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Role of Peptide Backbone Conformation on Biological Activity of Chemotactic Peptides*

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To investigate the role of peptide backbone conformation on the biological activity of chemotactic peptides, we synthesized a unique analog of *N*-formyl-Met-Leu-Phe-OH incorporating the $C^{\alpha,\alpha}$ disubstituted residue, dipropylglycine (Dpg) in place of Leu. The conformation of the stereochemically constrained Dpg analog was examined in the crystalline state by x-ray diffraction and in solution using NMR, IR, and CD methods. The secretagogue activity of the peptide on human neutrophils was determined and compared with that of a stereochemically constrained, folded type II β-turn analog incorporating 1-aminocyclohexanecarboxylic acid (Ac_6c) at position 2 (f-Met-Ac_6c-Phe-OMe), the parent peptide (f-Met-Leu-Phe-OH) and its methyl ester derivative (f-Met-Leu-Phe-OMe). In the solid state, the Dpg analog adopts an extended β-sheet-like structure with an intramolecular hydrogen bond between the NH and CO groups of the Dpg residue, thereby forming a fully extended (C5) conformation at position 2. The φ and ψ values for Met and Phe residues are significantly lower than the values expected for an ideal antiparallel β conformation causing a twist in the extended backbone both at the N and C termini. Nuclear magnetic resonance studies suggest the presence of a significant population of the peptide molecules in an extended antiparallel β conformation and the involvement of Dpg NH in a C5 intramolecular hydrogen bond in solutions of deuterated chloroform and deuterated dimethyl sulfoxide. IR studies provide evidence for the presence of an intramolecular hydrogen bond in the molecule and the antiparallel extended conformation in chloroform solution. CD spectra in methanol, trifluoroethanol, and trimethyl phosphate indicate that the Dpg peptide shows slight conformational flexibility, whereas the folded Ac_6c analog is quite rigid. The extended Dpg peptide consistently shows the highest activity in human peripheral blood neutrophils, being ~8 and 16 times more active than the parent peptide and the folded Ac_6c analog, respectively. However, the finding that all four peptides have ED_{50} (the molar concentration of peptide to induce half-maximal enzyme release) values in the 10^{-8}–10^{-9} M range suggests that an induced fit mechanism may indeed be important in this ligand-receptor interaction. Moreover, it is also possible that alterations in the backbone conformation at the tripeptide level may not significantly alter the side chain topography and/or the accessibility of key functional groups important for interaction with the receptor.

The discovery that formylmethionine- and formylmethionine-containing peptides were chemoattractants for polymorphonuclear leukocytes (Schiffmann *et al.*, 1975) initiated a series of studies aimed at identifying their structure-activity relationships (Showell *et al.*, 1976; Freer *et al.*, 1980). As a result, the tripeptide *N*-formyl-L-methionyl-L-leucyl-L-phenylalanine (f[1]-Met-Leu-Phe-OH) emerged as the prototypic chemotactic tripeptide. It has been demonstrated that the *N*-formyl-Met at position 1 and the Phe at position 3 are crucial for optimal activity, whereas alterations to the Leu at position 2 are well tolerated, provided the substituted amino acid contains a bulky, hydrophobic side chain group (Freer *et al.*, 1982). Formyl-Met-Leu-Phe-OH is considered a pan-activator of neutrophils (PMNs) as it stimulates a wide range of PMN functions from chemotaxis and lysosomal enzyme release to superoxide generation (Becker, 1987). These effects are mediated by specific cell surface receptors (Aswanikumar *et al.*, 1977; Williams *et al.*, 1977) and a membrane protein which binds *N*-formylpeptides has recently been cloned from HL-60 cells (Boulay *et al.*, 1990).

Based on NMR (Becker *et al.*, 1979) and x-ray diffraction data (Morffew and Tickle, 1981), a model which suggests an extended antiparallel β-sheet as the receptor-bound conformation of f-Met-Leu-Phe-OH (Freer *et al.*, 1982) has been proposed. Additional support for this hypothesis has come from several investigations (Bismara *et al.*, 1985; Valensin *et al.*, 1986; Toniolo *et al.*, 1989a). However, the flexibility of the parent peptide has been established (Bakir and Stevens, 1982; Edmundson and Ely, 1985; Gavuzzo *et al.*, 1989) and recent molecular modeling suggests that folded conformations of chemotactic peptides may be energetically favored (Semus *et al.*, 1988; Feller and Zimmerman, 1989). Furthermore, conformation-activity studies have revealed that the rabbit peritoneal PMN is able to recognize stereochemically constrained, folded chemotactic peptide analogs (Iqbal *et al.*, 1984; Sukumar *et al.*, 1985). These folded analogs are nearly as potent or more potent than the parent peptide, depending on the size of the hydrophobic side chain group at position 2, and the nature of the C terminus. Additional work has led to the

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[1] The abbreviations used are: f, formyl; Ac_6c, 1-aminocyclohexanecarboxylic acid; $(CH_3)_4Si$, tetramethylsilane; 2D COSY, two-dimensional correlated spectroscopy; Dpg, dipropylglycine; ED_{50}, molar concentration of peptide to induce half-maximal enzyme release; HPLC, high performance liquid chromatography; LDH, lactate dehydrogenase; NOE, nuclear Overhauser enhancement; PMN, polymorphonuclear neutrophilic leukocyte; TEMPO, 2,2,6,6-tetramethylpiperidine-1-oxyl; TFE, trifluoroethanol.

proposal of a folded type II β-turn as the receptor bound conformation (Raj, 1986; Toniolo *et al.*, 1989b). These findings formed the rationale for our present investigation in which we describe the biological activity of a stereochemically constrained, extended chemotactic peptide analog as compared to a folded type II β-turn analog in an attempt to assess the role of backbone conformation on secretagogue potency in the human peripheral blood neutrophil. The activity of the peptides suggests that the chemotactic peptide receptor in human neutrophils has a definite preference for an extended peptide ligand. The results also emphasize that the difference in the backbone conformation of the tripeptides may not significantly alter the topography of the side chains and accessibility of the functional groups for interaction with the receptor.

EXPERIMENTAL PROCEDURES

Selection of Peptides—*N*-Formyl-Met-Dpg-Phe-OMe was selected to generate a stereochemically constrained extended conformation based on theoretical and crystal structure studies which indicated that the Dpg residue is able to stabilize the extended backbone conformation (Benedetti *et al.*, 1984; Bonora *et al.*, 1984; Toniolo and Benedetti, 1988). *N*-Formyl-Met-Ac_6c-Phe-OMe was chosen for investigation since it has been found to be more active than the parent peptide in stimulating lysosomal enzyme release from rabbit peritoneal leukocytes, and its backbone conformational preference for a type II β-turn has been established (Toniolo *et al.*, 1989b). Formyl-Met-Leu-Phe-OH and its methyl ester derivative were also synthesized and studied because of their high biological activity and their crystal and solution conformational properties have previously been established (Bakir and Stevens, 1982; Raj and Balaram, 1985). All four peptides were used as conformational probes to study the influence of peptide backbone conformation on secretagogue activity.

Peptide Synthesis and Purification—All chemicals and solvents were of the highest purity available and used without further purification. Tertiarybutyloxycarbonyl-L-methionine was purchased from Bachem (Torrance, CA). Phe-OMe hydrochloride, 1-hydroxybenzotriazole, and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide were from Sigma. Ac_6c was obtained from Fluka Chemicals (Ronkonkoma, NY) and thionyl chloride, ethylcyanoacetate, and 1-bromopropane were from Aldrich Chemical Co. The peptides under study were synthesized as outlined in Fig. 1 by solution phase procedures using standard carbodiimide/1-hydroxybenzotriazole-mediated coupling (Konig and Geiger, 1970). The Dpg-OEt was synthesized as described by Hardy and Lingham (1983). The peptides were purified and analyzed on a Rainin Dynamax-60A reversed-phase C_{18} column (10 × 250 mm) coupled to a guard column (10 × 50 mm) employing an acetonitrile-water or methanol-water (each with 0.1% trifluoroacetic acid) linear gradient elution (flow rate: 2.0 ml/min) mode with detection at 230 and 240 nm. The HPLC trace of the purified Dpg analog is shown in Fig. 2. The purity of the peptides was also checked on thin-layer chromatography by three different solvent systems ($CHCl_3$/MeOH (95:5), *n*-1-butanol/acetic acid/H_2O (4:1:1), and $CHCl_3$/EtOH (9:1)) using iodine stain for detection. For biological assays the purified peptides were stored in glass ampules, sealed under nitrogen, and kept at −70 °C until the day of the assay.

X-ray Crystallographic Analysis—Single crystals of *N*-formyl-L-Met-Dpg-L-Phe-OMe were grown by slow evaporation from a mixture of benzene and hexane, $C_{24}H_{37}N_3O_5S \cdot 2/3(C_6H_6)$ fw = 531.6. The tripeptide crystallizes in an orthorhombic unit cell with dimensions of a = 12.204 Å, b = 20.226 Å, c = 23.961 Å; space group I222. There are eight peptide molecules per unit cell of volume 5914.8 $Å^3$. X-ray diffraction data was collected on an Enraf-Nonius CAD4 diffractometer equipped with a graphite monochromator using CuK^{α} radiation (1 = 1.54 Å), and all calculations were performed on a VAX computer using SDP/VAX (Frenz, 1985). The crystal structure was solved by the application of direct methods using SHELXS-86 (Sheldrick, 1985) and successive weighted Fourier maps. Full-matrix least squares were used for refinement, with atoms being treated isotropically in the initial stages and with anisotropic parameters in the final stages of refinement. Hydrogen atoms were not included in the calculations. The final weighted *R* factor for 2586 reflections where I > 2s was 0.085. The structure shown in Fig. 3*A* was plotted with the ORTEP computer program (Johnson, 1965). (A list of atomic coordinates and structure factor tables are available upon request.)

Spectroscopic Studies—The 500-MHz ^1H and 125-MHz ^{13}C NMR spectra were recorded using a Varian VXR-500 NMR spectrometer equipped with a SUN 3/110 computer. The concentration of peptide used for the two-dimensional correlated spectroscopy (2D COSY) and difference nuclear Overhauser enhancement (NOE) studies were 25 and 10 mM in $(CD_3)_2SO$ and $CDCl_3$, respectively. Peptide concentration for all other NMR studies was 2 mM. In the NOE studies, the perturbed and normal spectra were recorded sequentially in different parts of the memory, each with 16 K data points. The perturbed and normal spectra were obtained with low-power on-resonance saturation of a peak and by off-resonance shifting of the irradiation frequency, respectively. IR spectra were recorded on a Bio-Rad FTS-40 spectrometer. Solutions of the peptide were prepared in dry $CHCl_3$ and spectra recorded using pathlengths from 1 to 5 mm. CD spectra were recorded on a Jasco J-600 spectropolarimeter interfaced with an IBM PS/2 microcomputer with measurements carried out at 22 °C using a 0.1-mm pathlength and 2 mM peptide concentrations.

Neutrophil Isolation—Whole blood was collected by venipuncture from healthy human volunteers and diluted (6:1) in acid citrate dextrose anticoagulant. The neutrophils (PMN) were isolated using Histopaque gradients followed by dextran sedimentation and hypotonic lysis as described by Metcalfe *et al.*, 1986. PMN were washed twice and resuspended in Dulbecco's phosphate-buffered saline with 0.1% bovine serum albumin and 0.1% glucose, pH 7.3. Cell purity was assessed by Wright-Giemsa stain and was routinely 95–97% PMN.

Enzyme Release Assays—Peptide-induced degranulation (release of β-glucuronidase) was assessed as a measure of biological activity. Lactate dehydrogenase (LDH) released from the cytoplasm was measured to determine cell viability at the end of each assay. This value never exceeded 5% of the total LDH. The secretagogue activity of each peptide was tested at eight different concentrations using a timed assay. Briefly, PMNs (1 × 10^7/ml) in Dulbecco's phosphate-

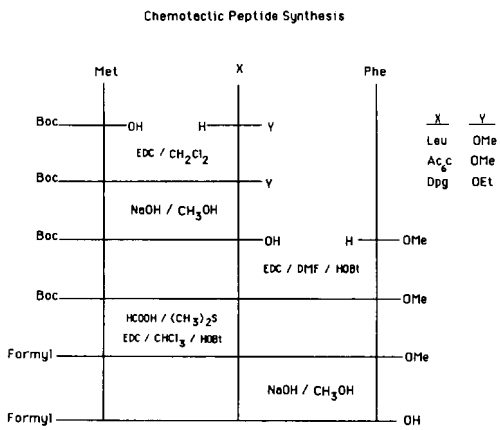

FIG. 1. **Scheme employed for solution-phase synthesis of *N*-formylated chemotactic peptides.** 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide (*EDC*)/1-hydroxybenzotriazole (*HOBt*) mediated coupling procedures were employed using methylene chloride (CH_2Cl_2) and *N*,*N*-dimethyl formamide (*DMF*) to solubilize Boc-amino acids and Boc-protected dipeptides, respectively.

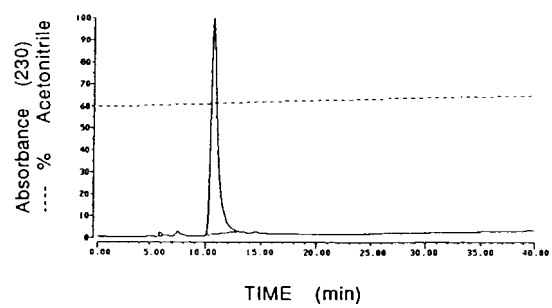

FIG. 2. **HPLC trace of the purified f-Met-Dpg-Phe-OMe on a Rainin Dynamax-60A reversed phase C_{18} column (10 × 250 mm) using acetonitrile and water as the solvent system (flow rate: 2 ml/min).** The peptide (100 μg) was loaded into an injection loop of 100-μl capacity and detected at 230 nm. The gradient used is shown as a *dashed line*.

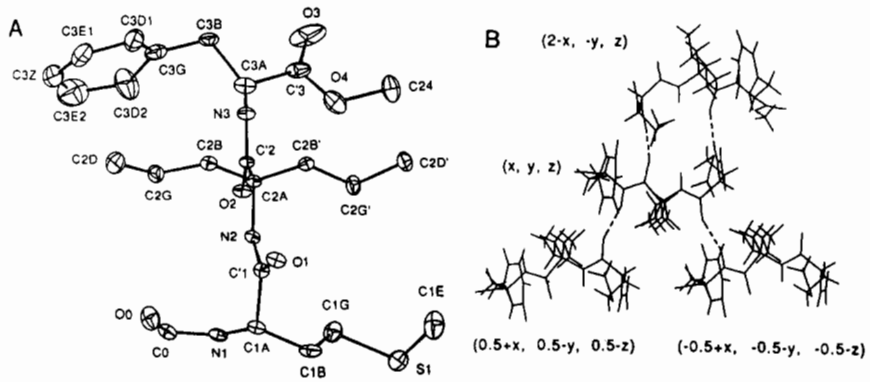

FIG. 3. *A*, a perspective view of the molecular conformation of f-Met-Dpg-Phe-OMe showing the atomic numbering scheme used. Hydrogen atoms are not included in the fig. for clarity. *B*, the intermolecular association of the peptide molecules involving the asymmetric unit (x, y, z) as viewed from the crystallographic c axis. The intermolecular hydrogen bonds (*dashed lines*) formed between Met NH (N1) and Dpg CO (O2) groups ($2 - x$, $-y$, z) of symmetry related molecules forming antiparallel β-strands and between Phe NH (N3) and Met CO (O1) groups ($-0.5 + x$, $-0.5 - y$, $-0.5 - z$, and $0.5 + x$, $0.5 - y$, $0.5 - z$) of symmetry-related molecules giving rise to parallel β-strands are shown.

buffered saline with cytochalasin B (final assay concentration of 5 μg/ml) were aliquoted (0.75 ml) into 1.75-ml polypropylene tubes containing 0.6 ml of Dulbecco's phosphate-buffered saline and allowed to equilibrate at 37 °C in a shaking water bath for 5 min. The peptide stimulus was added (0.15 ml) and the mixture incubated for 20 min before being placed on ice and subsequently centrifuged at 800 × *g* for 5 min at 4 °C to pellet the cells. PMN supernatants were then tested in duplicate for the presence of these enzymes as previously described (Metcalfe *et al.*, 1986). Maximum stimulated release was calculated as a percentage of the total enzyme present. Total β-glucuronidase and LDH was determined by adding 0.3% (v/v) Triton X-100 and vortexing for 2 min prior to centrifugation. Controls run for each experiment included spontaneous release (no peptide stimulus) and total release of both enzymes in the presence and absence of cytochalasin B, and spontaneous release at the highest $(CH_3)_2SO$ concentration run in the experiment. This value never exceeded 0.5% $(CH_3)_2SO$. To control for donor variability and to get a relative measure of their activity all four peptides were tested in each experiment and their potency relative to the parent peptide calculated as ED_{50} f-Met-Leu-Phe-OH/ED_{50} test peptide. Pilot studies were run where total enzyme release was assessed in the presence and absence of high concentrations of each peptide (10 μM) to insure that the peptides were not interfering with the detection of enzyme activity. Additionally, kinetics experiments revealed that the degranulation response was essentially complete by 5 min; however, we chose a 20-min incubation time for convenience since the spontaneous release of both LDH and β-glucuronidase did not increase significantly over this time interval. Dose-response curves were generated from each experiment using the Sigma-Plot program (Jandel Scientific Corp., Corte Madera, CA) and the ED_{50} values taken directly from the graphs. (See Fig. 11 for a composite graph from five experiments showing the average values of enzyme release for each peptide at eight different concentrations.) The *error bars* represent the standard error of the means. A third order polynomial gave the best fit to these data with R values above 0.99 for each of the four curves shown.

RESULTS AND DISCUSSION

Molecular Structure

The molecular structure of f-Met-Dpg-Phe-OMe is shown in Fig. 3*A* and the relevant torsional angles are provided in Table I. The peptide molecules assume an extended β conformation with a twist both at the N and C termini of the peptide backbone. The Dpg residue at position 2 has the ϕ and ψ values of 173.1° and 179.0°, respectively, and adopts an extended conformation with a short contact between the NH and CO groups giving rise to a C5 ring structure (N2H- - -O2 = 2.074 Å; τ angle (N2-C2A-C'2) = 105.4°) (Toniolo, 1980, 1989). Similar C5 structures have been observed for the Dpg residue in the crystal structures of homooligopeptides containing dipropylglycines (Benedetti *et al.*, 1984; Toniolo and Benedetti, 1988). The ϕ and ψ values for the Met and Phe residues (Table I) are much lower than the ϕ and ψ values (−130°, ±135°, respectively) observed for oligopeptides exhibiting ideal extended β-sheet structures (Karle *et al.*, 1983, 1988). The lower ϕ and ψ values of the Met and Phe residues cause a twist in the extended backbone of the peptide. Twisted antiparallel β-sheet structures have been observed in the crystals of oligopeptides containing L-cystine (Karle *et al.*, 1989; Raj *et al.*, 1990) and in globular proteins (Chothia, 1973; Salemme and Weatherford, 1981; Richardson, 1981). Interestingly, the symmetry of the Dpg residue results in the disposition of a hydrophobic propyl chain adjacent to both the Met and Phe side chain groups even though the Met and Phe side chains lie on opposite sides of the peptide backbone.

TABLE I

Relevant torsional angles (°) for f-Met-Dpg-Phe-OMe

The torsional angles for rotation about bonds of the peptide backbone (ϕ, ψ, ω) and side chains (χ^1, $\chi^{1'}$, χ^2, $\chi^{2'}$, χ^3) are described as in IUPAC-IUB Commission on Biochemical Nomenclature (1970).

	Met	Dpg	Phe
ϕ	−75.8 (6)[a]	173.1 (4)	−96.7 (6)
ψ	−30.4 (6)	179.0 (4)	33.6 (6)
ω	178.9 (4)	173.1 (4)	−178.3 (6)[b]
χ^1	57.4 (6)	−62.3 (5)	−58.7 (7)
$\chi^{1'}$		53.2 (5)	
χ^2	178.2 (4)	176.8 (6)	106.5 (9)
$\chi^{2'}$		175.4 (5)	−65 (1)
χ^3	−74.6 (7)		

[a] Estimated standard deviations are in parentheses.
[b] With 04C24 of the carboxymethyl group.

The hydrogen bonding pattern between the tripeptide molecules is shown in Fig. 3*B* and the geometry of the hydrogen bonds is provided in Table II. There are four intermolecular hydrogen bonds involving the asymmetric unit (x, y, z). Association of the peptide molecules in the crystal is characterized by a network of intermolecular hydrogen bonds formed between Met NH (N1) and Dpg CO (O2) groups ($2 - x$, $-y$, z) of symmetry related molecules forming antiparallel β-strands and between Phe NH (N3) and Met CO (O1) groups ($-0.5 + x$, $-0.5 - y$, $-0.5 - z$, and $0.5 + x$, $0.5 - y$, $0.5 - z$) of symmetry related molecules giving rise to parallel β-strands. The N1- - -O2 and N3- - -O1 distances, 2.854 and 2.915 Å,

TABLE II

Geometry of the hydrogen bonds observed in the f-Met-Dpg-Phe-OMe crystal structure

D	H	A	D-H	D...A	H...A	D-H...A	Code[a]
				Å		°	
N1	N1H2	O2	0.951	2.854	1.944	159.2	1
N3	N3H24	O1	0.972	2.915	2.000	156.0	2

[a] (1) N1H2 (x, y, z)...O2 ($2-x, -y, z$) and N1H2 ($2-x, -y, z$)...O2 (x, y, z); (2) N3H24 (x, y, z)···O1 ($0.5+x, 0.5-y, 0.5-z$) and N3H24 ($-0.5+x, -0.5-y, -0.5-z$)···O1 (x, y, z).

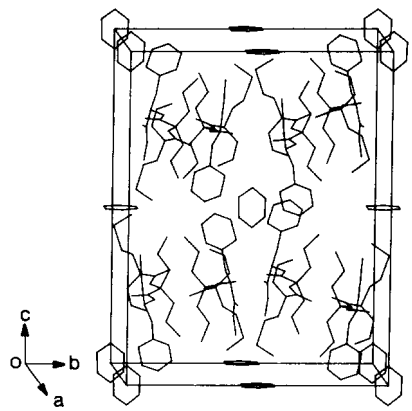

FIG. 4. **Crystal packing of f-Met-Dpg-Phe-OMe and benzene solvent molecules in the unit cell.** Eight peptide molecules in the orthorhombic unit cell are shown along with the benzene solvent molecules. Alternating hydrophobic and hydrophilic channels exist within the lattice along both the crystallographic a and c axes.

respectively, agree well with the average value observed in the crystals of chemotactic peptide analogs (Eggleston, 1988, 1990; Gavuzzo *et al.*, 1989). It is quite unusual to observe that the Dpg CO group which shows a short intramolecular NH- - -O contact with the Dpg NH group, is also involved in intermolecular association. The much lower ϕ and ψ angles observed for Met (−75.8°, −30.4°) and Phe (−96.7°, 33.6°) residues in the crystal structure may presumably be due to the involvement of the NH groups of these residues in strong intermolecular hydrogen bonding within the lattice structure. The twist observed in the peptide backbone can be attributed to these intermolecular hydrogen bonds as well as other hydrophobic/hydrophilic crystal packing forces.

Crystal Packing

The crystal packing of peptide and benzene solvent molecules in the unit cell is shown in Fig. 4. Alternating hydrophilic and hydrophobic channels exist within the lattice along both the crystallographic a and c axes. At the edges and at the center of the unit cell, there is a clustering of aromatic rings from the Phe side chains and the benzene molecules along the a axis, which results in the formation of aromatic channels passing through the lattice in this direction. In contrast, hydrophilic channels exist above and below the central aromatic channel along the a and c axes where parallel and antiparallel hydrogen bonds are formed between adjacent peptide molecules.

NMR Studies in Solution

Assignment of Resonances—The 500 MHz ^{1}H- and 125-MHz ^{13}C NMR spectra of the Dpg analog shown in Fig. 5 are fully consistent with its primary structure. The singlet resonance that occurs at 8.1–8.2 ppm both in $CDCl_3$ and $(CD_3)_2SO$ was recognized as the formyl proton by its characteristic chemical shift while the Dpg NH resonance was unambiguously recognized as the other singlet resonance at 7.38 ppm in $(CD_3)_2SO$. In $CDCl_3$, it was buried under the aromatic resonances, and it was assigned by solvent titration experiment (Fig. 6*A*). The Met and Phe doublet resonances in these solvents were assigned by spin decoupling experiments. In $(CD_3)_2SO$, the spin system connectivities of NH, $C^\alpha H$, $C^\beta H_2$, $C^\gamma H_2$, and $C^\delta H_3$ resonances were also established from the 2D COSY spectrum (Fig. 7).

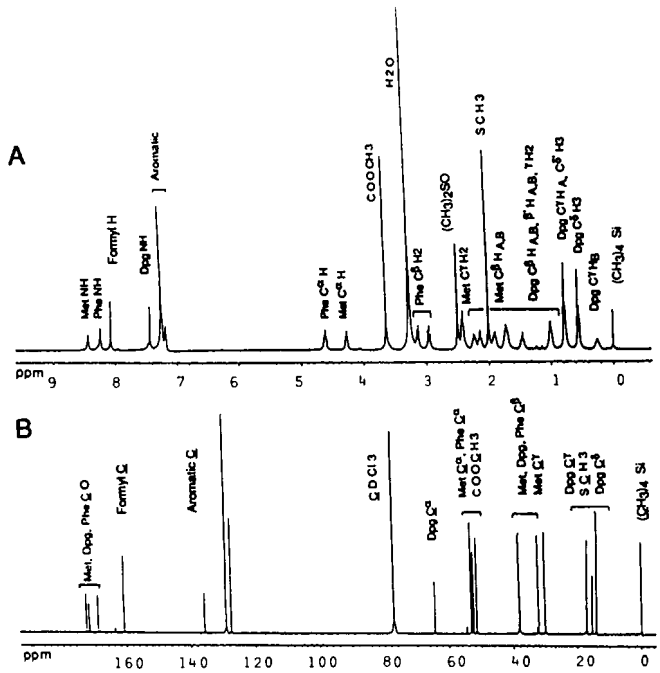

FIG. 5. **500 MHz ^{1}H (*A*) and 125 MHz ^{13}C (*B*) NMR spectra of f-Met-Dpg-Phe-OMe (2 mM) in $(CD_3)_2SO$ at 25 °C.**

Delineation of Hydrogen-bonded NH Groups—The involvement of NH groups in intramolecular hydrogen bonding was probed using solvent and temperature-induced NH chemical shifts, paramagnetic radical-induced line broadening, and hydrogen-deuterium exchange effects on NH resonances (Kopple and Schamper, 1972; Ohnishi and Urry, 1972; Pitner and Urry, 1972; Wuthrich, 1976; Kessler, 1982). The results are summarized in Fig. 6 and ^{1}H NMR parameters are provided in Table III. A peptide concentration of ~2 mM was used to avoid the influence of peptide aggregation on the ^{1}H NMR parameters (Raj and Balaram, 1985). In the solvent titration experiment (Fig. 6*A*), the Met and Phe NH resonances show substantial downfield shifts (2.1 and 2.2 ppm, respectively) with increasing concentrations of $(CD_3)_2SO$, while the Dpg NH resonance shows only a slight downfield shift of 0.3 ppm (Table III). In $(CD_3)_2SO$, the temperature coefficient ($d\delta/dT$) of the Dpg NH is quite low (0.0009 ppm/K) indicating solvent-shielding of this NH group, whereas the Met and Phe NH groups show relatively high $d\delta/dT$ values of 0.0048 and 0.0054 ppm/K, respectively, suggestive of NH groups which are freely accessible to the solvent (Fig. 6B). Likewise, addition of the paramagnetic radical probe 2,2,6,6-tetramethylpiperidine-1-oxyl (TEMPO) to the peptide in $CDCl_3$ solution results in a dramatic broadening of the Met and Phe NH resonances, whereas the Dpg NH resonance is much less affected (Fig. 6*C*). Furthermore, the rate of deuterium-hydrogen exchange in $(CD_3)_2SO$ is substantially faster for the Met and Phe NH resonances than for the Dpg NH resonance (Fig. 6*D*). These findings suggest that both the Met and Phe NH groups are freely accessible to the solvent and not involved in hydrogen bonding while the Dpg NH is inaccessible to the solvent and

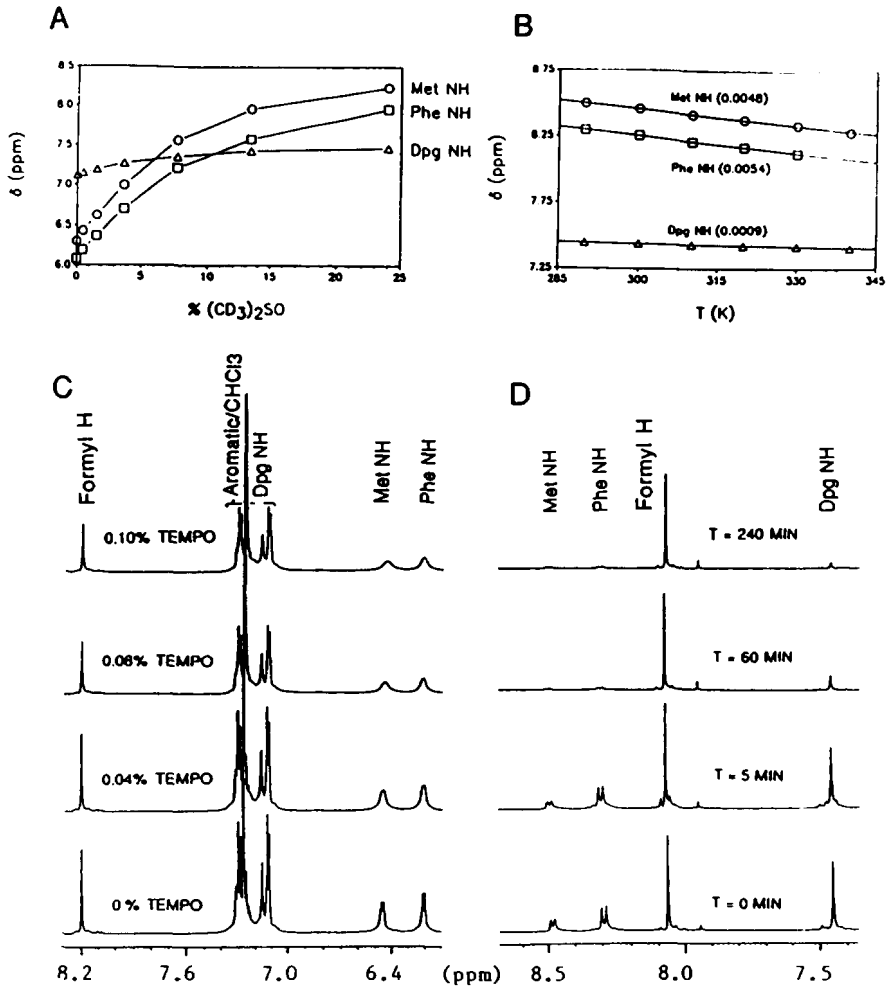

FIG. 6. **Delineation of hydrogen-bonded NH groups in f-Met-Dpg-Phe-OMe.** *A*, solvent dependence of NH chemical shifts in $CDCl_3$-$(CD_3)_2SO$ mixtures as a function of solvent concentration. *B*, temperature dependence of NH chemical shifts in $(CD_3)_2SO$. The temperature coefficients, $d\delta/dT$ (ppm/K) of the NH resonances are shown in the *traces*. *C*, line-broadening of NH resonances with increasing concentration of TEMPO radical in $CDCl_3$ containing 0.6% $(CD_3)_2SO$. TEMPO concentration is indicated on the *traces*. *D*, the effect of hydrogen-deuterium exchange on NH resonances in $(CD_3)_2SO$. Time intervals after the addition of 3% D_2O (v/v) are indicated on the *traces*. A peptide concentration of 2 mM was used in all experiments.

is presumably hydrogen bonded both in $(CD_3)_2SO$ and $CDCl_3$. This shielding is consistent with the crystal structure analysis which has established the intramolecular short contact between the Dpg NH and the Dpg CO groups as part of a C5 ring structure. The $J_{NH\text{-}C^{\alpha}H}$ values (7.6–8.1 Hz) observed for Met and Phe residues in $CDCl_3$ and $(CD_3)_2SO$ shown in Table III suggest values for ϕ of $\sim -90°$ and $\sim -150°$ (Karplus 1963; Pardi *et al.*, 1984). Both these values and the absence of intramolecular hydrogen bonding of the Met and Phe NH resonances provide evidence in favor of an extended peptide backbone structure in $(CD_3)_2SO$ and $CDCl_3$. In addition, the ϕ value (−90°) for the Phe residue deduced from NMR data is in close agreement with the value obtained from the crystal structure. Furthermore, the unusual chemical shift difference between the Dpg $C^{\beta}H_A$ and $C^{\beta}H_B$ in $CDCl_3$ and $(CD_3)_2SO$ (~1 ppm) as well as $C^{\gamma}H_A$ and $C^{\gamma}H_B$ resonances in $(CD_3)_2SO$ (Fig. 5*A*) suggests a ring current effect from the adjacent Phe side chain (Bovey *et al.*, 1988). This is also consistent with the crystal structure (Fig. 3*A*) where the Phe side chain is in a position to exert a substantial shielding effect on these protons. Taken together, these data indicate that the Dpg analog has a preference for an extended β structure in solvents of widely varying polarities and hydrogen bonding capabilities and the solution conformation is in good agreement with the crystal structure.

NOE Studies

NOE studies were carried out to provide additional information on the backbone conformation (Rao *et al.*, 1983). Extended antiparallel β structures can be recognized by the observation of NOEs between the $C_i^{\alpha}H$ and $N_{i+1}H$, since the distance between these nuclei is <3 Å (Kuo and Gibbons, 1980; Billeter *et al.*, 1982). The results are summarized in Table IV. Fig. 8 shows a representative one-dimensional difference NOE spectra in $CDCl_3$ where the Phe NH has been irradiated and an intensity enhancement of 3.6% for Dpg $C^{\beta}H_B$ and 3.0% for the $C^{\beta'}H_B$ is observed. Irradiation of the Dpg NH resonance gives rise to 4.1% ($(CD_3)_2SO$) and 7.8% ($CDCl_3$) of NOEs on the Met $C^{\alpha}H$ resonance. In the reverse experiment, the irradiation of the Met $C^{\alpha}H$ gives rise to NOEs on the Dpg NH (Table IV). NOEs are also observed between the formyl proton and the Met NH both in $CDCl_3$ and $(CD_3)_2SO$, suggesting that the formyl H and Met NH are in *cis* geometry as observed in the crystal structure (Fig. 3*A*). The observed interresidue NOEs are consistent with an extended antiparallel β conformation where NOE connectivities are expected between the formyl H and Met NH, between the Met $C^{\alpha}H$ and Dpg NH, and between Phe NH and Dpg $C^{\beta}H$, $C^{\beta'}H$ (Kuo and Gibbons, 1980; Billeter *et al.*, 1982). A graphic representation of the through-space connectivities and the proposed antiparallel β conformation shown in Fig. 8 are consistent with the NOE data in $CDCl_3$ and $(CD_3)_2SO$ solutions.

Fourier Transform Infrared Studies

The NH and CO stretching regions in the IR spectra of the Dpg analog in $CHCl_3$ solutions are shown in Fig. 9. Even at low concentrations (3×10^{-4} M) the peptide shows a weak band at 3359/cm (Fig. 9*A*) which is characteristic of a hydrogen bonded NH in addition to the free NH band at 3427/cm

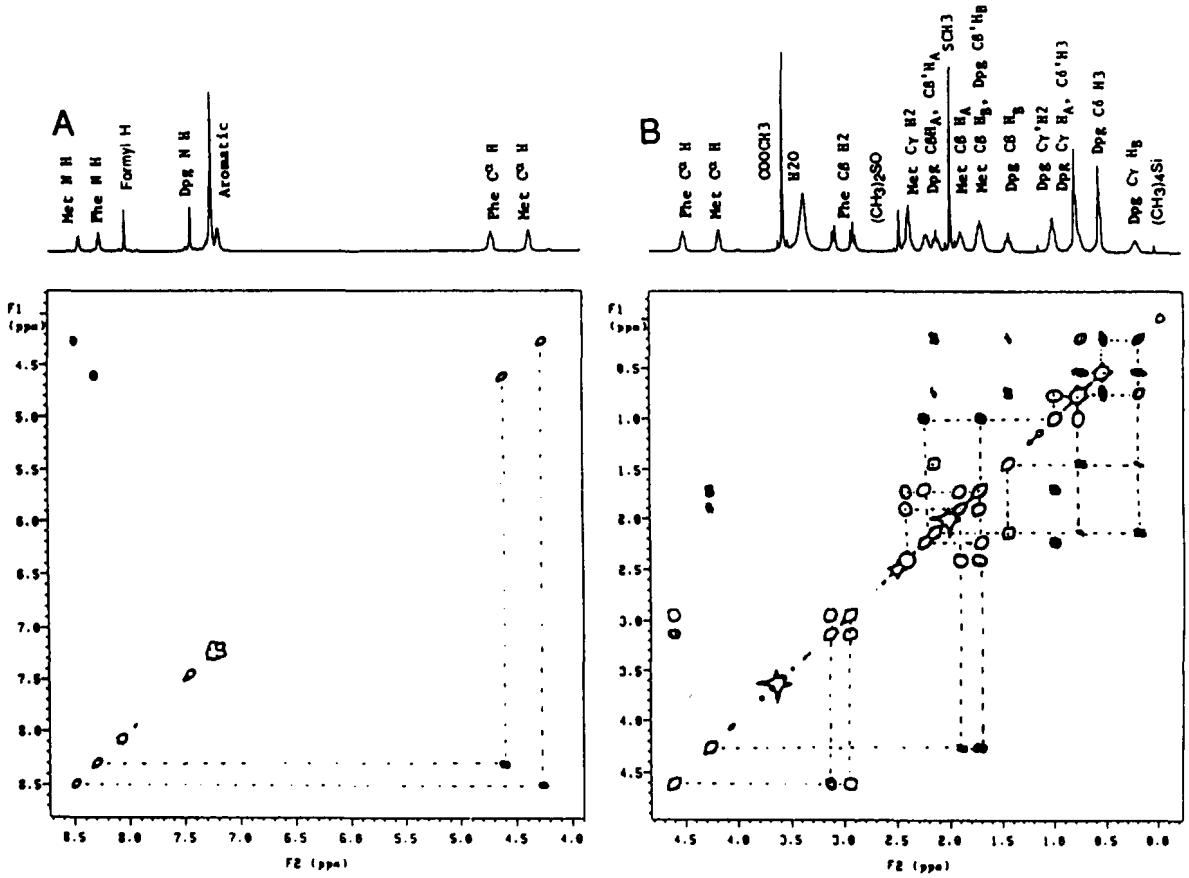

FIG. 7. **500 MHz ^{1}H NMR 2D COSY contour plot of f-Met-Dpg-Phe-OMe in $(CD_3)_2SO$ with spin system connectivities.** *A*, NH-C$^\alpha$H connectivities, 3.9–8.7 ppm; *B*, C$^\alpha$H-C$^\beta$H, C$^\gamma$H, and C$^\delta$H connectivities, 0–4.8 ppm (illustrated by *dashed lines*).

TABLE III

NH group[a] NMR parameters for f-Met-Dpg-Phe-OMe

Parameters[b]	Met-NH[c]	Dpg-NH	Phe-NH
$\Delta\delta$(ppm) = $\delta[(CD_3)_2SO] - \delta(CDCl_3)$	2.1	0.3	2.2
$d\delta/dT$(ppm/K) $(CD_3)_2SO$	0.0048	0.0009	0.0054
$J_{NH-C^\alpha H}$ (Hz) $CDCl_3$	7.8		7.8
$J_{NH-C^\alpha H}$ (Hz) $(CD_3)_2SO$	7.6		8.1

[a] Peptide concentration = ~2 mM.
[b] Assignments made as described in the text.

TABLE IV

One-dimensional difference NOE data[a] for f-Met-Dpg-Phe-OMe

Irradiated	Observed	NOE	
		$CDCl_3$	$(CD_3)_2SO$
		%	
Formyl H	Met NH	4.0	0.1
Met NH	Formyl H	3.7	8.7
Met C$^\alpha$H	Dpg NH	4.1[b]	1.5
Dpg NH	Met C$^\alpha$H	7.8[b]	4.1
Phe NH	DpgC$^\beta$H$_B$	3.6	2.2
	DpgC$^{\beta'}$H$_B$	3.0	1.6

[a] Only interresidue NOEs are tabulated.
[b] The percentage NOEs between the Met C$^\alpha$H and Dpg NH have been assessed in $CDCl_3$ with 0.6% $(CD_3)_2SO$, as the NH resonance in the off-resonance spectrum in $CDCl_3$ is buried under the aromatic resonances.

(Bonora *et al.*, 1984). The band observed at 3359/cm even at low concentrations suggests the presence of a weak intramolecular hydrogen bond in the peptide molecule (Bardi *et al.*, 1985; Raj *et al.*, 1988), which is consistent with both the crystal structure and the NMR data. In the carbonyl-stretching region (Fig. 9*B*) the weak band observed at 1692/cm provides further support for an extended structure (Miyazawa, 1967). However, an exciton split of only 29/cm is observed at high concentrations suggesting that β-sheet structures formed by aggregation of the peptide molecules are not fully antiparallel. The exciton split is increased to 45/cm at low concentration presumably due to the formation of a more orderly extended conformation of the peptide backbone (Krimm and Bandekar, 1986).

CD Studies

Circular dichroism studies were carried out on both the Dpg peptide and the Ac$_6$c peptide in methanol (MeOH), trifluoroethanol (TFE), and trimethyl phosphate (TMP) in an attempt to assess the solvent dependent conformational change of the peptide backbone. The CD spectra of the Dpg and Ac$_6$c analogs recorded between 175 and 250 nm in these solvents are shown in Fig. 10, and the CD parameters are summarized in Table V. Though CD is a sensitive technique for the prediction of secondary structure of polypeptides (Beychok, 1967), the limitations on the use of CD for conformational analysis of small linear peptides with aromatic residues, such as those studied here, have been reported (Toniolo and Bonora, 1976; Toniolo *et al.*, 1989a). However, CD studies do provide useful information on the relative conformational flexibility of these two constrained peptides. The Dpg peptide exhibits a weak positive CD band at ~215–220 nm (Fig. 10*A*) and an intense negative band at ~190–195 nm in MeOH and TMP. In TFE, it exhibits a positive shoulder at ~213 nm, a

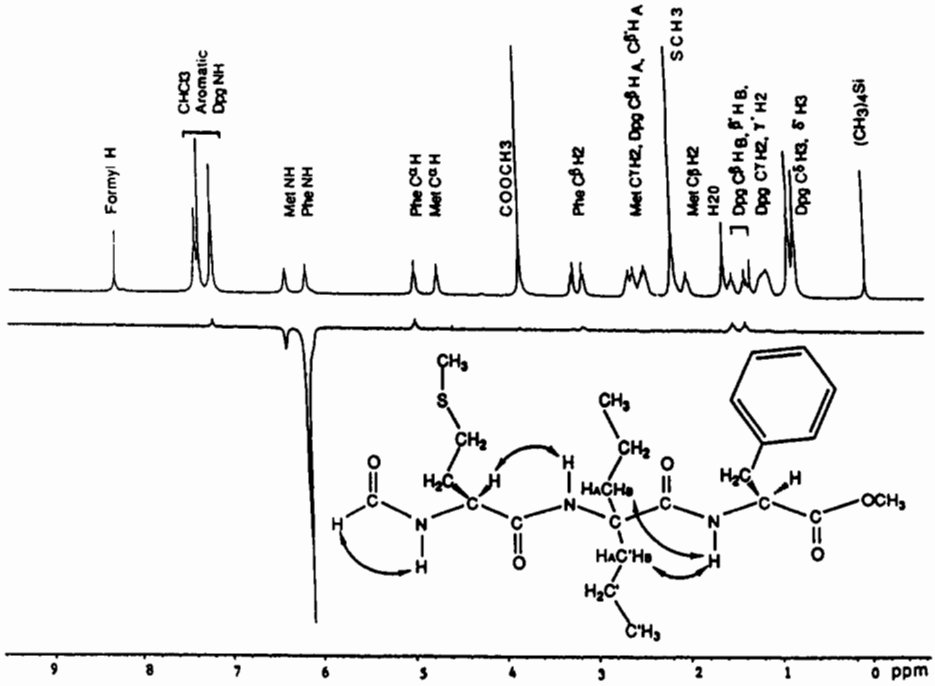

FIG. 8. **500 MHz ^{1}H NMR off-resonance spectrum of f-Met-Dpg-Phe-OMe in $CDCl_3$ (*top*).** The NOEs observed on Phe $C^{\alpha}H$, Dpg $C^{\beta}H_B$, and $C^{\delta'}H_B$ resonances are shown in the difference NOE spectrum (*bottom*) obtained by saturation of Phe NH. The proposed antiparallel β conformation of the peptide in solution is also shown. The *arrows* indicate the protons that give rise to interresidue NOEs.

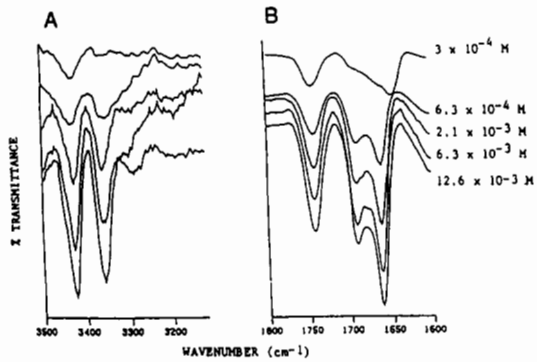

FIG. 9. **Partial IR spectra of f-Met-Dpg-Phe-OMe in dry $CHCl_3$ at various concentrations indicated against the *traces*.** *A*, NH stretching region showing free NH bands at 3420/cm and hydrogen-bonded NH band at 3359/cm. *B*, CO stretching region showing the strong bands at 1745 and ~1663/cm and a weak band at 1692/cm. Note the shift in the strong band from 1663 to 1647/cm as the peptide concentration is decreased to 3×10^{-4} M.

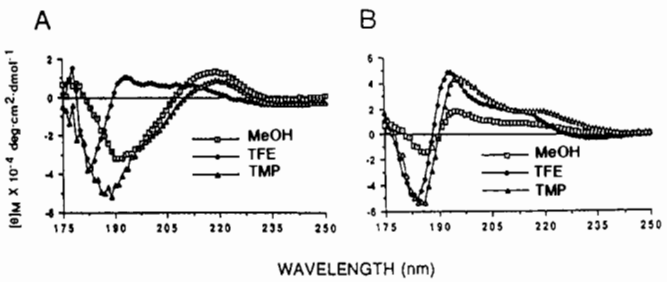

FIG. 10. **CD spectra of stereochemically constrained formylpeptides.** f-Met-Dpg-Phe-OMe (*A*) and f-Met-Ac_6c-Phe-OMe (*B*) in TFE, trimethyl phosphate (*TMP*), and methanol (*MeOH*) at 2 mM peptide concentration.

positive maximum at ~193 nm, and a strong negative minimum at ~183 nm. The reported CD spectrum of the parent chemotactic peptide, f-Met-Leu-Phe-OH which has been established to adopt an extended β conformation in the solid state by x-ray diffraction and in solution by NMR is quite

TABLE V

CD[a] parameters for chemotactic peptide analogs

λ (nm) $[\theta]_M$[b]	MeOH	TMP	TFE
f-Met-Dpg-Phe-OMe			
λ (nm)	219	220	213
$[\theta]_M$	+13051	+8551	+6121
λ (nm)	192	189	193
$[\theta]_M$	−32193	−52353	+10048
λ (nm)			183
$[\theta]_M$			−36248
f-Met-Ac_6c-Phe-OMe			
λ (nm)	209	220	212
$[\theta]_M$	+9041	+17524	+18892
λ (nm)	196	196	194
$[\theta]_M$	+18151	+44257	+47948
λ (nm)	186	186	184
$[\theta]_M$	−14047	−53845	−51031

[a] Peptide concentration: ~2 mM.

[b] $[\theta]_M$ is molar ellipticity expressed as deg cm^2 dmol^{-1}.

similar in TFE (Bakir and Stevens, 1982). The folded Ac_6c analog shows two weak shallow positive bands between 194 and 220 nm in all three solvents with a maximum at ~196 and a strong negative band at ~186 nm. Similar CD spectra observed for the pentapeptide Tyr-Pro-Gly-Asp-Val in water (Dyson *et al.*, 1988) and for pivaloyl-L-prolyl-α-aminoisobutyryl-*N*-methylamide in MeOH (Crisma *et al.*, 1984) have been interpreted in terms of a β-turn conformation, which is consistent with the previously established β-turn structure of f-Met-Ac_6c-Phe-OMe (Toniolo *et al.*, 1989b). Even though it is not safe to relate CD spectra of linear oligopeptides in terms of any specific conformation, it appears from CD studies that the stereochemically constrained, extended Dpg peptide shows some conformational flexibility, whereas the folded Ac_6c analog appears quite rigid.

Biological Activity Studies

The peptides were examined for their ability to induce the release of β-glucuronidase from cytochalasin B-treated human peripheral blood neutrophils. The dose-response curves for β-glucuronidase release and ED_{50} values (the molar concentra-

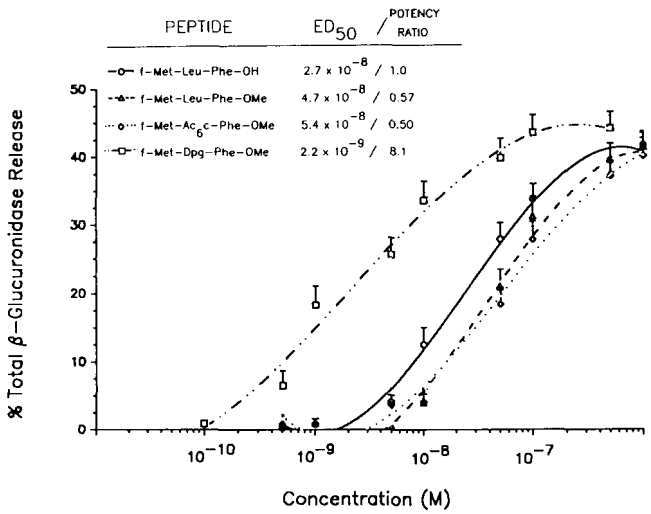

FIG. 11. **Dose-response curves generated using the SigmaPlot program (Jandel Scientific Corp., Corte Madera, CA) for formylpeptide-induced β-glucuronidase release from human neutrophils.** ED_{50} values taken from the curves and the potency ratio (ED_{50} of f-Met-Leu-Phe-OH/ED_{50} of test peptide) are also shown. The *error bars* represent the standard error of the means calculated from the percentage release values of five separate experiments. A third order polynomial gave the best fit to these data with R values above 0.99 for each of the four curves shown.

tion of the peptide which would induce half-maximal enzyme release) are provided in Fig. 11. The maximum formylpeptide-induced release of β-glucuronidase ranged from 40 to 45% of the total amount of enzyme detectable with f-Met-Dpg-Phe-OMe consistently showing 3–5% higher maximum release values than the other three peptides tested. In addition to its slightly higher efficacy, f-Met-Dpg-Phe-OMe was consistently the most potent secretagogue for the human PMN, being ~8- and 16-fold more active than the parent peptide and the folded Ac_6c analog, respectively. We also found f-Met-Ac_6c-Phe-OMe and f-Met-Leu-Phe-OMe to be consistently less active (~0.5 ×) than the parent peptide (f-Met-Leu-Phe-OH). It is worth mentioning that these same two peptides have been shown to be ~5 × more active than the parent peptide, when tested on rabbit peritoneal neutrophils (Sukumar *et al.*, 1985; Toniolo *et al.*, 1989b). The ED_{50} observed for f-Met-Leu-Phe-OMe (Fig. 11) is in good agreement with a previously published report (Belleau *et al.*, 1989). It is noteworthy that for these tripeptides the simple conversion of the C-terminal functional group from a carboxylic acid to the methyl ester results in a loss of secretagogue activity in human peripheral blood neutrophils. The finding that f-Met-Ac_6c-Phe-OMe is equipotent to f-Met-Leu-Phe-OMe suggests that the replacement of the Leu side chain at position 2 by a cyclohexane ring does not substantially alter peptide activity.

Conformation and Biological Activity

Despite the methyl ester function at the carboxyl terminus, f-Met-Dpg-Phe-OMe is ~8 × more potent than the parent peptide and 16 × more potent than f-Met-Ac_6c-Phe-OMe. The enhanced activity of the extended Dpg analog and the decreased activity of the folded Ac_6c analog relative to the parent peptide suggest that the extended backbone may be the preferred ligand conformation for the human neutrophil chemotactic peptide receptor. This is consistent with Freer's original proposal that the receptor-bound conformation of f-Met-Leu-Phe-OH is an extended β-structure (Freer *et al.*, 1982). However, our findings do not rule out the possibility that other factors are responsible for these alterations in biological activity.

In addition to peptide backbone conformation, the topography of the side chain groups will also influence activity. The side chain orientation may in part account for the increased potency of the Dpg analog. One can speculate that the inherent symmetry of bilateral propyl side chains of Dpg essentially increases the total number of side chains in the tripeptide from three in the parent peptide to four in the Dpg analog (Fig. 1*A*). This may increase the probability of having three side chain groups of the peptide aligned in a favorable orientation for binding to the receptor. In this regard it should be noted that when f-Met-Leu-Phe-OH was cocrystallized with a protein receptor the preferred orientation of the Met, Leu, and Phe side chains showed the three hydrophobic groups on one face of the peptide backbone swept back into a hydrophobic pocket (Edmundson and Ely, 1985). Since side chain group topography and peptide backbone conformation are intimately associated (Lyu *et al.*, 1990) one must also consider that these two factors together may affect the surface accessibility of some as of yet unidentified critical backbone functional group in the initial binding interaction between the peptide and receptor. This latter possibility may be very important if an induced fit mechanism is at work. Moreover, it may be possible for peptides with different backbone conformations to display similar side chain orientations (Hruby *et al.*, 1990). Other possibilities which cannot be ruled out on the basis of this work include susceptibility of the different peptides to enzyme degradation or the presence of more than one receptor on the human PMN.

In summary, we have synthesized a unique chemotactic peptide analog f-Met-Dpg-Phe-OMe and shown that the peptide prefers an extended backbone conformation both in solution and in the solid state with a C5 conformation at position 2. We have assessed the biological activity of this peptide in human neutrophils and found it to be a more potent secretagogue than the stereochemically constrained type II β-turn folded chemotactic peptide analog (f-Met-Ac_6c-Phe-OMe) and the unconstrained parent peptides (f-Met-Leu-Phe-OMe and f-Met-Leu-Phe-OH). While these findings are consistent with the hypothesis that a formylpeptide receptor prefers a ligand with an extended peptide backbone (Freer *et al.*, 1982), the biological activity of all four peptides (ED_{50} ~10^{-8}–10^{-9} M) provides indirect evidence in support of the induced fit mechanism originally proposed by Edmundson and Ely (1985). It should also be noted that the importance of side chain topography is as yet undefined for the formylpeptides. This may be important particularly at the tripeptide level where the orientation of the side chains in either a folded or extended tripeptide may be similar enough to activate the receptor. Finally, we have observed that in the human PMN model system f-Met-Leu-Phe-OMe and f-Met-Ac_6c-Phe-OMe are less active than f-Met-Leu-Phe-OH. This is in contrast to the findings in the rabbit peritoneal neutrophil model (Sukumar *et al.*, 1985; Toniolo *et al.*, 1989b) where the methyl ester derivatives are reported to be more potent than the parent peptide.

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