Conformational Effects on Peptide Aggregation in Organic Solvents: Spectroscopic Studies of Two Chemotactic Tripeptide Analogs

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Synopsis

The aggregation behavior of the chemotactic peptide analogs, Formyl-Met-Leu-Phe-OMe (1) and Formyl-Met-Aib-Phe-OMe (2), has been studied in chloroform and dimethylsulfoxide over the concentration range of 0.2-110 mM by 'H-nmr spectroscopy. Both peptides associate in CDCl₃ at concentrations ≥ 2 mM, while there is no evidence for aggregation in (CD₃)₂SO. Analog 1 adopts an extended conformation in both solvents favoring association to form β-sheet structures. A folded, y-turn conformation involving a 3 → 1 hydrogen bond between Met CO and Phe NH is supported by 'H-, 'C-nmr, and ir studies of analog 2. The influence of backbone conformation on the ease of peptide aggregation is demonstrated by ir studies in CHCl₃ and CD studies in dioxane.

INTRODUCTION

The conformational analysis of peptides in solution by spectroscopic techniques is often complicated by the problem of peptide aggregation. The influence of peptide association on 'H-nmr parameters and the demonstration of the presence of aggregates in chloroform solutions of small protected peptides by vapor-phase osmometry have been the focus of recent investigations. An analysis of aggregation effects on spectral parameters and the possible role of monomer conformation on the mode and facility of intermolecular association would be of value in developing spectroscopic methodology to probe the structural characteristics of peptide aggregates. With this end in view, we have chosen to examine the peptides Formyl-Met-Leu-Phe-OMe (1) (For = formyl, OMe = methoxy) and Formyl-Aib-Phe-OMe (2) by nmr ('H and 'C), and CD methods, in organic solvents, over a range of concentrations. These peptides constitute biologically-active analogs of the chemotactic tripeptide, For-Met-Leu-Phe-OH, which has been shown to stimulate neutrophil chemotaxis and selective release of lysosomal enzymes. Esterification of the C-terminal carboxylic acid group has marginal effects on biological potency. Earlier 'H- and 'C-nmr studies of For-Met-Leu-Phe-OH in dimethylsulfoxide have been interpreted in terms of an unassociated peptide in an extended β-sheet conformation. In peptide 2, the central Leu residue has been replaced by

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an α-aminoisobutyryl (Aib) residue in order to facilitate chain reversal, resulting in stabilization of a folded backbone conformation. This feature may be anticipated, in view of the well-established tendency of Aib residues to favor folded β-turn structures in small peptides. The choice of peptides 1 and 2 permits a study of aggregation effects on two peptides having sequences with markedly different tendencies to adopt folded conformations. A comparative study of peptides 1 and 2 is also relevant, since it has been shown that replacement of Leu by Aib at position 2 leads to retention of biological activity.

MATERIALS AND METHODS

The peptides For-Met-Leu-Phe-OMe (1), For-Met-Aib-Phe-OMe (2), For-Met-Leu-Leu-OMe (3), and For-Met-Aib-Leu-OMe (4) were synthesized by standard solution-phase procedures using dicyclohexylcarbodiimide (DCC) or DCC-1-hydroxybenzotriazole-mediated couplings. tert-Butyloxycarbonyl (Boc) and methyl ester groups were used for amino and carboxyl group protection, respectively. The Boc group was cleaved from the corresponding tripeptide methyl esters by treatment with 98% formic acid. Evaporation of formic acid, followed by addition of freshly distilled acetic anhydride to the residue, yielded the formyl peptides in good yields (75–90%). All peptides were purified by column chromatography on silica gel using CHCl₃ or 2% CH₃OH-CHCl₃ as eluants. Peptides were obtained as white solids, homogeneous by tlc, and yielded 270-MHz ¹H-nmr spectra fully consistent with their structures. Peptides were also found to be homogeneous by HPLC on a reverse-phase RP-18 Lichrosorb column, using methanol–water linear gradient elution and employing an LKB HPLC system (55–75% MeOH in 15 min; flow rate, 0.8 mL/min; retention time 1, 13.4 min; 2, 11.6 min).

¹H- (270-MHz) and ¹³C- (67.89-MHz) nmr spectra were recorded on a Bruker WH-270 spectrometer at the Sophisticated Instruments Facility, Bangalore, under conditions described elsewhere. Chemical shifts are expressed as δ (ppm) downfield from internal tetramethylsilane for both ¹H- and ¹³C-spectra. The ir spectra were recorded on a Perkin-Elmer model 297 spectrometer. Solutions were prepared in dry CHCl₃ and spectra recorded using pathlengths of 1–7.5 mm. CD spectra were recorded on a Jasco 5-20 spectropolarimeter using 1-mm-pathlength cells. CD band intensities are expressed as molar ellipticities [θ]M in deg cm² dmol⁻¹.

RESULTS AND DISCUSSION

¹H-NMR Studies

The dependence of peptide NH chemical shifts (δ) and their temperature coefficients (dδ/dT) on concentration, were determined in CDCl₃ and (CD₃)₂SO for 1 and 2. The NH resonances were readily

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assigned by spin decoupling experiments, which established the connectivities between the CαH, CαH and NH resonances for each residue. Figures 1 and 2 illustrate the variations in δ values obtained for the NH, CαH, and formyl resonances in 1 and 2 in the two solvents. The concentration dependences of the dδ/dT values of the NH groups are illustrated in Fig. 3. In peptide 1, the only JHNCαH value that showed a significant change with concentration in CDCl₃ was Met (1)NH, varying from 7.7 Hz at 0.2 mM to 9.2 Hz at 110 mM. Smaller changes were observed for Leu NH, while the JHNCαH value for Phe NH was concentration independent. In peptide 2, the J values were largely concentration independent over the range studied. In view of these observations, J values have not been used in subsequent analysis of aggregation effects.

A feature of the data in Figs. 1–3 is that δ and dδ/dT values for NH groups are concentration independent for both peptides in (CD₃)₂SO over the range 2–100 mM. This suggests that the peptides exist as solvated monomers in (CD₃)₂SO. In contrast, in CDCl₃, all three NH groups in 1 show pronounced concentration dependences, moving...
Fig. 2. Concentration dependence of chemical shifts (δ) for various resonances in For-Met-Aib-Phe-OMe (2) in (a) CDCl₃ and (b) (CD₃)₂SO. Abscissa expresses concentration as log C, where C is defined as in Fig. 1.

Fig. 3. Concentration dependence of temperature coefficients (dδ/dT) of NH resonances. (a) For-Met-Leu-Phe-OMe (1): (CD₃)₂SO; O, CDCl₃. (b) For-Met-Aib-Phe-OMe (2): (CD₃)₂SO; (CD₃)₂SO. Abscissa expresses concentration as log C, as in Fig. 1.
sharply downfield at concentrations $>1\,\text{mM}$. The CαH resonances of Met and Leu move slightly downfield at higher concentrations, while the Phe CαH peak is insensitive. The $d\delta/dT$ values for all NH groups in 1 are strongly concentration dependent in CDCl$_3$ (Fig. 3). The data clearly suggest that peptide association of 1 is significant in CDCl$_3$ at concentrations $\gtrsim 1\,\text{mM}$. In peptide 2, the concentration dependence of $6$ values differentiates the various NH groups. While the Phe NH shows a small but linear downfield shift with increasing concentration, the Aib and Met NH groups show a sharp discontinuity at $\sim 2.4\,\text{mM}$. No concentration dependence is observed for the $6$ values of the formyl (HCO) and CαH resonance. Thus, it appears that the Aib and Met NH groups are exposed and, at higher concentrations, participate in intermolecular hydrogen bonding. There is no convincing evidence for the involvement of the Phe NH in peptide association. Furthermore, at concentrations $<2.4\,\text{mM}$, 2 appears to be in an unaggregated state in CDCl$_3$ since the $6$ versus concentration curves are flat in this region.

The $d\delta/dT$ values for the NH groups in 2, in CDCl$_3$, show a pronounced concentration dependence, increasing with peptide concentration (Fig. 3). Low $d\delta/dT$ values in CDCl$_3$ ($<0.0024\,\text{ppm/K}$) may be attributed to solvent-exposed or -shielded NH groups, while high $d\delta/dT$ values ($>0.0035\,\text{ppm/K}$) are characteristic of NH groups involved in intermolecular hydrogen bonding. The results suggest that Met and Aib NH groups are involved in peptide association, while Phe NH does not appear to participate in a significant intermolecular interaction. Phe NH has an extremely low $d\delta/dT$ value in CDCl$_3$ at 2.4 mM but does show an increase at higher concentrations. This may be indicative of its participation in a weak intramolecular hydrogen bond, with the population of such species decreasing on heating. Further support for this conclusion is derived from paramagnetic radical-induced broadening of NH resonances in CDCl$_3$; as well as from solvent-titration experiments. Addition of the free radical, 2,2,6,6-tetramethylpiperidine-1-oxyl (TEMPO), causes almost equal line broadening of all three NH groups in the case of peptide 1 [Fig. 4(a)]. However, in peptide 2 [Fig. 4(b)], the Aib and Met NH groups are significantly more affected than Phe NH, suggesting that the latter is indeed, shielded from the solvent. The formyl proton resonance is unaffected by TEMPO addition and serves as an internal control. The dependence of NH chemical shifts on (CD$_{3j}$_2$SO$ Concentration in CDCl$_3$–(CD$_{3j}$_2$SO$ mixtures in 1 and 2 are presented in Fig. 5. While all three NH groups in 1 show a pronounced solvent dependence, in 2 the Phe NH is markedly less sensitive to the addition of the strongly hydrogen-bond–accepting component. Peptide 1 should have been significantly associated under the conditions of the TEMPO broadening experiment, while aggregation effects may be less important for 2. In peptide 2, the $d\delta/dT$ values measured in (CD$_{3j}$_2$SO$ are concentration independent. However, the Phe NH resonance does have a relatively low $d\delta/$
Fig. 4. Effect of increasing concentration of the free radical, TEMPO, on the linewidths of the NH and formyl proton resonances. TEMPO concentrations range from \(\sim 0.02\) to \(0.08\%\). (a) For-Met-Leu-Phe-OMe (1), (b) For-Met-Aib-Phe-OMe (2). Peptide concentration, \(\sim 11\) mM; solvent, CDCl₃.

\(dT\) value (\(\sim 0.0035\) ppm/K), which argues for at least a partial shielding of this NH group from the solvent. NH groups that are relatively strongly hydrogen bonded (solvent shielded) have \(\delta/dT\) values <0.003 ppm/K, while solventexposed groups are generally charac-

Fig. 5. Dependence of NH chemical shifts on \((\text{CD}_3)_2\text{SO}\) concentration in mixtures of CDCl₃ and \((\text{CD}_3)_2\text{SO}\). Peptide concentration \(-11\) mM. (a) For-Met-Leu-Phe-OMe (1), (b) For-Met-Aib-Phe-OMe (2).
characterized by $d\delta/dT > 0.004 \text{ ppm/K}$, in (CD$_3$)$_2$SO.$^{5,23,24}$ Values between 0.003 and 0.004 ppm/K are difficult to interpret with certainty, although they have been frequently assigned to weakly hydrogen-bonded NH groups.$^{25,26}$

The above results suggest that For-Met-Leu-Phe-OMe aggregates in CDCl$_3$. The results are consistent with the formation of $\beta$-sheet aggregates, both “in-register” and “out-register” (Fig. 6) as proposed for oligopeptides of norvaline.$^1$ In (CD$_3$)$_2$SO there is no evidence for folded intramolecularly hydrogen-bonded conformations. Extended structures, as proposed for the free acid,$^{12}$ are consistent with the nmr results. In For-Met-Aib-Phe-OMe (2), the Phe NH is partially shielded from the solvent and presumably intramolecularly hydrogen-bonded in CDCl$_3$ and (CD$_3$)$_2$SO. The Aib and Met NH groups are solvent-ex-

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![Diagram](attachment:diagram.png)

Fig. 6. Schematic representation of aggregation modes For For-Met-Leu-PheOMe (1) in chloroform: (a) antiparallel $\beta$-sheets “in register,” (b) antiparallel $\beta$-sheets “out of register,” and (c) parallel $\beta$-sheet aggregates.
posed and form intermolecular hydrogen bonds at high peptide concentrations in CDCl₃. The involvement of the Phe NH in intramolecular hydrogen bonding favors a folded conformation for 2. Two possible conformations are illustrated in Fig. 7. The β-turn structure [Fig. 7(a)] involves a 4 → 1 hydrogen bond between the formyl CO group and Phe NH, while the γ-turn conformation [Fig. 7(b)] is stabilized by a 3 → 1 hydrogen bond between Met CO and Phe NH. The γ-turn is defined as a structure involving a single 3 + 1 hydrogen bond, as suggested in Ref. 27.1 The β-turn structure, with Aib as the i + 2 residue, has been crystallographically observed in a large number of Aib-containing peptides.13-16 The γ-turn (C₇) conformation has been observed in the crystal structure of the cyclic tetrapeptide, dihydrochlamydocin,29 and has been suggested on the basis of ir studies of several Aib-containing dipeptides.30-32 In general, β-turn structures, with backbone conformational angles of φ ~ ± 60° ± 20° and ψ ~ ± 20° ± 20°, are favored for the Aib residue.13-16 The γ-turn (φ ~ ± 70°, ψ ~ ± 70°) is less frequently observed. However, β-turn structures in Aib peptides have been generally characterized by strong intramolecular hydrogen bonds, as evidenced by low dδ/dT values in (CD₃)₂SO. The β-and γ-turn structures are distinguishable if the identity of the hydrogen-bonded carbonyl group can be determined.

**13C-NMR Studies**

The 67.89-MHz ¹³C-nmr spectra of peptides 1 and 2 showed four carbonyl carbon resonances between 160 and 175 δ. The chemical shifts (δ) in CHCl₃ and CH₃OH solutions are listed in Table I. A solvent titration experiment,27,28 carried out in CHCl₃-CH₃OH mixtures of varying composition, confirmed the absence of any crossover of peaks.
on going to the more strongly hydrogen-bonding donor solvent, CH$_3$OH (peptide concentration $\sim 0.09 \text{M}$). The formyl (HCO-) peak is easily assigned to the high-field resonance at 161–164 $\delta$ by virtue of its appearance as a doublet in the $^1$H-off-resonance-decoupled $^{13}$C spectrum. In peptide 2, the Aib CO group is assigned to the low-field resonance [CO(1)], as comparisons with a number of related peptides establishes a downfield shift of the CO resonance on $\alpha$-alkylation. The Met and Phe CO groups were assigned by comparison of the shifts in the peptides, For-Met-Leu-Phe-OH and For-Met-Leu-Phe-NH$_2$ (data not shown). The Phe CO shows small but significant shifts between the ester, acid, and amide derivatives. A similar assignment of the CO resonances in 1 was not possible. However, the data in Table I show that all the CO groups in 1 experience a similar downfield shift (−2.1 to 2.6 ppm) on going from CHCl$_3$ to CH$_3$OH, suggesting almost equal degrees of exposure to solvent. A specific assignment is therefore unimportant for conformational arguments. On the contrary, in 2, the Met CO group experiences a substantially smaller downfield shift, suggesting its relative inaccessibility to solvent.\textsuperscript{37,38} The similarity of the solvent shifts for the formyl CO in 1 and 2 suggests that this group is exposed (not hydrogen-bonded) in both peptides. This observation strongly argues against the $\beta$-turn conformation [Fig. 7(a)] as a preferred structure for 2. The $^{13}$C results thus suggest that a y-turn (C$_y$) conformation involving a 3 $\rightarrow$ 1 hydrogen bond (Met CO--HN Phe) is consistent with both $^1$H- and $^{13}$C-nmr data.

The $^{13}$C chemical shifts of the CO carbons in 2 show a monotonic downfield shift with increasing CH$_3$OH concentration in CHCl$_3$–CH$_3$OH mixtures. The absence of discontinuous chemical-shift changes argues against solvent-induced conformational transitions.

**IR Studies**

Figure 8 shows the NH stretching region of the IR spectra of 1 and 2 in CHCl$_3$ solutions over the concentration range of 1.6–50 mM. At high concentrations of 1, two bands are observed at $-$3320 and $-$3430...
Fig. 8. The ir spectra (NH stretching bands) in CHCl₃ at various peptide concentrations indicated against the traces: (a) For-Met-Leu-Phe-OMe (1), (b) For-Met-Aib-Phe-OMe (2). The arrow marks the position of the solvent absorption band.

The absorption intensity corresponding to hydrogen-bonded [νNH(hb)] and free [νNH(f)] NH groups, respectively. The νNH(hb) peak disappears at concentrations < 6 mM, suggesting that peptide 1 exists as unassociated species below this concentration in CHCl₃. Furthermore, there is no evidence for formation of an intramolecular hydrogen bond, supporting the conclusions of the nmr studies. In peptide 2, the appearance of the NH bands is significantly different from the case of 1. At equivalent concentrations, the intensities of the 3320-cm⁻¹ band is less in 2 when compared with 1. Further, a third NH stretching peak is observed at 3385 cm⁻¹. While the 3320-cm⁻¹ peak disappears at low concentrations (< 10 mM), the 3385-cm⁻¹ band is largely concentration-independent and is observed even at 1.6 mM. This band may therefore be assigned to an intramolecularly hydrogen-bonded NH group. A plot of the ratio, νNH(hb)/νNH(f) [νNH(hb) = 3320 cm⁻¹], shows a steep concentration dependence above 2 mM in the case of 1. No such dependence is observed for 2 (not shown). Figure 9 shows the NH-absorption bands in the ir spectra of 1 and 2 for the solid state. In 1, only a single band is observed at -3290 cm⁻¹, suggesting that all NH groups are involved in intermolecular hydrogen bonding. In contrast, 2 exhibits a shoulder at
Fig. 9. NH stretching bands in the ir spectra of peptides in the aggregated state for (a) Nujol mull and (b) KBr pellet: ···, For-Met-Leu-Phe-OMe (1); —, For-Met-Aib-Phe-OMe (2).

3385–3390 cm\(^{-1}\), in addition to the low-frequency \(\nu_{\text{NH}}(\text{hb})\) peak at \(-3290\) cm\(^{-1}\) in both nujol mull and KBr pellet spectra. The ir results suggest that 2 adopts a folded conformation in solution, stabilized by an \textit{intramolecular} hydrogen bond and that this interaction is maintained in the solid state. The position of the intramolecularly hydrogen-bonded \(\nu_{\text{NH}}\) peak at 3385 cm\(^{-1}\) suggests a relatively weak H-H-\(\cdot\)-O interaction, consistent with a \(\gamma\)-turn (\(C_\gamma\)) conformation.\(^{30-32,39,40}\) In earlier studies of Aib-containing peptides, it has been observed that the stronger \(\beta\)-turn interaction is characterized by a \(\nu_{\text{NH}}\) band at lower frequency (\(-3340\) cm\(^{-1}\)).\(^{30-32}\) \(\nu_{\text{NH}}\) bands at 3380–3400 cm\(^{-1}\) have often been assigned to \(C_\gamma\) structures.\(^{30,31,39,40}\) It should, however, be noted that a range of strengths is possible for \(\beta\)-turn hydrogen bonds. The ir data alone cannot, therefore, be used to argue for a \(C_\gamma\) structure, but in conjunction with the \(^{13}\text{C-nmr}\) data, there is reasonable circumstantial evidence in favor of a \(\gamma\)-turn conformation.

\textbf{CD Studies}

There has been only a single report of a CD study of the parent chemotactic peptide, For-Met-Leu-Phe-OH in the solvents trifluoroethanol (TFE) and 96.4\% \(\text{H}_2\text{SO}_4\). This suggested the possibility of folded conformational states.\(^{41}\) Figure 10 shows the CD spectra of peptides 1, 2, For-Met-Leu-Leu-OMe (3), and For-Met-Aib-Leu-OMe (4) in various solvent systems. The CD parameters are summarized in Table 11. Peptides 3 and 4 were examined in order to evaluate the extent of Phe side-chain contribution to the observed CD bands.\(^{42}\) Peptides 1 and 3 exhibit a negative band at \(-227–235\) nm and a positive one at 210–217 nm in methanol, dioxane, and TFE. The positive and negative
Fig. 10. CD spectra of peptides in various solvents. (Left) For-Met-Leu-Phe-OMe (1) in (a) MeOH, (b) dioxane, (c) TFE; For-Met-Leu-Leu-OMe (3) in (d) MeOH and (e) dioxane. (Right) For-Met-AibPhe-OMe (2) in (a) MeOH, (b) dioxane, (c) TFE; For-Met-AibLeu-OMe (4) in (d) MeOH and (e) dioxane. Peptide concentration, $-2.2 \text{ mM}$.
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*a [σ]M is molar ellipticity expressed as deg cm2 dmol⁻¹.

b The peptide concentration in all cases is 1 mg/mL (~2.2 mM).
bands are significantly weaker in peptide 3, which lacks the Phe residue. While an altered backbone conformation cannot be ruled out, this observation suggests that aromatic side-chain contributions to the observed Cotton effect may be substantial. Qualitatively similar CD spectra are observed for peptide 2, and the differences in the spectra of 2 and 4 (Fig. 10) again suggest Phe side-chain contributions. While definitive conformational conclusions cannot be safely drawn from the CD data, it is possible to probe aggregation effects. Dioxane was chosen as a solvent in view of its transparency in the 200–250-nm region and its relatively poor solvating (hydrogen-bonding) power, which should promote molecular association. CH$_3$OH and CH$_3$OH–H$_2$O mixtures were also studied for comparison. Figure 11 shows the dependence of the ratio of the two CD bands on concentration for peptides 1 and 2. A dramatic solvent dependence is observed for 1 in dioxane, whereas no effect is seen for 2. The data suggest that 1 aggregates in dioxane at concentrations > 3 mM. No concentration effects are detectable for both peptides in methanol and methanol–water mixtures.

CONCLUSIONS

1. For-Met-Leu-Phe-OMe (1) and For-Met-Aib-Phe-OMe (2) aggregate in solvents of low polarity such as chloroform at concentrations > 2 mM. Aggregation effects are more pronounced for 1.
2. Both peptides appear to be unassociated in strongly polar, hydrogen-bonding solvents like dimethylsulfoxide and methanol. This behavior is concentration independent.

3. The concentration dependence of the spectroscopic data suggests that aggregation is a more facile process in an extended peptide conformation as exemplified by 1, as compared with a folded peptide exemplified by 2.

4. Peptide 1 favors nonintramolecularly hydrogen-bonded, presumably extended conformations in both low-polarity solvents and in polar solvents. The spectral data for 2 in chloroform and dimethylsulfoxide are consistent with a significant population of folded peptide conformations. A y-turn (C$_7$) conformation stabilized by a 3'→1 hydrogen bond between the Met CO and Phe NH groups is compatible with the reported spectroscopic results.

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References