β-Turns in Model Dipeptides. An Infrared Quantitative Analysis with NMR Correlation

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Abstract: An IR method is proposed for estimating the β-folding ratio of model dipeptides RCO-X-Y-NHR′ in CH_2Cl_2. When R′ = Me is substituted for R′ = *i*-Pr, a frequency shift of nearly 20 cm^{-1} of the NH(R′) stretching vibration is induced. By subtraction of the two spectra, all NH stretching absorption bands are cancelled out, except the NH(R′) contribution. After determination of the molar extinction coefficient of the residual free NH(Me) band at 3450 cm^{-1} in CH_2Cl_2, the percentage of β-folded conformers was estimated for some dipeptide sequences containing alanine, glycine, proline, serine, and histidine. Results concerning the more or less frequent occurrence of these residues in β-turn agree with Chou and Fasman's statistical analysis of proteins. Quite a good correlation is found between β-folding ratios and the difference of the NMR chemical shifts of the C-terminal NH proton measured in $(C^2H_3)_2SO$ and C^2HCl_3, and this could be used for longer peptides. NMR temperature coefficients for NH protons do not appear to be correlated with β-folding ratios.

The chain reversal of the so-called β-turn[1] is one of the most frequent conformational units in proteins[2] and peptides.[3] Its role as a nucleus for tertiary folding and site for post-translational enzymatic modifications[6] in proteins has been emphasized. In the case of natural peptides, its role in structure–activity relationships is well documented.[1]

The amino acid residues which are preferentially incorporated in β-turns have been characterized by statistical treatment of the data on crystallized proteins.[2,7,8] The conclusion reached was that short polar residues (Ser, Asp, Asn, ...) favor β-turn formation and that Pro and Gly residues occur preferentially in the so-called $(i + 1)$th and $(i + 2)$th positions, respectively. These conclusions were then quantified in the well-known Chou and Fasman analysis,[9] which was successfully applied to a number of globular proteins. However, its application to low molecular weight peptides seems questionable, and the protein dataset is too limited to provide reliable statistical analysis of all 400 dipeptide sequences.[7,8] This justifies the interest taken in the model dipeptides RCO-X-Y-NHR′ which constitute the smallest molecules where a β-turn can occur.

Following Venkatachalam's pioneering work,[10] many conformational energy calculations were made on model dipeptides,[11-16] their conclusion being that the propensity to β-turn formation depends upon the nature, the position, and the relative configuration of both X and Y residues. However, β-folded conformers have often been predicted to be less stable than other conformers,[12,17,18] a prediction which seems to be at variance with a number of experimental facts.[1,3,19] Therefore, the predicted propensity to β-turn formation in model dipeptides needs to be confronted with experimental data.

For several years we have been carrying out extensive IR, ^1H NMR, and X-ray investigations of model dipeptides[19] and propose here an experimental estimation of β-folded conformers based on the NH stretching absorption spectra in CH_2Cl_2 solution.

Method

The IR spectra of model dipeptides with the Pro-Y sequence (Y = Gly, L-Ala, D-Ala) have been reported elsewhere and interpreted in terms of three rapidly interconverting conformers in CCl_4 solution:[19,20] (i) an open conformer with free NH and CO vibrators; (ii) a γ-folded C_7C_5 conformer, characterized by a bifurcated $i \leftarrow i + 2 \rightarrow i + 2$ hydrogen bond (Figure 1a); and (iii) a β-folded C_{10} conformer, characterized by an intramolecular $i + 3 \rightarrow i$ interaction (Figure 1b).

The relative stability of these conformers depends on the solvent. Figure 2 shows how the γ-folded conformer of the L-Pro-L-Ala sequence is converted into the β-folded form when going from CCl_4 to CH_2Cl_2. In the latter solvent, the three-component equilibrium in CCl_4 is simplified and becomes a two-component equilibrium between open and β-folded conformers. The amount of each component is theoretically related to the intensity of the NH stretching absorption bands. However, the band overlap would imply a decomposition of the spectra, making it too imprecise for quantitative analysis. We therefore worked out a method of quantitative analysis by subtracting the spectra of the two homologous dipeptides with different R′ C-terminal groups.

In earlier studies[21,22] we pointed out that substituting the C-terminal methyl group (R′ = Me) for the isopropyl one (R′ = *i*-Pr) induces a NH stretching frequency shift of nearly 20 cm^{-1}. The fact that the N-terminal CO stretching absorption remains unmodified indicates that this substitution has a negligible influence on the above-mentioned conformational equilibrium. Therefore, the subtraction of the NH absorption spectra of homologous dipeptides RCO-X-Y-NHR′ with methyl and isopropyl R′ groups should cancel out all but the C-terminal NH contribution, which should appear as positive and negative residual bands (Figure 3). Because of its sharp profile and its minimum overlap with the

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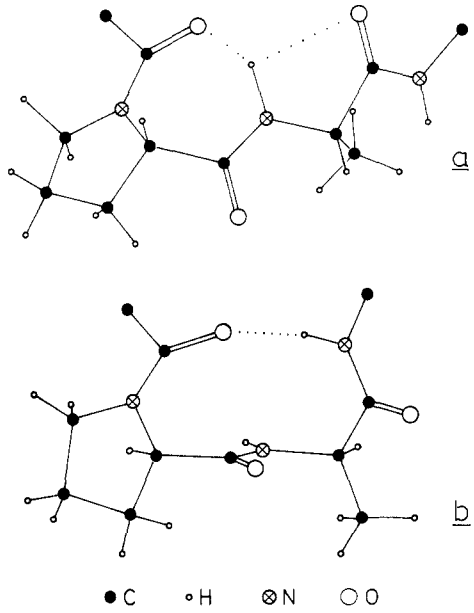


Figure 1. Representation of the γ-folded (a) and βI-folded (b) conformers of the L-Pro-L-Ala model dipeptide. (Intramolecular hydrogen bonds in dotted lines.)

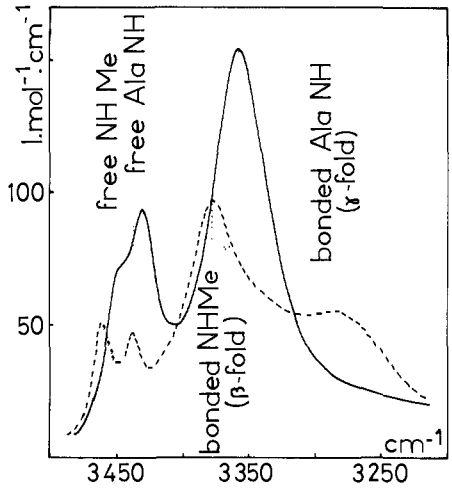
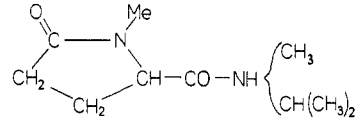


Figure 2. NH stretching absorption spectrum for *t*-BuCO-L-Pro-L-Ala-NHMe in CH_2Cl_2 (solid line, 5×10^{-3} mol L^{-1}) and CCl_4 (broken line, 5×10^{-4} mol L^{-1}).

low-frequency component typical of the $i + 3 \rightarrow i$ hydrogen bond, the residual free NH(Me) absorption band at 3450 cm^{-1} in CH_2Cl_2 is directly related to the amount of open conformers, provided the molar extinction coefficient ϵ_0 is known.

We propose the methylamide and isopropylamide of *N*-methyl-DL-pyroglutamic acid as calibrating compounds for the estimation of ϵ_0.



The cis disposition of the intracyclic amide link prevents any intramolecular interaction. The absorption bands at 3450 and 3430 cm^{-1} are therefore typical stretching of free NH(Me) and NH(*i*-Pr) bonds, respectively, and their difference provides us with a molar extinction coefficient ϵ_0 at 3450 cm^{-1} equal to 130 L mol^{-1} cm^{-1}.

Experimental Section

Model dipeptides were synthesized by classical procedures with dicyclohexylcarbodiimide or benzotriazolyloxytris(dimethylamino)phosphonium hexafluorophosphate as coupling reagents and the *tert*-butyloxycarbonyl group (BOC) as intermediary N-protection for the synthesis of L-X-L-Y and L-X-D-Y derivatives. The methylamide and isopropylamide groups were introduced into the BOC-amino acid by the mixed anhydride or 4-nitrophenyl ester method. The pivaloyl group was introduced by means of pivaloyl chloride.

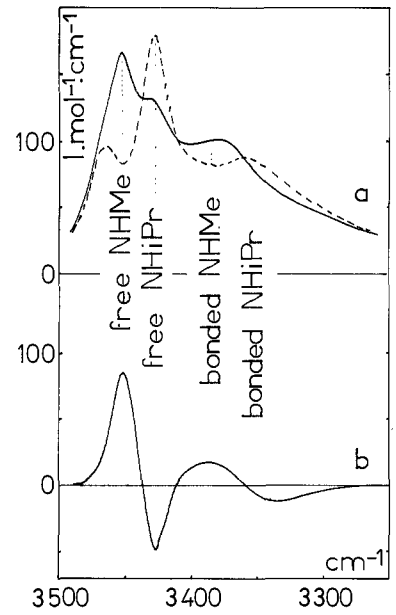


Figure 3. NH stretching absorption spectra (a) and difference spectrum (b) for the homologous *t*-BuCO-Gly-DL-Ala-NHR′ model dipeptides with R′ = Me (solid line) and R′ = *i*-Pr (broken line) in CH_2Cl_2 (5×10^{-3} mol L^{-1}).

The methylamide and isopropylamide of *N*-methyl-DL-pyroglutamic acid were prepared by aminolysis of the corresponding methyl ester. N-Methylation of the L-pyroglutamic methyl ester was carried out by the action of sodium metal in benzene and methyl iodide and provided the racemized *N*-methyl-DL-pyroglutamic methyl ester.

IR spectra were run at room temperature on a PE 580 spectrometer connected to an Inter Data 6/16 computer. The 1580–1800 and 3200–3500 cm^{-1} frequency domains investigated correspond to the CO and NH stretching vibrations, respectively. Spectra were run at various concentrations to check that solute–solute interaction was negligible for the following concentrations:

CH_2Cl_2: 5×10^{-3} mol L^{-1}
$CHCl_3$, CH_3CN: 1×10^{-2} mol L^{-1}
$(CH_3)_2SO$: 2×10^{-2} mol L^{-1}

^1H NMR spectra were scanned in the Fourier transform mode on a JNM FX 100 spectrometer equipped with a JNM SD HC heteronuclear ^{14}N decoupling unit giving sharp NH proton signals with an accuracy greater than 0.005 ppm with reference to internal tetramethylsilane.

Results

All model dipeptides have the general formula *t*-BuCO-X-Y-NHMe (or *i*-Pr), and the dipeptide sequences we have examined are listed in Table I. The pivaloyl group presents the advantage of favoring good solubility (except for the L-Ala-L-Ala derivative) in CH_2Cl_2 and preventing cis disposition of the *t*-BuCO-Pro fragment.[19] His is known to exist in both neutral and cation states at the physiological pH, and the L-Pro-L-His derivative was therefore studied in both states. The cation form was associated with the weak proton-accepting PF_6^- anion.[23]

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Table I. Percent [β-Turn] in X-Y Model Dipeptides in CH_2Cl_2

	Y							
X	L-Pro	L-Ala	L-Ser	L-His	Gly	D-Ala	D-Ser	D-Pro
L-Pro	87 (100; 70)[a]	67	90	84 (35)[b]	85	90	100	100
L-Ala	60 (80; 60)[a]	43[c]	74		69	52		79
Gly	46	35			33			
L-Ser		42			46			

[a] Cis and trans conformers, respectively (see text for reported values). [b] β-Folding ratio of the L-Pro-L-His$^+$, PF_6^- sequence. [c] The L-Leu-L-Leu dipeptide was substituted for the L-Ala-L-Ala derivative, insoluble in most solvents.

Figure 3 reproduces the NH stretching absorption spectra of the homologous *t*-BuCO-Gly-DL-Ala-NHMe (or *i*-Pr) peptides and their difference. When Me is replaced by *i*-Pr, the absorption band at 3450 cm^{-1} is shifted and overlaps the band at 3430 cm^{-1} which is mainly due to the middle NH bond. Thus, the residual contribution ϵ at 3450 cm^{-1} of the free C-terminal NH(Me) bond is obtained by spectral difference.

The percentage of β-folded conformers given by $100(1 - \epsilon/\epsilon_0)$ is closely related to the nature, the position, and the relative configuration of both residues (Table I).

The highest percentages are found for sequences having Pro in the X position and/or a polar residue (His$^+$ excepted) in the Y position. The influence of Gly varies with its position, and the Y position is by far the most favored. These observations are in fairly close agreement with Chou and Fasman's conclusions,[2] and model dipeptides appear to adequately mimic the main medium-range interactions responsible for β-folding.

The introduction of Pro in the Y position induces a cis–trans isomerism of the middle amide link in the homochiral L-Ala-L-Pro (15% cis) and L-Pro-L-Pro (50% cis) sequences.[19,24] The β-folding ratio is therefore an average value of the *cis* (βVI-turn) and *trans* (βI-turn) dispositions, and this will be discussed later. In all the other X-L-Pro sequences investigated, the cis conformer is very infrequent in chlorinated solvents,[19,24] and these sequences are less favorable to β-folding than Pro-X sequences.

The influence of serine depends on its position. The high percentage of βI-turn in X-L-Ser sequences was previously attributed to an attractive interaction between the proton donating Ser NH bond and the Ser O^γ accepting site.[25-27] This was also found to be true for the L-Pro-L-Thr sequence.[28] The interaction N—H···O^γ still occurs in Ser-Y derivatives,[26,29] but it now competes with the $i + 3 \rightarrow i$ interaction typical of β-bend and the percentage of β-folded conformers therefore decreases. The influence of the Ser position agrees with the theoretical predictions of Zimmerman and Scheraga[18] and the observations of Kolaskar et al.[7,8] However, the stabilizing influence of Ser in the Y position has not yet been accounted for by the ECEPP calculations,[18] probably because of the neglected N—H···O^γ interaction, the importance of which has recently been emphasized after ab initio calculations.[30,31]

The β-folding ratio of the L-Pro-L-His sequence is closely related to the neutral or cation form of His and falls from 84% for L-Pro-L-His to 35% for L-Pro-L-His$^+$. IR, ^1H NMR, and X-ray experiments[32] have shown that the βI-turn of L-Pro-L-His is stabilized by an additional interaction of the amide His NH proton donating bond with the imidazole His N^π accepting site. This interaction is obviously absent in L-Pro-L-His$^+$ and is replaced by a His$^+$ N^π—H···O=C(*t*-Bu) hydrogen bond which competes with the $i + 3 \rightarrow i$ interaction typical of β-folding.

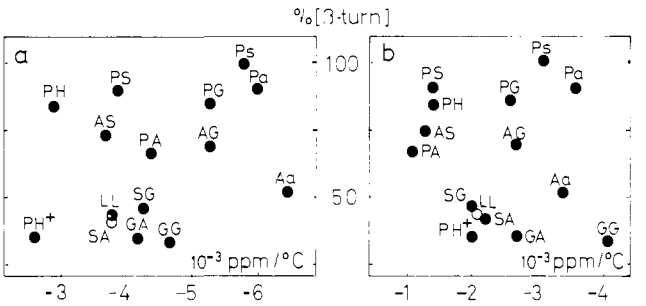

Figure 4. Absence of correlation between β-folding ratios in CH_2Cl_2 and NMR temperature coefficients $\Delta\delta/\Delta T$ in C^2H_3CN for the C-terminal (a) and middle (b) NH protons of the model dipeptides identified in the monoletter code of the dipeptide sequence (small letters stand for D residues).

Discussion

The folded conformers detected by IR spectroscopy are those containing the $i + 3 \rightarrow i$ interaction closing a ten-membered cycle. Folded forms having no intramolecular hydrogen bond are considered as open conformers, although the peptide backbone could be arranged to allow the peptide chain in longer peptides to fold back on itself. Such "open reverse turns" are frequently observed in proteins and eight classes of β-turns, four of which are without $i + 3 \rightarrow i$ interaction, have been listed.[33] However, we must keep in mind the low accuracy of this type of crystal structure and consider the very few exemples of "open reverse turn" occurring in the crystal structures of linear peptides with no ionic charge.[34] We therefore think that "open reverse turns" are probably due to long-range interactions which are obviously absent in model dipeptides.

The above IR quantitative analysis cannot be applied to any sort of peptide in any kind of solvent. It is restricted to C-terminal dipeptide sequences with a terminal amide function. The solvent activity on NH bonds must be low enough to prevent the free NH stretching absorption band from being shifted to low frequencies. For this reason aprotic solvents are excluded, and inert or slightly acidic chlorinated solvents are the only ones acceptable. Finally, in longer peptides than those investigated here, the percentage of β-turns can be overestimated, due to the eventual participation of the C-terminal NH bond in particular intramolecular interactions with the peptide backbone and/or the side chains. For all these reasons, this method only applies to very low molecular weight peptides soluble in chlorinated solvents and possessing a terminal amide function.

The results listed in Table I can be compared to those obtained by more classical methods such as the solvent and temperature dependence of amide proton NMR chemical shifts.[35] Slight variations are generally attributed to solvent-shielded NH protons and large variations to solvent-exposed NH protons. Although

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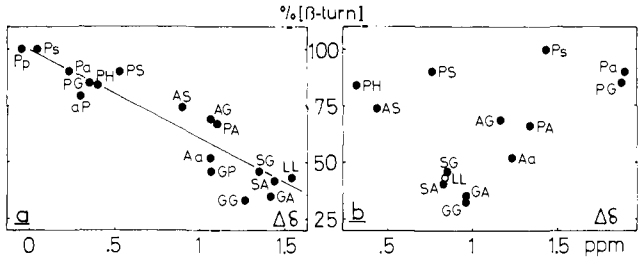

Figure 5. Correlation between β-folding ratios in CH_2Cl_2 and the chemical shift differences $\Delta\delta = \delta((C^2H_3)_2SO) - \delta(C^2HCl_3)$ for the C-terminal (a) and middle (b) amide protons of model dipeptides (same symbols as in Figure 4). The straight line in part a corresponds to the linear relationship in the least-squares approximation.

solvent shielding does not necessarily result from intramolecular interaction,[36,38] the correlation of NMR temperature coefficients with the percentage of hydrogen-bonded conformers has been promoted.[39-41] We decided to measure the NH proton chemical shifts for the above model dipeptides in two common solvents (C^2HCl_3 and $(C^2H_3)_2SO$) and to estimate the NMR temperature coefficients in C^2H_3CN. IR spectroscopy reveals that β-folded conformers are retained in CH_3CN to approximately the same extent as in $CHCl_3$ or CH_2Cl_2. The influence of $(CH_3)_2SO$ results in limited destabilizing effects on highly β-folded L-Pro-X sequences but noticeable destabilization of the other β-folded sequences. Temperature coefficients $\Delta\delta/\Delta T$ and chemical shift differences $\Delta\delta = \delta((C^2H_3)_2SO) - \delta(C^2HCl_3)$ are plotted in Figures 4 and 5 against the β-folded ratios listed in Table I.

There seems to be no correlation with the temperature coefficients for both NH protons (Figure 4). Given the high and small $\Delta\delta/\Delta T$ values generally attributed to solvent-exposed and solvent-protected NH protons, an eventual correlation should correspond to experimental points approximately disposed in a straight line with negative or positive slope for the C-terminal (Figure 4a) and middle (Figure 4b) NH proton, respectively. On the other hand, one notes that LD and LG sequences (G standing for the achiral Gly residue), which are preferentially folded in a βII turn,[3,19,25] correspond to high C-terminal NH(R′) values usually attributed to free NH protons.[35,40] A possible "end effect" can be invoked here but does not hold for the middle NH proton. These examples are arguments among others[36-38] indicating that NMR temperature coefficients should not be used as entirely reliable evidence of intramolecular hydrogen bonding.

The chemical shift difference $\Delta\delta$ with the solvent shows rather good correlation with %[β-turn] for the only C-terminal NH proton (Figure 5a). The absence of correlation for the middle NH proton (Figure 5b: experimental points expected to fit an approximative line with positive slope) is probably the consequence of the above-mentioned intramolecular interaction involving the proton-donating Ser or His NH bond in βI-folded conformers.

The correlation for the C-terminal amide proton (Figure 5a) between the percentage of β-folding in CH_2Cl_2 and the chemical shift difference

$$\Delta\delta = \delta((C^2H_3)_2SO) - \delta(C^2HCl_3)$$

can be represented by a linear relationship by using the least-squares approximation

$$\%[\beta\text{-turn}] = 100 - 39\Delta\delta$$

(correlation factor $r = -0.93$; standard deviation $\sigma = 8\%$).

A similar correlation is also found when the chemical shift difference $\Delta\delta'$ arises from the introduction of 10% $(C^2H_3)_2SO$ in C^2HCl_3, a mixture which induces less conformational perturbation than neat $(C^2H_3)_2SO$.[19] The above relation then becomes

$$\%[\beta\text{-turn}] = 100 - 62\Delta\delta'$$

(correlation factor $r = -0.94$; standard deviation $\sigma = 8\%$).

These correlations can be applied to estimate the folding ratios for the cis and trans conformers of the L-Ala-L-Pro and L-Pro-L-Pro sequences. Cis conformers exhibit lower values of $\Delta\delta$ (0.32 and −0.08 ppm, respectively) than trans conformers (0.99 and 0.75 ppm, respectively), indicating a higher folding ratio in the former case (Table I). We suggest that these correlations could provide an estimation of the folding ratio in CH_2Cl_2 for a given dipeptide sequence in longer peptides provided the NH bond of the $(i + 3)$th residue does not interact with the side function of a neighboring polar residue. As a supporting argument, we note that the chemical shift difference $\delta((C^2H_3)_2SO) - \delta(C^2HCl_3)$ for some β-folded peptides mentioned in the literature[42-44] are within the range −0.36 to 0.38 corresponding to %[β-turn] = 100 ± 15%.

Conclusion

The C-terminal NH stretching frequency of model dipeptides RCO-X-Y-NHR′ depends on the nature of R′. The substitution of R′ = Me for R′ = *i*-Pr induces a frequency shift of nearly 20 cm^{-1}, resulting in the cancellation of all NH stretching absorption bands excepting the NH(R′) contribution when the spectra of homologous derivatives with different R′ end groups are subtracted.

The molar extinction coefficient of the residual free NH(Me) contribution at 3450 cm^{-1} in CH_2Cl_2 is provided by the methyl- and isopropylamide of *N*-methylpyroglutamic acid, which cannot accommodate folded conformations because of the cis disposition of the intracyclic amide link. The resulting β-folding ratios of model dipeptides agree with the qualitative conclusions of Chou and Fasman's statistical analysis of crystallized proteins, concerning the more or less favored occurrence of the residues in β-turn. This argues that short-range interactions in dipeptide sequences are of primary importance in β-folding.

The comparison of IR results with the temperature and solvent dependence of the NMR chemical shifts of the C-terminal NH proton reveals a rather good linear correlation with the solvent effect, suggesting that it could be applied to longer peptides. Surprisingly, no correlation appears with temperature coefficients which are sensitive to the βI- or βII-folding mode of model dipeptides.

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Registry No. *t*-BuCO-L-Pro-L-Ala-NHMe, 53933-24-7; *t*-BuCO-Gly-DL-Ala-NHMe, 94891-07-3; *t*-BuCO-Gly-DL-Ala-NHPr-*i*, 94891-08-4.

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