in the temperature scan is linear

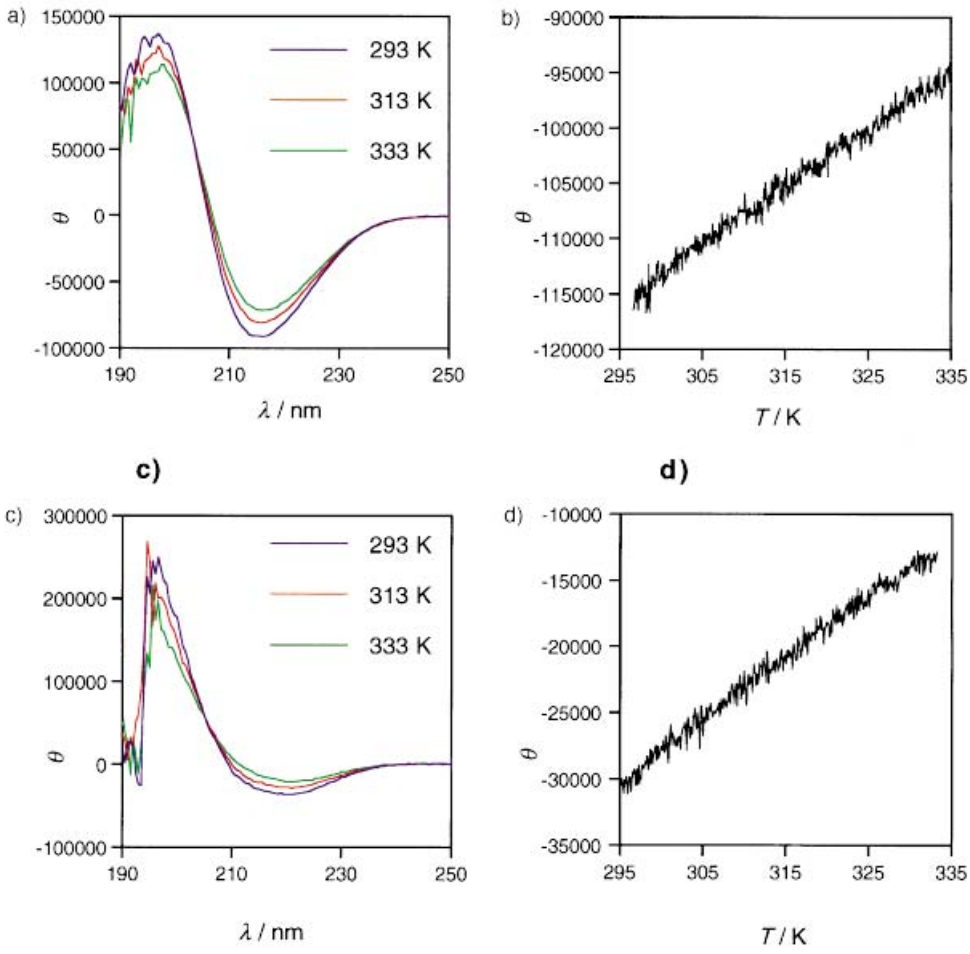

Fig. 5. *Temperature-dependent CD spectra in methanol*. *a*) CD Spectra of the β-peptide **1** at three different temperatures. The typical CD pattern associated with a 3_{14} helix is present at all temperatures, and no breakup of the helical structure is indicated. *b*) CD Temperature scan of the β-peptide **1** in methanol for the *Cotton* effect at 215 nm. The decrease of intensity is linear and, again, no cooperative breakup of the structure is observed. This scan is reversible (data not shown), so the unfolding and folding route of the helix must be reversible. *c*) CD Spectra of the β-peptide **2** at different temperatures. The intensity of the negative *Cotton* effect is much smaller reflecting the smaller stability of the 3_{14} helix for this peptide according to NMR spectroscopy [9]. *d*) Temperature scan for the β-peptide **2**. Note that the decrease in intensity at 215 nm is much larger than in *b*. However, the scan is linear in both cases, reflecting that no breakup of the helical structure is observed.

for both peptides, and the CD pattern assigned to the 3_{14}-helical structure is maintained up to 333 K. It is not possible to correlate the decrease of the CD signal for β-peptide **1** to the experimental data obtained by NMR such as the J values, because slight local changes may have a dramatic impact on NMR data. Furthermore, it is important to note that CD does not indicate any *melting*, *i.e.*, cooperative breakup of the whole helical structure, for the two peptides.

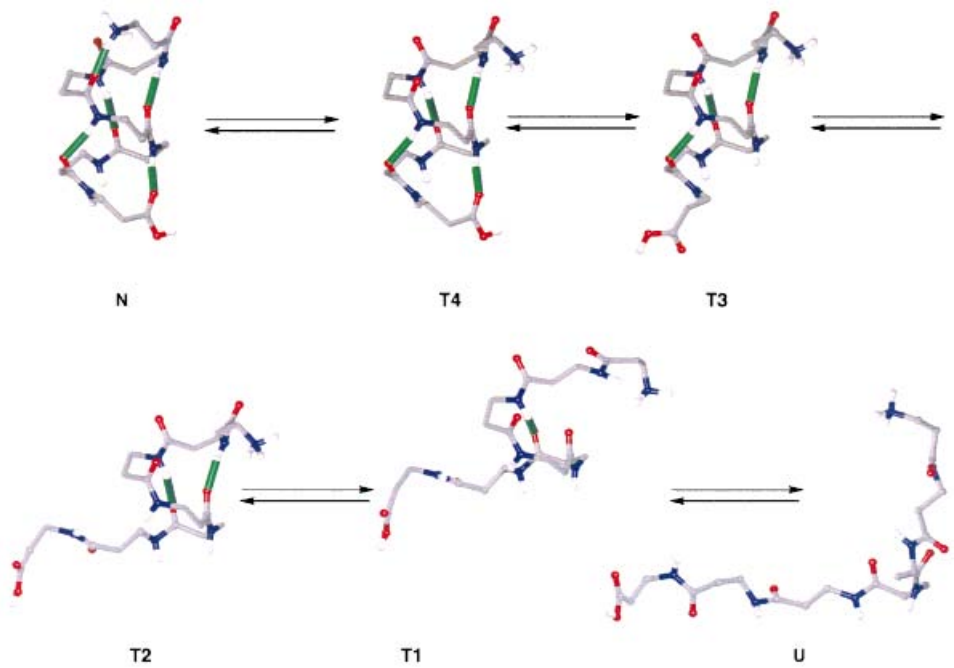

Fig. 6. *A model for the unfolding of β-peptides based on the experimental evidence discussed herein*. **N** and **U** denote the 'native' and unfolded states, whereas **T4**–**T1** represent transitional states or intermediates clustered according to the number of H-bonds. At room temperature, only **N**, **T4**, and **T3** are populated, but upon increase of the temperature, **T2**, **T1**, and **U** also get populated. It is proposed that a preferred backbone conformation of the central C–C bond in β-amino acids is a major contribution to the stability of the helical structure of β-peptides (thus in **T1** only one H-bond and no hydrophobic effects are present, but still three amino acids are arranged in a *synclinal* conformation).

2.3. *A Model for β-Peptide Folding*[6]). The above findings led us to propose a model for the thermal unfolding and folding of β-peptides, and a schematic representation is shown in *Fig. 6*. First, no melting was observed in the NMR and CD experiments. Therefore, the usual two-state model [29][30] is not applicable to the β-peptide folding process. Moreover, our data indicate that many states get populated during unfolding[7]). These states can be clustered into at least six families displayed in *Fig. 6*, according to the number of their H-bonds. Beside the folded 'native' state (**N**) and the fully unfolded state (**U**), there are transitional states or intermediates specified after the number of their H-bonds (T4–T1). According to the NMR measurements [20] and to MD simulations [21], rather mobile termini are observed at room temperature. Therefore, several states, *i.e.*, **N**, **T4**, and **T3**, in *Fig. 6* are populated. Upon increase of the temperature, the experimental results indicate the population of additional states, *i.e.*, **T2**, **T1**, and **U**. For example, the disappearance of NH resonances of β^3-HVal[1] and

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[6]) Folding and unfolding for β-peptides is reversible according to CD spectroscopy (see *Sect. 2.2*) and MD simulations [22][23].

[7]) In addition to the experimental data presented here, MD simulations support this model [21–23].

β^3-HAla[2] is consistent with a shift of the population from the 'native' state **N** to **T4**, **T3**, and **T2**. Furthermore, the decreasing J coupling constants imply that states are populated with the NH–C(β)–H bonds arranged to give small J values.

The temperature corresponding to an equal population of U and N could not be determined, because NMR and CD data of unfolded or random coil structures remain unknown. However, there is no predominant conformation at any of the elevated temperatures studied.

3. Conclusion. – The experimental evidence that the helical structure of a small β-peptide is largely intact at 353 K and above in the polar H-bonding solvent CD_3OH marks just another striking difference between the two worlds of α- and β-peptides. The exceptional thermodynamic stability complements the earlier results (stable secondary structures in solution and the resistance to proteases), and all these findings push the door wide open for possible applications in drug design. The most fundamental question, namely the reason for this remarkable stability of β-peptides, can now be addressed.

Three factors may contribute: *1*) the H-bonding is thought to be essential for structure formation. However, there is no big energetic difference between an intramolecular and an intermolecular H-bond (with CH_3OH).

2) The so-called hydrophobic effect between the bulky aliphatic side chains is a contributing source of helix stability at room temperature [5]. However, it is safe to state that in conformations **T3**, **T2**, and **T1** (populated at higher temperatures), this hydrophobic effect must be very small, and since there is no *cooperativity* observed, such long-range effects (a requirement for cooperativity) cannot be large.

3) A *preferred backbone conformation* around the central C(α)–C(β) bond has been referred to as a helix-inducing and stabilizing contribution [5]. It may be concluded from the experimental evidence discussed here that this effect is the major source of stability at least at elevated temperatures, because even in **T1** three amino acids must be in this *synclinal* arrangement. This conformational effect is not cooperative and thus compatible with the fact that no melting is observed, and that there is a continuous shift of populations to the right in *Fig. 6*. In contrast to H-bonding and to the hydrophobic effect, which are also present in α-peptides and proteins, this staggering effect is unique to β-peptides[8]) (having an additional C–C bond!), and, hence, may account for the exceptional stability of β-peptide helical structures.

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Experimental Part

Synthesis. The synthesis of compounds **1** and **2** is described in [20] and [9].

NMR Spectroscopy. Sample: 8 mg of **1** were dissolved in 0.7 ml of CD_3OH. Spectra were recorded on a *Bruker AMX2-500* (500 MHz) spectrometer in a sealed tube. Measurement at $T = 298.45$ K: solvent signal (CD_3OH) eliminated by presaturation, 88 K data points, 128 scans, spectral width 8064.52 Hz, transformed to 64 K data points. Measurements at $T = 313.35$ K, $T = 323.55$ K, and $T = 333.15$ K: 96 scans, other parameters unchanged from 298.45 K. Measurement at 343.25 K: same parameters as 298.45 K. Measurement at 353.35 K:

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[8]) The striking stability of γ-peptide helices [31][32] may be due to a similar *preferred backbone conformation* effect.

160 scans, other parameters unchanged from 298.45 K. Measurement at 363.25 K: 160 scans, 0.1 Hz line broadening, other parameters unchanged from 298.45 K. Measurements at 372.75, 384.05, and 392.85 K: 0.1-Hz line broadening, other parameters unchanged from 298.45 K.

CD Spectroscopy of **1** *and* **2**. The CD spectra were recorded at 293, 313, and 333 K under N_2 on a *Jasco J-720* spectropolarimeter, equipped with a computer-controlled cooling bath (*Neslab RTE-111*). All spectra were obtained at peptide concentrations of 1.36 mM for **1** and 0.97 mM for **2** in methanol using a 0.2-mm quartz cell. They represent the mean spectra of three recordings from 250 to 190 nm, at a scan rate of 20 nm/min, using a 2-s response and a sensitivity of 10 mdeg. Background spectra were recorded under identical experimental conditions. The difference spectrum was generated by subtracting the background spectrum from the corresponding peptide spectrum.

The temp. scans were performed at peptide concentrations of 0.25 mM for **1** and 0.39 mM for **2** in methanol using a 0.5-mm quartz cell. The denaturation profile from 293 to 333 K was recorded at 215 nm using 0.1-K increments at a heating rate of 20 K/h.

To establish the reversibility of reaction, additional spectra of **1** and **2** after cooling down to 293 K were recorded. The temp. scan of **1** and **2** were tested by measuring the back scans from 333 to 293 K.

All data were expressed in terms of molar ellipticity $[\Theta]$ [degcm^{-2}/dmol].

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