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FT-IR SPECTROSCOPY GUIDED ANALYSIS OF THE CIRCULAR DICHROISM SPECTRA OF POLYPEPTIDES

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In memory of G.Snatzke, virtuoso of chiroptical spectroscopy

Abstract: The curve-fitted Fourier-transform infrared spectrum of linear turn-forming peptides was found to contain an amide I component band near or below 1640 cm^{-1}, similarly to the IR spectra of cyclic β-turn models. This band, termed *β-turn band*, is due to the C=O stretching vibrational mode of the acceptor amide group involved in a strong 1←4 H-bond. H-bondings are chiral constraints because they stabilize helical or folded conformations with only one (right or left) sense of handedness. This makes the combination of CD and FT-IR spectroscopy a unique tool for characterizing the absolute conformation of polypeptides.

The conformational analysis of small peptides plays an important role in the understanding of protein's folding but it also has challenging spectroscopic aspects. The most widely used techniques that provide structural information on the conformation in solution are circular dichroism (CD), Fourier transform infrared (FT-IR) and NMR spectroscopy. The conformation of proteins shares similar structural motifs, called secondary structural elements, such as α-helix, β-sheet, β-turns etc. Theoretical calculations revealed that for each amino acid residue only a limited number of ϕ,ψ backbone torsional angle combinations exists[1], which results in a finite number of secondary structures. Conformation of small peptides in aqueous solution is often summarized as "random"[2], which term reflects a mixture of conformers. The informational content of data from different spectroscopic techniques is in some extent complementary. CD and FT-IR spectroscopy are relative methods, unless isotopic labelling is used in the latter case. On the

other hand, CD and FT-IR have the capacity of time-resolving most conformational changes of the peptide chain, while NMR can detect only the proline cis-trans izomerization with rates of the order of tenth of a second.

The CD spectrum of a polypeptide is primarily determined by the spatial arrangement of the amide chromophores around the chiral centers (C^{α} atoms of the amino acids) therefore CD reflects backbone conformation. The analysis is generally inexpensive and fast. It is based on the comparison and empirical correlation of the measured CD spectra with known spectra of the major secondary structural elements. When a single structure is predominant, CD spectroscopy is generally accepted to be diagnostic of this conformation. However, globular proteins are composed from segments with various secondary structures. Thus, the CD spectra (180 nm $\leq \lambda \leq$ 240 nm) of proteins are generally treated as a linear combination of the corresponding spectra for the various types of secondary structures[3].

The theory and practice of chiroptical spectroscopy of peptides, polypeptides and proteins is reviewed in several excellent articles[2-4]. In these papers the scope and limitations of the different CD curve analyzing methods are discussed in detail. Apparently, neglecting the chiral contribution of the aromatic, disulfide and other chromophores, the major problem is the selection of the reference or basis spectra. The CD spectra of synthetic polypeptides were initially used[6,7] as references. Other approaches make use of the CD of proteins where the secondary structures were previously determined by X-ray diffraction analysis[2,4]. Two proposed methods[4,8-10] also utilize the CD spectra of proteins of known structure, but no attempt is made to derive basis spectra for the specific types of secondary structures. The convex constraint analysis (CCA), an alternative mathematical approach was developed in our laboratories[11].

By the end of the 80's it has become evident that the information, derived from the CD spectra alone, gives only a rough and blurred picture on the steric structure of proteins and peptides. Methods of CD curve analysis are not powerful enough to differentiate subtypes of folded (turn) secondary structures or reflect variations and distortions of the basic conformations. Small and mid-sized peptides are usually present as mixtures of conformers in solution (see equation [1]). Thus, the complete conformational set is required, to calculate the probability occurrence (p_i) of a structure based on it's total energy (E_i).

$$p_i = \frac{e^{-E_i}}{\Sigma\, e^{-E_i}} \qquad [1]$$

Although calculations can yield all the minimum energy related conformations for small peptides in vacuum (environment-free state), the spectroscopic identification of conformers or conformational regions of larger peptides in solution is still problematic. The general approach of the conformational analysis of small and/or mid-sized peptides (<30 residues), is to decrease the number of the possible conformations by special environmental effects (temperature, solvent etc.) or structural constraints (cyclization, H-bonds, ion pairs etc.). Crystals, usually incorporating only one single conformer, can be obtained from proteins, cyclic peptides or protected linear models. It is, however, difficult to grow crystals from unprotected linear mid-sized peptides. The information derived from X-ray analysis is extremely valuable, but the solid state structure is not necessarily identical with the dominant conformation adopted in solution. The CD spectrum, containing time-resolved structural information, is rather complex due to the presence of multiple conformations and/or the dynamic aspects of backbone conformation in solution. Moreover, the CD spectrum provides no clue to structure-sequence correlation. Therefore, the deconvolution of the complex CD spectrum must be followed by the assignment of the component CD curves to individual conformation(s), using external data, such as FT-IR and/or NMR (NOE) structural constraints.

It is the assignment of the basis spectra to the different types of folded structures (turns) which meets the most serious problems[2]. The characteristic Cotton effects of the peptide group are around 220 nm ($n\pi^*$ transition), 210 nm ($\pi\pi^*$ [||] transition) and above 190 nm ($\pi\pi^*$ [⊥]transition) Taking into account that in the majority of the known secondary structures the $n\pi^*$ band has a negative sign, it is basically the chiral interaction between the two $\pi\pi^*$ transitions which governs the CD spectrum of each form. The mechanism of this interaction, called "exciton splitting", is determined by the relative spatial orientation of the amide groups, ordered periodically in an α-helix and β-sheet, or aperiodically in turns. The α-helix (ϕ=-55°, ψ=-45°) and the 3_{10}-helix (ϕ=-60°, ψ=-30°) have similar average backbone torsional angle values. The type I β-turn comprises three amide groups whose relative position is determined by the torsion angles of the i+1 and i+2 residues (ϕ_{i+1}=-60°, ψ_{i+1}=-30°, ϕ_{i+2}=-90° and ψ_{i+2}=0°)[12]. There are several indications in recent literature[13-16] that the type I and III β-turns also give helix-like (class C)[17] CD spectra. The same difficulty arises from the similar shape and band positions of the CD spectrum of β-pleated sheet conformation (ϕ_β=-150°, ψ_β=+150°)and the class B CD pattern[17] of the type II turn (ϕ_{i+1}=-60°, ψ_{i+1}=+120°, ϕ_{i+2}=+80°and ψ_{i+2}=0°)[12]. Obviously, the performance of CD

spectroscopy can be significantly improved by using other constraints which may help to narrow the conformational space allowed by the CD spectrum.

The homo-nuclear Overhauser [^{1}H-(^{1}H)-NOE] experiment is the most frequently applied NMR technique for determining the folding pattern of the peptide or protein backbone[18]. It was shown that interproton distances can be determined on the basis of quantitative NOE even for multiple spin systems[18] as accurate as ±0.1Å. However, in the case of conformational mixtures these experimentally determined interproton distances reflect an average structure[19] due to the time-scale of the NMR experiment. Therefore the application of NOE based structural constraints has serious limitations and requires sophisticated calculations, when used to assist the assignment of the deconvoluted CD curves to the equilibrium conformers of the molecule.

The amide groups in periodically ordered conformations are linked together by H-bonds of similar average geometry. Therefore the vibrational spectroscopy yielding information on these natural constraints, may be of great help in assigning CD based conformations. (Vibrational circular dichroism (VCD) spectroscopy represents a combination of the two methods.)

Results and discussion;

Molecular forces playing an essential role in determining the shape of peptide molecules can be studied by IR and Raman spectroscopies. The bands reflecting H-bond formation and skeletal conformation are hidden behind the complex band system of the molecule. Assignment of the experimental bands to specific vibrational modes may be ambiguous and involve a great deal of arbitrariness. Bands showing measurable vagrancies in position and intensity in dependence of solvent, temperature etc. are traditionally assigned to "conformation dependent" modes. Conformational speculations should be preceded by "well established" normal coordinate analysis: the determination of the experimental force constants. At this time such an analysis is unfortunately not feasible for a larger peptide, therefore only a small portion of the total spectrum is informative from a conformational point of view.

A simple and effective technique of vibrational analysis of peptides is FT-IR spectroscopy. The amide group gives rise to several intensive IR bands, whose fine structure may be correlated with the various types of secondary structures and their relative amounts. Due to the difficulties outlined above, most studies focus on the amide I region (1620-1700 cm^{-1}) of the spectrum. Mathematical procedures of band narrowing by Fourier transforms (Fourier self-deconvolution and Fourier-derivation)[20,21] are used to enhance the visual

separation of individual bands in the broad contour of the amide I band. Absorptions in the amide I (amide I' in D_2O) region are due to stretching vibrations of the backbone C=O groups. The frequency of these vibrations has been shown to be sensitive to hydrogen bonding characteristics of the backbone amide groups. H-bondings also reflect molecular geometry and can be used as a constraint to distinguish ideal (nondistorted) secondary structures from their variants usually featuring H-bonds of decreased strength. The correlation between the position of composite amide I bands and the different secondary structures is based on the works of Krimm and Bandekar[22], Byler and Susi[23] and Mantsch et al.[20]. The component band centered between 1650-58 cm^{-1} in the IR spectra of proteins in D_2O solution is generally accepted to represent α-helical segments. The aperiodic (unordered) conformation is associated with absorption around 1644 cm^{-1} (1640-48 cm^{-1}), while IR bands between 1620-40 cm^{-1} are assigned to the β-sheet (extended) conformation. An additional weak band between 1670 and 1695 cm^{-1} was suggested to be indicative of the antiparallel orientation of β-strands. Generally, there are more than one weak bands above 1660 cm^{-1} in the amide I region of the infrared spectra of proteins. It has been proposed to assign bands around 1665, 1670, 1683, 1688 and 1694 cm^{-1} to turns (see ref. 20 and related references therein). More recently, a band near 1640 cm^{-1} was assigned to the repeating 1←4 H-bonds of 3_{10} helices[24,25].

According to X-ray crystallographic, NMR and CD experiments, the bridged cyclic peptides (cyclo[Gly-Pro-Xxx-Gly-NH-$(CH_2)_n$-CO][Xxx = Gly, Ser(O^tBu), Ser; n=2,4] contain a predominant type I or type II β-turn encompassing the Pro-Xxx sequence[14,15]. The turns are fixed by a 1←4 intramolecular H-bond. The pseudohexapeptide models (n=4) feature a second ten-membered (C_{10}) H-bonded ring comprising the achiral δ-amino valeric acid moiety. Detailed FT-IR spectroscopic studies, focusing on the NH, amide I and II regions of the spectra, have revealed[26,27] that the component band near 1640 cm^{-1} in both the crystal and solution spectra, can be correlated with the acceptor C=O of the strong 1←4 intramolecular H-bond. Bands above 1660 cm^{-1} are due to the "free" (more or less solvent-exposed) amide C=O groups.

To answer the question whether the β-turn-band can be detected or not in the FT-IR spectra of linear β-turn models, comparative NMR, CD and FT-IR spectroscopic studies were performed on peptides **1** and **2**.

Boc-Tyr-Pro-Gly-Phe-Leu-OH **1**

Boc-Tyr-Gly-Pro-Phe-Leu-OH **2**

Peptide **1** was shown[28] to have a significant antagonist effect against [Met^5]-enkephalin in the mouse vas deferens bioassay.

Peptide 1 and 2 show significantly different CD spectra in TFE known to promote the adoption of H-bonded secondary structures. Peptide 2 exhibits a CD curve with no definite extrema (Fig.1). This indicates an averaging of the backbone and aromatic contributions of opposite sign in the predominant conformer population(s) of 2. In acetonitrile-water mixtures quite a similar spectral behavior is observed. However, in neat acetonitrile (c=0.38 mg/ml) the appearance of a spectrum with a definite positive band at 197.5 *nm* and a strong negative band at 214.5 nm (with a negative shoulder) may be the sign of increased chiral contribution of the peptide backbone likely adopting fixed but nontypical conformation(s).

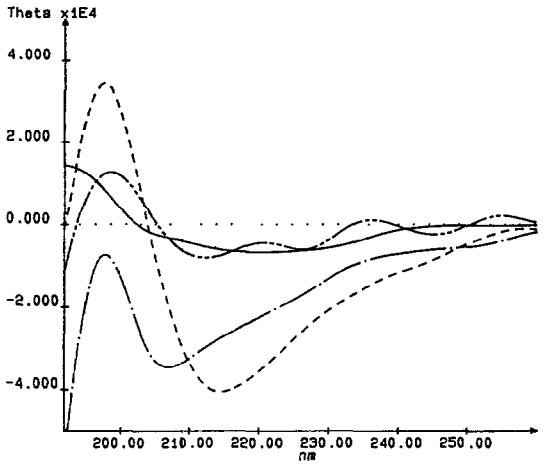

Figure 1. *CD spectra of Boc-Tyr-Gly-Pro-Phe-Leu-OH (2) in TFE (————), water containing 15% TFE (-.——.——.-), acetonitrile (- - - -) and acetonitrile - water 1:1 (— —— —); c=0.37-0.7 mg/ml.*

In TFE peptide 1 shows a class C' CD spectrum which has been suggested to reflect the adoption of certain subtypes of II β-turns[2] (Fig.2). This spectral feature is more or less preserved in TFE-water or acetonitrile-water mixtures. Surprisingly, an expressed concentration dependence was observed in pure acetonitrile (Fig.2). In dilute solution the spectrum shows resemblance to that of the β-conformation or the B CD pattern[17] of type II β-turns[13-16]. Obviously, the assignment of CD spectra to any type of ordered backbone conformation is obscured by the conformation-dependent chiral contribution of the aromatic side chains of phenylalanine and tyrosine. Fig. 1 and 2 suggest that the L_a band of phenylalanine near 210 nm[29] has no significant chiral contribution. The bands appearing between 200 nm and 210 nm are likely due to the amide chromophores because the influence of aromatic groups is generally ignorable in this spectral region. The red-shifted L_a band of tyrosine near 225 nm[29] is positive in the CD spectra of 1 (Fig.2). Its intensity is,

however, influenced by the coupling with the peptide transitions. As shown in Fig. 1, the L_a band of tyrosine does not have a definite contribution to the CD spectra of 2. It is difficult to predict even the sign of the L_a band of tyrosine in the averaged spectra in TFE or acetonitrile-water 1:1 mixture. The shapes of the spectra of 2 in water or acetonitrile are more compatible with an L_a band of negative sign. As expected, because of the overlapping of the peptide and aromatic bands, the CD spectra of 1 and 2 are not informative. They clearly reflect, however, the conformational mobility of both peptides and the higher tendency of 1, with the turn-forming Pro-Gly core, to adopt a folded structure.

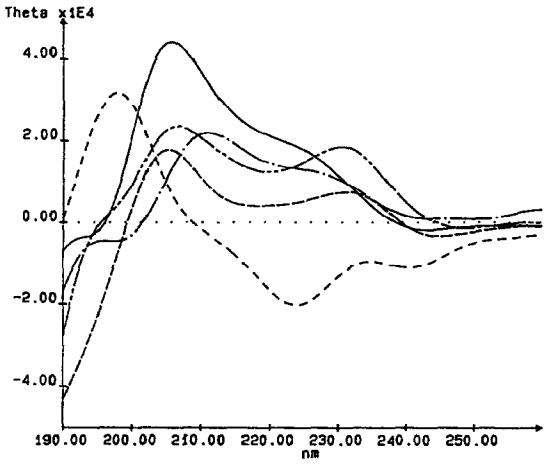

Figure 2. *CD spectra of Boc-Tyr-Pro-Gly-Phe-Leu-OH (1) in TFE (————), water containing 40% TFE (-.——.——.-), acetonitrile (c=0.84 mg/ml) (— —— ——) and acetonitrile (c=0.28 mg/ml) (- - - -) as well as acetonitrile/water 1:1 (— —— —); c=0.6 mg/ml. (No concentration dependence was observed in TFE in the above range of concentration.)*

Contrary to the CD spectra, the amide I region of the FT-IR spectra is void of aromatic bands. The spectra in TFE of 1 and 2 show significant differences (Figs.3a and b). (Beside the recorded spectra, the component curves and the difference between the experimental and sum curves are also shown in the middle.) In the curve fitted spectrum of 1 composite bands show up at 1682, 1666 and 1638 cm^{-1} while in that of 2 at 1675, 1661, 1644 and 1630(w) cm^{-1}. (Note that no composite band is present in the IR spectrum of 2 between 1640 and 1635 cm^{-1}.) The component band near 1640 cm^{-1} is also present in the IR spectrum of 1 in chloroform but absent in DMSO[31]. This solvent is known to destroy H-bonds[30] if they are not buried (e.g.in cyclic peptides) or otherwise protected from the solvent. In the infrared spectrum of 1 in acetonitrile (Fig. 3c), component amide I bands appear at 1682, 1666 and 1634 cm^{-1}. (Bands above 1700 cm^{-1} are due to the vibrations of C=O's of the COOH and urethane

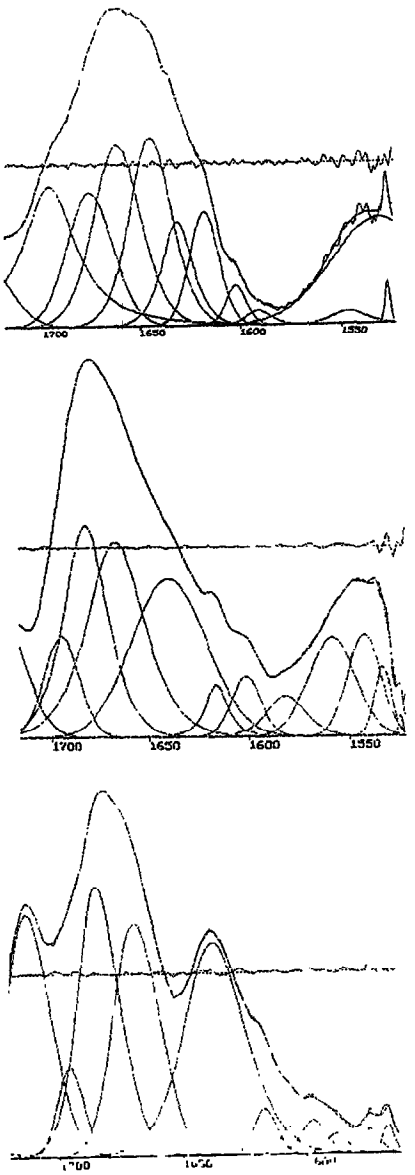

Figure 3. *The amide I region of the FT-IR spectra of Boc-Tyr-Gly-Pro-Phe-Leu-OH (2) in TFE (A) and Boc-Tyr-Pro-Gly-Phe-Leu-OH (1) in TFE (B) and acetonitrile (C), c< 0.5 mg/ml.*

groups.) The component band at 1634 cm^{-1} is broad and has an increased intensity (Figure 3c). This may be the sign of the strength of the 1←4 H-bond of the turn and/or the formation of a second H-bond initiating an antiparallel β-conformation.

Infrared spectroscopic studies reported herein give support to the earlier proposals[26,27] that the characteristic amide I band of H-bonded type I and II β-turns appears at 1640±2 cm^{-1} in D_2O, $CHCl_3$ and DMSO. In halogenated alcohols (e.g. TFE) or acetonitrile this band, termed *β-turn band*, may be shifted down to 1634 cm^{-1}. IR data on 1 and 2 show that under favorable structural and solvational conditions the population of H-bonded β-turns may be high enough even in linear peptides to give rise to a *β-turn band* of well defined position and intensity.

The conformational assignment mainly based on IR studies is strongly supported by 500 MHz ^{1}H NMR data[31] in CD_3CN of 1 and 2. For 1 the NOESY spectrum shows characteristic cross-peaks between the $H^{\alpha}_{Pro}/NH_{Gly}$, $H^{\alpha}_{Gly}/NH_{Gly}$, NH_{Phe}/NH_{Gly}, $H^{\alpha}_{Gly}/NH_{Phe}$, $H^{\delta}_{Pro}/NH_{Tyr}$ and $H^{\delta}_{Pro}/H^{\alpha}_{Tyr}$ protons. The *cisoid* orientation of the C^{α}_{Pro}-H^{α}_{Pro}, N_{Gly}-H_{Gly} and C^{α}_{Gly}-H^{α}_{Gly} N_{Gly}-H_{Gly} bonds is in good agreement with the strong $H^{\alpha}_{Pro}/NH_{Gly}$ and $H^{\alpha}_{Gly}/NH_{Gly}$ NOE's (Fig.4). Such NOE pairs are diagnostic of a type II β-turn (or an extended-like backbone conformation). The coupling constant of Gly ($J_{N\alpha}$ = 5 Hz) and the $H^{\delta}_{Pro}/NH_{Tyr}$ cross-peak in the NOESY spectrum is also compatible with the adoption of a type II β-turn conformation. In addition to the above NOE-derived structural information, a cross-peak between the delta protons of leucine and beta protons of the Boc protecting group was also observed, which may suggest the steric vicinity of these groups in the folded backbone conformation. In contrast to 1, NMR data obtained from 2 do not support the occurrence of significant population of folded conformers in CD_3CN solution.

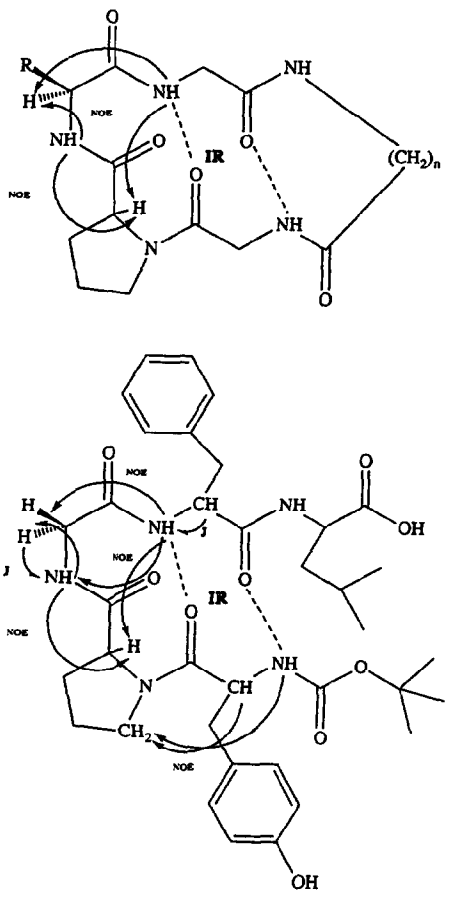

Figure 4. *Schematic representation of the conformations of cyclo[Gly-Pro-Xxx-Gly-NH-$(CH_2)_n$-CO] [Xxx = Gly, Ser(O^tBu), Ser; n=2,4]* **(A)** *and Boc-Tyr-Pro-Gly-Phe-Leu-OH (1)(B). The conformational constraints used for the assignment of the CD spectra are also shown (IR; FT-IR data, NOE; data from ^{1}H-{^{1}H}-NOE experiments and coupling constants).*

IR studies, reported earlier[26,27] and discussed in this paper, clearly show that bands above 1665 cm^{-1} cannot be correlated with the acceptor C=O of H-bonded β-turns but may be due to carbonyls of its second (central) and third (H-bond donating) amide groups.

NMR experiments briefly outlined above strongly suggest that the IR band, showing up near or right below 1640 cm^{-1} is indicative of significant population of a 1←4 H-bonded β-turn. Furthermore, the intensity of the *β-turn band* may be high enough even in the spectra of linear peptides in TFE generally used in CD spectroscopic studies. Thus, IR spectroscopy provides constraints characteristic of the orientation and distance of amide groups in crucial conformational position. These constraints are readily accessible and therefore may be of great help in CD-based conformation studies of polypeptides. NMR and CD spectroscopy cannot distinguish between the α-helix (i to i+4 H-bonding) and the 3_{10} helix (i to i+3 H-bonding).In the IR spectrum the appearance of the *β-turn band*, characteristic of the formation of strong i to i+3 (1←4) H-bondings, near or below 1640 cm^{-1} at the upper borderline of β-sheets, allows to differentiate between these two types of helices (α- and 3_{10}). Polypeptides having a helix-like or type C CD spectrum[17] but showing an amide I IR band near 1640 cm^{-1} (instead of 1655 cm^{-1}) are expected to adopt a 3_{10} helix (or repeats of type I, type II' and or type III β-turns) rather than an α-helical structure. On the other hand, peptides showing the 1640 cm^{-1} *β-turn band* but featuring a type B or type C' CD spectrum instead of type C[17] likely adopt a strongly H-bonded type II β-turn. The CD spectra of Boc-Tyr-Pro-Gly-Phe-Leu-OH (1) in TFE and acetonitrile (Fig.2) represent the latter case. The B character of the spectrum in dilute acetonitrile solution and the C'->B spectral transition may indicate the stabilization of the β-turn.

H-bondings are chiral constraints because in helical, folded or twisted conformations they stabilize conformations with only one sense of chirality or handedness. In a α-helix (3.6_{13}-helix) the 1←5 type H-bonds fix a right-handed winding of the backbone. In a 3_{10} helix repeating 1←4 H-bonds are found but the helix has the same right-handedness. IR spectroscopy reflects the strength of intramolecular H-bondings which strongly depends on environmental effects. Thus, the position of the amide I bands may indicate distortions caused by solvation, association etc. This makes the combination of the CD and FT-IR spectroscopy unique in characterizing the *absolute conformation* of the polypeptides built up from amide units located in homochiral configurational environment.

Experimental:

The synthesis and characterization of compounds 1 and 2 have been reported earlier[28]. CD spectra were recorded on a Jobin-Yvon dichrograph VI. Spectrograde solvents and 0.02 cm cells were used. CD is expressed in molar ellipticity ($[\theta]$,deg*cm^2/dmol). FT-IR spectra were measured on a Nicolet 170 SX spectrometer at a resolution of 4 cm^{-1} using KBr cells (0.041 cm). Unless otherwise stated, peptide concentration ranged between 0.5 and 1 mg/cm^3. Infrared spectra of the solvents were obtained under identical conditions and subtracted from the spectra of the samples. The spectra were analyzed by a normalized least squares curve fitting program, using products of Gaussian and Lorentz curves (Holly et al. unpublished)[31] and by the Fourier self-deconvolution method of Mantsch et al.[21].

ACKNOWLEDGEMENTS

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11. The CCA (convex constraint algorithm) gives at least theoretically the possibility of a quantitative CD analysis. This procedure operating conversely than a linear combination, aimes to determine simultaneously the weights p_i and the pure component curves $g_i(\lambda)$. Such a minimization

of the following expression is feasible when a set of measured $f_j^m(\lambda)$ values is available

$$\left[\sum_{j}^{N} f_j^m(\lambda) - \sum_{j}^{N} f_j^c(\lambda)\right]^2 = \left[\sum_{j}^{N} f_j^m(\lambda) - \sum_{i}^{N}\sum_{j}^{P} p_{ij} * g_i(\lambda)\right]^2 \rightarrow \text{minimized}$$

Three constraints are applied during CCA deconvolution;

a) $\sum_{i=1}^{P} p(i,j) = 1 \qquad j=1,2,..,N$

b) $p(i,j) \geq 0$

c) the points of $\{w(i,j),\ i=1,..,P\},\ j=1,...,N$ must be embedded in a simplex of the P-dimensional Euclidean space with the smallest volume. For details see A.Perczel, G.Tusnady, M.Hollosi and G.D.Fasman *Protein Engineering* **1991**, 4, 669

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$$\frac{1}{d_i^6} = \sum_{j=1}^{all.conf} p_j \frac{1}{d_{ij}^6} \qquad [2]$$

where p_j is the probability of the j-th conformation in which the analyzed interproton distance is d_{ij}. Therefore the correct interpretation of distance information based on NOE experiments requires the knowledge of the marker distances (d_{ij}) in all the individual conformers.

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