The Conformational Properties of the Delta Opioid Peptide [D-Pen^2,D-Pen^5]enkephalin in Aqueous Solution Determined by NMR and Energy Minimization Calculations

Victor J. Hruby,*[1a,c] Lung-Fa Kao,[1a] B. Montgomery Pettitt,[1b,c] and Martin Karplus[1c]

Contribution from the Department of Chemistry, University of Arizona, Tucson, Arizona 85721, the Department of Chemistry, University of Houston, Houston, Texas 77004, and the Department of Chemistry, Harvard University, 12 Oxford Street, Cambridge, Massachusetts 02138. *Received June 26, 1987*

Abstract: The conformational properties of the highly potent delta opioid receptor selective cyclic peptide [D-Pen^2,D-Pen^5]enkephalin (DPDPE) have been investigated by use of one- and two-dimensional nuclear magnetic resonance (NMR) spectroscopy, molecular modeling based on the NMR results, and molecular mechanics energy minimization. A new method for elimination of H_2O (HOD) signals was used which, in conjunction with 2D methods, made possible a complete assignment of all hydrogen atoms in DPDPE. Additional computer simulations allowed an accurate determination of all ^3J and ^2J coupling constants. NOESY experiments gave direct evidence for transannular interactions of the Tyr^1 and Phe^4 aromatic rings with the β,β-dimethyl groups of D-Pen^2. Utilizing NMR parameters in conjunction with model building, extensive energy minimization studies led to two pairs of very similar energy-minimized conformations for DPDPE. Each pair of conformations primarily differed by the sign of the disulfide helicity. One pair was of lower energy and satisfied all of the NMR criteria. Its conformation is distinguished by an amphiphilic conformation with a type IV β-turn and transannular interactions between the aromatic side chains of Tyr^1 and Phe^4 with the β,β-dimethyl groups of D-Pen^2. The factors which stabilize this conformation are discussed, and the possible relationship of this conformation to the high delta opioid receptor selectivity is suggested.

The peptide neurotransmitters [Leu^5]enkephalin (H-Tyr-Gly-Gly-Phe-Leu-OH) and [Met^5]enkephalin (H-Tyr-Gly-Gly-Phe-Met-OH) are the natural endogenous ligands for the opioid receptors.[2] As with many peptide hormones and neurotransmitters the endogenous opioid peptides are short, linear, and highly conformationally flexible molecules with many accessible conformations. Efforts to determine preferred conformations of the enkephalins by spectroscopic methods, X-ray crystallography, and energy calculations have led to many different conclusions (for reviews see ref 3, especially ref 3c). Furthermore, like other peptide hormones and neurotransmitters, the opioids interact with multiple receptors, of which the μ (mu), δ (delta), and κ (kappa) receptors (and possibly others) are generally accepted as opioid receptors which appear to mediate analgesia and the many other biological activities of opioids.[2b,3a,b] Efforts to understand the role(s) of these receptors in the multiple biological activities of the opioids have been hampered by the lack of highly receptor selective ($\geq$1000 fold) peptide (or non-peptide) agonists and antagonists for the individual receptors. While conformational flexibility and lack of receptor specificity may play a biological role (for a discussion see 4a), they pose problems for developing an understanding of the physical–chemical basis for opioid function.

To overcome these problems in small linear peptides such as the enkephalins, we have incorporated conformational constraints including amino acid side chain to side chain cyclization and geminal dimethyl substitution in medium-sized rings.[4] Conformational analysis of such highly selective and conformationally restricted analogues should provide valuable insight into the topological and structural requirements for the different opioid receptors. The studies have led to the development[5] of cyclic analogues of enkephalin such as H-Tyr-D-Pen-Gly-Phe-D-Pen-OH ([D-Pen^2,D-Pen^5]Enk; DPDPE)[6] (Figure 1) which we[5c,7] and others[8] have found to be the most δ receptor selective compound presently available. Utilizing a related approach, Schiller and co-workers[9] have developed highly μ receptor selective cyclic

(1) (a) University of Arizona. (b) University of Houston. (c) Harvard University. Part of this work was done while V.J.H. was on a Guggenheim Fellowship at Harvard University (1984–1985). A preliminary report on some aspects of these studies was presented at the Ninth American Peptide Symposium, Toronto, June 23–28, 1985. Hruby, V. J.; Kao, L. F.; Hirning, L. D.; Burks, T. F. In *Peptides: Structure and Function*; Deber, C. M., Hruby, V. J., Kopple, K. D., Eds.; Pierce Chemical Co.: Rockford, IL, 1985; pp 487–490.

(2) (a) Hughes, J.; Smith, T. W.; Kosterlitz, H. W.; Fothergill, L. A.; Morgan, B. A.; Morris, H. R. *Nature (London)* **1975**, *258*, 577. (b) For recent reviews see: *Opioid Peptides: Molecular Pharmacology, Biosynthesis, and Analysis*; Rapaka, R. S., Hawks, R. L., Eds.; NIDA Research Monograph 70; Rockville, 1986.

(3) (a) *Opioid Peptides: Medicinal Chemistry*; Rapaka, R. S., Barnett, G.; Hawks, R. L., Eds.; NIDA Research Monograph 69; Rockville, 1986. (b) *The Peptides: Analysis, Synthesis, Biology. Vol. 6. Opioid Peptides: Biology, Chemistry, Genetics*; Udenfriend, S., Meienhofer, J., Eds.; Academic Press; New York, 1984. (c) Schiller, P. W. *The Peptides: Analysis, Synthesis, Biology. Vol. 6 Opioid Peptides: Biology, Chemistry, Genetics*; pp 219–268.

(4) (a) Hruby, V. J.; Hadley, M. E. In *Design and Synthesis of Organic Molecules Based on Molecular Recognition*; Van Binst, G., Ed., Springer-Verlag: Heidelberg, 1986; pp 269–286. (b) Hruby, V. J. *Life Sciences* **1982**, *31*, 189. (c) Hruby, V. J. In *Conformationally Directed Drug Design*; Vida, J. A., Gordon, M. Eds.; ACS Symposium Series 251; American Chemical Society: Washington, DC, 1984; pp 9–27. (d) Hruby, V. J. *Trends Pharm. Sci.* **1985**, *6*, 259. (e) Hruby, V. J. In *Perspective in Peptide Chemistry*; Eberle, A., Geiger, R., Wieland, T., Eds.; S. Karger: Basel, 1981; pp 207–220.

(5) (a) Mosberg, H. I.; Hurst, R.; Hruby, V. J.; Galligan, J. J.; Burks, T. F.; Gee, K.; Yamamura, H. I. *Biochem. Biophys. Res. Commun.* **1982**, *106*, 506. (b) Mosberg, H. I.; Hurst, R.; Hruby, V. J.; Galligan, J. J.; Burks, T. F.; Gee, K.; Yamamura, H. I. *Life Sciences* **1983**, *32*, 2565. (c) Mosberg, H. I.; Hurst, R.; Hruby, V. J.; Gee, K.; Yamamura, H. I.; Galligan, J. J.; Burks, T. F. *Proc. Natl. Acad. Sci. U.S.A.* **1983**, *80*, 5871.

(6) All amino acids are of the L configuration (except for Gly) unless otherwise indicated. Abbreviations for amino acids and other abbreviations are those recommended by the IUPAC-IUB. Other abbreviations include the following: Pen, penicillamine, β,β-dimethylcysteine; NMR, nuclear magnetic resonance; NOESY, nuclear Overhauser enhancement spectroscopy: COSY, correlation spectroscopy; Acc, α-aminocyclohexane carboxylate.

(7) (a) Galligan, J. J.; Mosberg, H. I.; Hurst, R.; Hruby, V. J.; Burks, T. F. *J. Pharmacol. Exp. Therap.* **1984**, *229*, 641. (b) Porreca, F.; Mosberg, H. I.; Hurst, R.; Hruby, V. J.; Burks, T. F. *J. Pharmacol. Exp. Therap.* **1984**, *230*, 341. (c) Akiyama, K.; Gee, K. W.; Mosberg, H. I.; Hruby, V. J.; Yamamura, H. I. *Proc. Natl. Acad. Sci. U.S.A.* **1985**, *82*, 2543.

(8) (a) James, I. F.; Goldstein, A. *Mol. Pharmacol.* **1984**, *25*, 337. (b) Corbett, A. D.; Gillan, M. G. C.; Kosterlitz, H. W.; McKnight, A. T.; Paterson, S. J.; Robson, L. E. *Br. J. Pharmac.* **1984**, *83*, 271. (c) Cotton, R.; Kosterlitz, H. W.; Paterson, S. J.; Rance, M. J.; Traynor, J. R. *Ibid.* **1985**, *84*, 927.

(9) (a) DiMaio, J.; Shiller, P. W. *Proc. Natl. Acad. Sci. U.S.A.* **1980**, *77*, 7162. (b) Schiller, P. W.; DiMaio, *J. Nature (London)* **1982**, *297*, 74. (c) DiMaio, J.; Nguyen, T. M. D.; Lemieux, C.; Schiller, P. W. *J. Med. Chem.* **1982**, *25*, 1432. (d) Schiller, P. W.; DiMaio, J. In *Peptides: Structure and Function*; Hruby, V. J., Rich, D. H., Eds.; Pierce Chemical Co.: Rockford, 1983; pp 269–278.

0002-7863/88/1510-3351$01.50/0

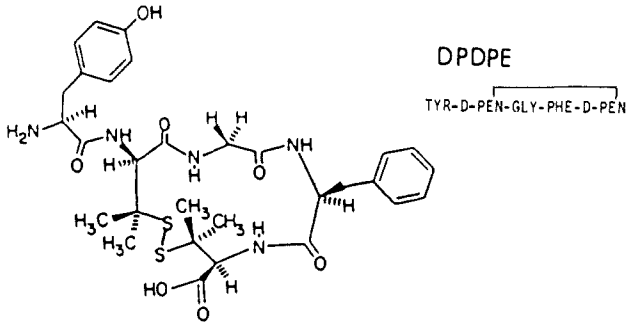

Figure 1. Structure of [D-Pen^2,D-Pen^5]enkephalin (DPDPE).

enkephalins, and we have developed cyclic conformationally constrained somatostatin-like analogues with high μ receptor selectivity and potency and little somatostatin-like binding activity.[10]

We report here on a detailed nuclear magnetic resonance (NMR) study of [D-Pen^2,D-Pen^5]Enk in aqueous solution and an examination of the conformational properties of DPDPE which are suggested by the NMR results and molecular mechanics calculations. The two approaches lead to a low-energy conformational family with closely related topological properties that are consistent with the available data. These results are discussed in terms of possible conformational and structural features which may be related to the receptor selectivity of DPDPE.

Experimental Section

Peptide Synthesis and Analysis. [D-Pen^2,D-Pen^5]enkephalin (DPDPE) was prepared as described previously[5c] by the solid-phase peptide method and purified by partition chromatography on Sephadex G-25 followed by HPLC on a Vydac C-18 reverse phase column with a Perkin-Elmer series 3B instrument. Purity was assessed by thin-layer chromatography in three different solvent systems, by analytical reverse phase high pressure liquid chromatography, by amino acid analysis, and by fast atom bombardment mass spectrometry, and the peptide used in this study was greater than 98% DPDPE.

Nuclear Magnetic Resonance (NMR) Spectroscopy. A 16-mM sample of the peptide was made by dissolving 7 mg of DPDPE in approximately 0.6 mL of 100% D_2O (low paramagnetic impurities, Aldrich) or in a solution of H_2O/D_2O (90:10 v/v) which was adjusted to pH 3.1 with acetic acid-d_4. Samples were degassed by several freeze–thaw cycles and sealed in NMR tubes under vacuum.

Proton spectra were recorded with a Bruker AM-250 spectrometer equipped with an Aspect-3000 computer. Chemical shifts were measured in parts per million (ppm) downfield from internal 3-(trimethylsilyl)-propionic acid-d_4 acid sodium salt (TSP). To overcome the problem of trying to observe 16 mM proton signals from the peptide in the presence of ~100 M H_2O proton signal, solvent suppression in 1-D and 2-D COSY[11,12] and NOESY[13] experiments was performed utilizing the recently developed DEFT-COSY and DEFT-NOESY pulse sequences.[14] The applied pulse sequences were

$$D\text{–}\theta^\circ\text{–}\tau_s\text{–}180^\circ\text{–}\tau_s\text{–}90^\circ(\mathrm{pH_1})\text{–}t_1\text{–}t_f\text{–}90^\circ(\mathrm{pH_2})\text{–}t_f\text{–FID (DEFT-COSY)} \quad (I)$$

and

$$D\text{–}\theta^\circ\text{–}\tau_s\text{–}180^\circ\text{–}\tau_s\text{–}90^\circ(\mathrm{pH_1})\text{–}t_1\text{–}90^\circ(\mathrm{pH_2})\text{–}t_m\text{–}90^\circ(\mathrm{pH_3})\text{–FID (DEFT-NOESY)} \quad (II)$$

where D is the recycle delay, θ° is a 30–80° pulse, τ_s is the pulse delay, t_1 is the evolutionary time, t_f is a fixed delay, and t_m is the mixing time. Since presaturation of the solvent is not utilized in this approach, NMR signals in the neighborhood of the solvent peak are not distorted, and accurate chemical shifts and coupling constants can be more readily determined. The solvent suppressions were achieved by adjusting D and τ_s values to satisfy eq[14] 1

$$(1 - \exp(-\tau_s/T_1))^2/\exp(-2T_s/T_1)(1 - \exp(-D/T_1)) = \cos\theta \quad (1)$$

where T_1 is the spin-lattice relaxation time of the solvent, such that the logitudinal and transverse magnetization of the solvent (HOD or H_2O) is nulled prior to the 2-D pulse sequence. One-dimensional spectra with solvent suppression were obtained by setting $t_1 = 6 \times 10^{-6}$ s. Two-dimensional COSY and NOESY experiments with solvent suppression were acquired with spectral widths of ±1050 Hz (256 t_1 values) in W_1 and 2100 Hz (1 K points) in W_2 by using the appropriate set of D, θ°, and τ_s values. In the NOESY experiments, the buildup of NOE intensities was investigated by nine 2D DEFT-NOESY experiments with several different mixing times, t_m, of 20, 40, 80, 120, 200, 350, 500, 700, and 900 ms. At shorter mixing times it was difficult suppressing completely the coupling constants. The strongly coupled α and β regions of the Tyr^1 and Phe^4 resonances were treated as two three-spin systems and were analyzed by computer simulation with the Bruker Instrument PANIC.81 computer program.[15] The temperature dependencies of the amide proton resonances in H_2O were determined by five measurements over a range of 20–60 °C by using a saturating pulse[16] at the H_2O resonance. It should be noted that the D-Pen^5 amide proton overlaps with the Phe^4 aromatic resonances and hence the temperatures dependencies were determined by examination of the cross peak (Figure 3a) in several DEFT-COSY experiments over the temperature range.

Table I. DPDPE NMR Parameters for $^3J_{NH\text{-}\alpha CH}$ and Their Correlations with ϕ Angles[a]

residue	$^3J_{NH\text{-}\alpha CH}$	possible ϕ angles[b]
D-Pen^2	8.1	−60, 90,[c] 150
Gly^3	8.5	60, −150, −90[c]
	4.2	120, 180, −60, 10
Phe^4	6.8	90, −160, −80,[c] 30
D-Pen^5	8.4	−70, 90,[c] 150

[a] See ref 22. [b] Angles used for model building of starting structures were ±25° those shown here. [c] Correspond to ϕ angles for most low-energy structures of DPDPE.

Energy Calculations. All energy calculations were carried out with the CHARMM Program.[17] The empirical energy functions used to obtain the energy minimized structures include harmonic potential energy terms for bond lengths, bond angles, bond dihedral angles, improper dihedral angles, van der Waal and electrostatic terms for nonbonded interactions, and a hydrogen bonding potential. The computational methodology and the general form of the potential functions correspond to those described previously[17] except as noted below.

The structural parameters for D-penicillamine (bond lengths and bond angles, etc.) were obtained by fitting X-ray crystallographic data of penicillamine[18] and the penicillamine residues in penicillins.[19] The disulfide bond length parameter was set equal to 2.04 Å. The energy terms for the bond lengths, bond angles, torsional angles, and improper torsional angles in the penicillamine residue were taken from those used for cysteine except for the γ_{CH_3}–β_C–γ'_{CH_3} group which was taken from leucine.[17] Solvent molecules were not explicitly included in the calculations, but the effect of bulk solvent H_2O was approximated by employing a dielectric constant of 80 which is appropriate for a small molecule such as DPDPE.[20] Both trans and cis peptide bonds were allowed in all cases. The neutral non-zwitterionic structure of DPDPE was used.

The starting structures for conformational energy minimization included a series of models built with minimal constraints or with structural parameters obtained on the basis of measured NMR parameters. The NMR data was used as a guide for building a variety of plausible starting structures. The subsequent extensive calculations then were used to either refine or reject the final conformers on the basis of energy analysis (see below). Space-filling models were used to generate structures, which

(10) (a) Pelton, J. T.; Gulya, K.; Hruby, V. J.; Duckles, S.; Yamamura, H. I. *Peptides* **1985**, *6*, 159. (b) Pelton, J. T.; Gulya, K.; Hruby, V. J.; Duckles, S. P.; Yamamura, H. I. *Proc. Natl. Acad. Sci. U.S.A.* **1985**, *82*, 236. (c) Pelton, J. T., Kazmierski, W.; Gulya, K.; Yamamura, H. I.; Hruby, V. J. *J. Med. Chem.* **1986**, *29*, 2370.
(11) Aue, W. P.; Bartholdi, E.; Ernst, R. R. *J. Chem. Phys.* **1976**, *64*, 2229.
(12) Nagayama, K.; Kumar, A.; Wüthrich, K.; Ernst, R. R. *J. Magn. Reson.* **1980**, *40*, 321.
(13) Jeener, J.; Meier, B. H.; Bachmann, P.; Ernst, R. R. *J. Chem. Phys.* **1979**, *71*, 4546.
(14) Kao, L.-F.; Hruby, V. J. *J. Magn. Reson.* **1986**, *70*, 394.
(15) Bruker Instruments, Inc., Manning Park, Billerica, MA 01821.
(16) Wider, G.; Hosur, R. V.; Wüthrich, K. *J. Magn. Reson.* **1983**, *52*, 130.
(17) Brooks, B. R.; Bruccoleri, R. E.; Olafson, B. D.; States, D. J.; Swaminathan, S.; Karplus, M. *J. Comput. Chem.* **1983**, *4*, 187.
(18) Dexter, D. D.; van der Veen, J. M. *J. Chem. Soc., Perkin Trans. 1* **1978**, 185, and references therein.
(19) See, for example: (a) Rosenfield, R. E., Jr.Parthasarthyl, R. *Acta Crystallogr., Sect. B: Struct. Crystallog. Cryst. Chem.* **1975**, *B31*, 75, 462. (b) Cameron, A. F.; McElhatton, J.; Campbell, M. M.; Johnson, G. *Acta Crystallogr., Sect. B: Struct. Crystallogr. Cryst. Chem.* **1979**, *B35*, 1263.
(20) Pettitt, B. M.; Karplus, M. *Chem. Phys. Lett.* **1985**, *121*, 194.

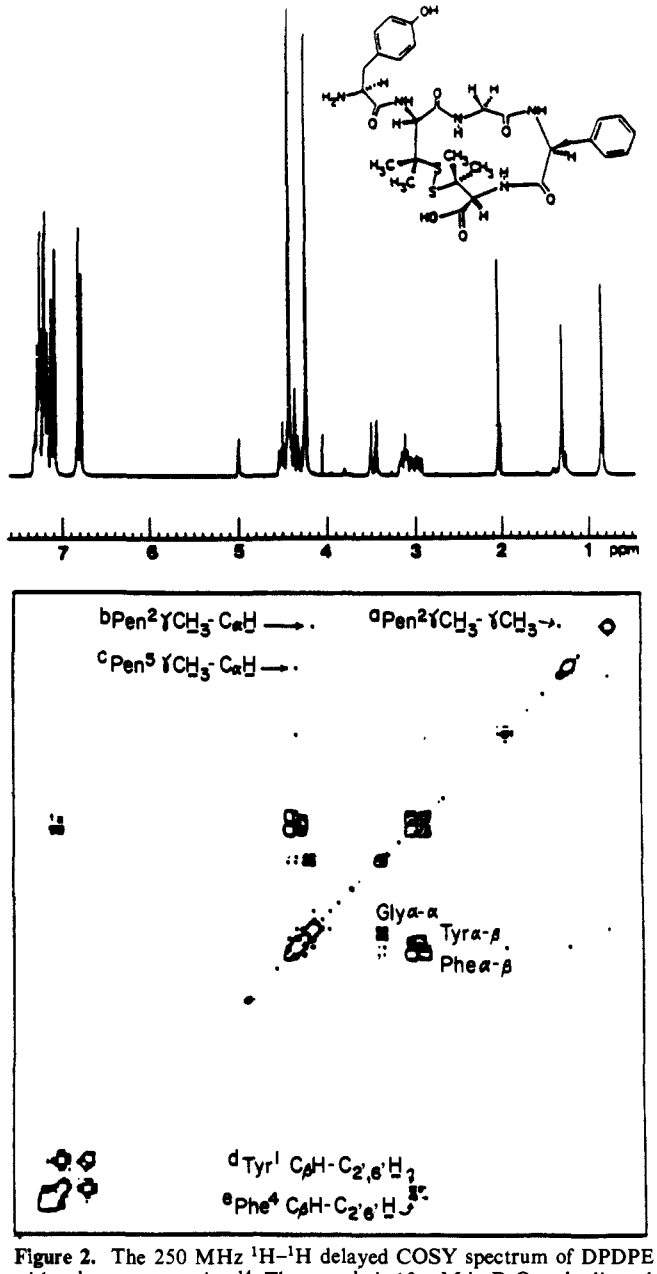

Figure 2. The 250 MHz ^1H–^1H delayed COSY spectrum of DPDPE with solvent suppression.[14] The sample is 10 mM in D_2O and adjusted to pH 3.0 with acetic acid-d_4. An 1800 Hz spectral width in W2 was collected into 2K data points. The pulse sequence I was applied with $\theta = 63.5°$, $D = 3$ s, $\tau_s = 2.6$ s, $\tau_f = 0.1$ s, $t_1 = 6 \times 10^{-6}$ s, 16 scans. A total of 256 FIDs with $\Delta t_1 = 0.556$ ms were accumulated. A sine-bell window was multiplied to the FIDs. A 1024 × 1024 data matrix was acquired within a total time of 11 h. Long-range couplings are as follows: (a) Pen² CH_3–CH_3, (b) Pen² $C_\alpha H$–CH_3, (c) Pen⁵ $C_\alpha H$–CH_3, (d) Tyr¹ $C_\beta H$–$C_{2,6}H$ (aromatic), (e) Phe⁴ $C_\beta H$–$C_{2,6}H$ (aromatic).

were transferred to the computer with the aid of the CHEMGRAPH program and a Lundy graphics terminal. In all cases, any structural constraints imposed while building plausible starting structures were relaxed during the calculation, and the energy minimization was allowed to proceed freely without any conformational constraints. Low-energy conformations from the calculations were checked to see if they were consistent with the NMR parameters. To attempt to cover the conformational space available to DPDPE, the following ranges of starting structures were examined. The starting conformations all had a ring closure through the disulfide bond with S–S bond lengths of 2.04 ± 0.40 Å. Consistent with ring closure, standard reverse turn conformations involving the D-Pen², Gly³, Phe⁴, and D-Pen⁵ residues were built including C_5, C_7, type I, I′, II, II′, III, and III′ β-turns[21] with the appropriate hydrogen bonds. Side chain χ_1 conformations for Tyr¹ and Phe⁴ in most cases were started at either trans (±180°) or gauche (−) (−60°) conformations (consistent with the NMR data—vide infra), and both right-handed and left-handed C–S–S–C dihedral angles were allowed. Model building also was performed by using ϕ angles consistent with the $^3J_{NH-\alpha CH}$ coupling constants[22] from the NMR data (Table I). All possible ϕ angles were examined in a systematic manner to obtain a structure which permitted ring closure. Then the allowed ϕ angles were again examined to determine if others were compatible with this structure. Not all possible combinations were energy minimized because they often were not compatible with construction of a 14-membered ring. Examples of a small number of the starting structures examined are shown in Table II.

(21) (a) Lewis, P. N.; Momany, F. A.; Scheraga, H. A. *Biochim. Biophys. Acta* **1973**, *303*, 211. (b) Rose, G. D.; Gierasch, L. M.; Smith, J. A. *Adv. Protein Chem.* **1985**, *37*, 1.

Table II. Table of Selected Starting Conformations for DPDPE[a]

structure	residue (dihedral angles)			
	D-Pen²	Gly³	Phe⁴	D-Pen⁵
	(Φ_2, Ψ_2)	(Φ_3, Ψ_3)	(Φ_4, Ψ_4)	(Φ_5)
I	(−60, −120)	(−60, −140)	(−75, 60)	(65)
II	(−95, −120)	(−60, −150)	(−60, 60)	(70)
III	(110, 140)	(170, −80)	(180, 25)	(−70)
IV	(80, −160)	(−40, 10)	(−70, −25)	(65)
V	(80, 50)	(−85, −20)	(−60, −40)	(75)
VI	(90, 20)	(−60, −70)	(−80, −70)	(85)
VII	(90, 90)	(−90, −60)	(−150, 70)	(70)
VIII	(160, 80)	(−80, −30)	(−120, −50)	(85)
IX	(120, 130)	(60, −90)	(90, 10)	(60)
X	(120, 140)	(170, 10)	(40, 90)	(150)
XI	(90, 170)	(130, −90)	(−160, 40)	(85)
XII	(95, 80)	(−90, −35)	(−80, −30)	(90)
XIII	(95, 25)	(−110, −15)	(−80, −60)	(80)
XIV	(115, 50)	(−110, 15)	(−135, 70)	(70)
XV	(90, 15)	(−100, −20)	(−75, −50)	(85)

[a] All the ω values in the examples illustrated here were in the neighborhood of 180°, but structures with ω near 0° were examined. No specific Ψ angle was set for Tyr¹.

Table III. Chemical Shifts of Pen², Pen⁵ Hα and CH_3 Groups of DPDPE, [D-Cys², D-Pen⁵]enkephanlin, and [D-Pen², D-Cys⁵]enkephalin at pH 3.1 and 6.0

DPDPE, pH 6.0	DPDPE pH 3.1	[D-Cys², D-Pen⁵]Enk, pH 3.1	[D-Pen², D-Cys⁵]Enk, pH 3.1
	Cα protons		
4.26 (Pen²)	4.25 (Pen²)		4.22 (Pen²)
4.15 (Pen⁵)	4.43 (Pen⁵)	4.47 (Pen⁵)	
	CH_3 groups		
0.84 (Pen²)	0.89 (Pen²)		0.88 (Pen²)
1.26 (Pen⁵)	1.33 (Pen⁵)	1.38 (Pen⁵)	
1.33 (Pen⁵)	1.38 (Pen⁵)	1.43 (Pen⁵)	
1.54 (Pen²)	1.49 (Pen²)		1.43 (Pen²)

Results

NMR Chemical Shift Assignments and Coupling Constants for DPDPE. A prerequisite for elucidation of the solution conformation of the peptide is the complete and unambiguous assignments of NMR chemical shifts and coupling constants. Often a major problem in aqueous NMR studies is the interference from solvent (HOD/H_2O) which may be present. A contour plot and the projected 1-D spectrum of COSY experiment with solvent suppression is shown in Figure 2. By the DEFT-COSY pulse sequence the residual solvent HOD peak at $\delta = 5.0$ ppm and acetate peak at $\delta = 2.04$ ppm were suppressed to an intensity comparable to the peptide signals. The insertion of a fixed delay t_f[23] in the pulse sequence allows us to observe long range ^1H–^1H couplings (Figure 2 a–c) as well as the vicinal couplings in the COSY experiment. Ambiguities in the assignments of the four

(22) (a) Bystrov, V. *Prog. Nucl. Magn. Reson. Spectrosc.* **1976**, *10*, 41. (b) Hruby, V. J. In *Chemistry and Biochemistry of Amino Acids, Peptides and Proteins*; Weinstein, B., Ed.; Marcel Dekker: New York, 1974; Vol. 3, pp 1–188. (c) Karplus, M. *J. Am. Chem. Soc.* **1963**, *35*, 2870. (d) Karplus, S.; Karplus, M. *Proc. Natl. Acad. Sci. U.S.A.* **1972**, *69*, 3204.

(23) Bax, A.; Freeman, R. *J. Magn. Reson.* **1981**, *44*, 542.

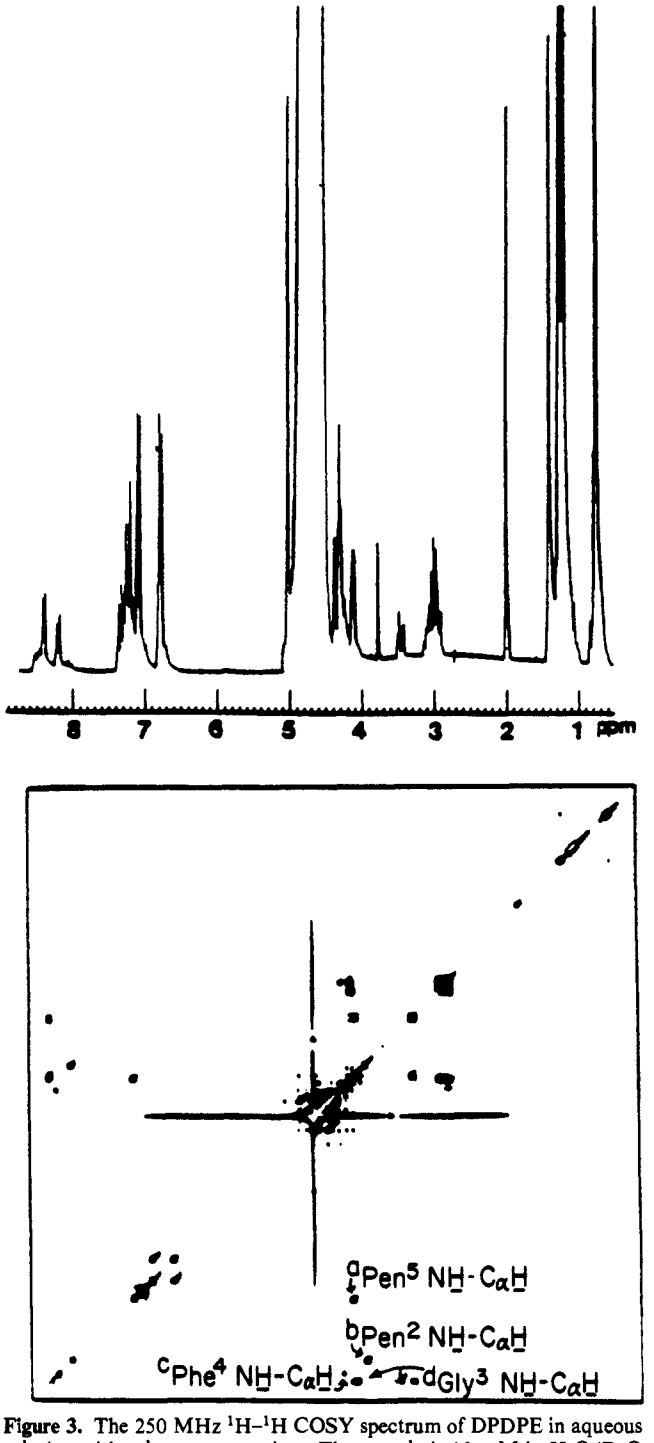

Figure 3. The 250 MHz ^1H–^1H COSY spectrum of DPDPE in aqueous solution with solvent suppression. The sample is 10 mM in H_2O/D_2O (90:10 v/v). A 2100 Hz spectral width in W2 was collected into 2K data points. The pulse sequence I was applied with $\theta = 64.3°$, $D = 2.5$ s, $\tau_s = 0.8$ s, $\tau_f = t_1 = 6 \times 10^{-6}$ s, 64 scans. A total of 256 FIDs with $\Delta t_1 = 0.476$ ms was accumulated. A 1024 × 1024 data matrix was acquired within a total time of 21.6 h. Vicinal peptide amide NH proton to α-proton couplings are as follows: (a) Pen_5 HN–C_αH, (b) Pen^2 HN–C_αH, (c) Phe^4 HN–C_αH, (d) Gly^3 HN–C_αH.

methyl groups and two α protons in D-Pen^2 and D-Pen^5 residues were removed by the pH titration experiments, examination of other D-penicillamine-containing enkephalin analogues, previous NMR studies of related constrained cyclic enkephalins,[24] and use

(24) Mosberg, H. I.; Schiller, P. W. *Int. J. Peptide Protein Res.* **1984**, *23*, 462. While this paper was in preparation, additional data on penicillamine-containing enkephalin analogues was reported by Mosberg (Mosberg, H. I. *Int. J. Peptide Protein Res.* **1987**, *29*, 282) including DPDPE. The chemical shifts and assignments are consistent with those reported here.

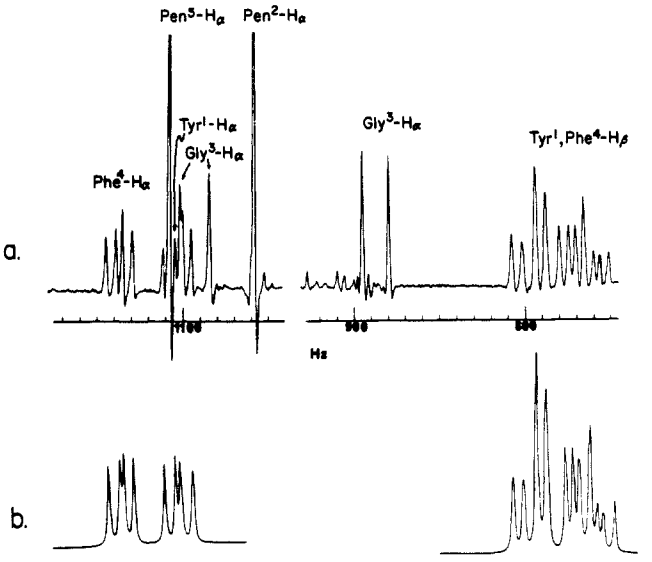

Figure 4. (a) Observed 250 MHz ^1H NMR spectrum of the α and β proton region in DPDPE. (b) Calculated spectrum for the α and β proton resonances of Tyr^1 and Phe^4 in DPDPE (see text for method).

of the delayed COSY experiments. Acetic acid-d_4 and NaOD were used in titration to obtain pHs of 3.1 and 6.0 for DPDPE in aqueous solution; at pH 3.1 DPDPE bears a net charge of +1, while at pH 6.0 net charge is near −1. The results are shown in Table III. It can be seen that the α proton of DPDPE at 4.43 ppm was shifted 0.28 ppm toward higher field when the pH changed from 3.1 to 6.0, whereas the proton at 4.25 ppm is essentially unaltered. Since the D-Pen^2 residue is an internal residue its C-α proton is expected to be unaffected by the pH change; the D-Pen^5 residue is C-terminal so that its C-α proton is expected to be shifted as the C-terminal carboxylate is titrated. Thus, the Hα at 4.43 ppm can be assigned to D-Pen^5 and the Hα at 4.25 ppm to D-Pen^2. The chemical shifts of D-Pen^2 CH_3 and D-Pen^5 CH_3 in DPDPE did not show significant pH dependencies and can only be partially assigned by comparing the Pen-CH_3 chemical shifts in [D-Cys^2,D-Pen^5]enkephalin and [D-Pen^2,D-Cys^5]enkephalin (Table III), i.e., only the resonance at 0.89 can be assigned to Pen^2. However, the remaining ambiguities were removed by the long range couplings observed in the DEFT-COSY experiments. Since the D-Pen^2-Hα and D-Pen^5-Hα are unambiguously assigned (Table III), the long range couplings observed in Figure 2a–c can be assigned to D-Pen^2 CH_3-CH_3 (a), D-Pen^2 Hα-CH_3 (b), and D-Pen^5 Hα-CH_3 (c), respectively. The amide protons in DPDPE were assigned by a DEFT-COSY experiment in a solution consisting of 90% H_2O and 10% D_2O (Figure 3). Under these conditions it would be impossible to detect many of the signals in a 16 mM solution in the presence of huge H_2O signal (with proton concentration of ~100 M) unless the water resonance were suppressed. By using the present pulse sequence[14] a factor of 100–150 can be achieved in reduction of the H_2O intensity. This still leaves the water signal as the most intense peak in a spectrum. However, all the amide resonances can be observed, and the vicinal coupling constants ^3J[HN–αCH] can be obtained for the α protons, many of which are near the water resonance. Often these ^3J[HN–αCH] values would be perturbed when a method such as presaturation[16] is used. In a 1-D experiment water suppression can be achieved by setting $t_1 = 6 \times 10^{-6}$ s in the DEFT-COSY pulse sequence. The complete assignment of chemical shifts and coupling constants for DPDPE were obtained. Resonances of strongly coupled α and β regions of Tyr^1 and Phe^4 were simulated by using the Bruker software program PANIC.81 (Figure 4), and the resulting best-fit chemical shifts and coupling constants were obtained.

The chemical shift difference for the two nonequivalent Gly Hαs in DPDPE and various cyclic and linear peptides is given in Table IV. Generally a small chemical shift difference was

Table IV. Nonequivalence of Glycine α-Hydrogens in Cyclic Enkephalins, Oxytocin Analogues, and Linear Peptides

peptide	δGly-Hα	Δδ	ref
H-Tyr-D-Pen-Gly-Phe-D-Pen-OH (DPDPE)	3.42, 4.22	0.80	this paper
H-Tyr-D-Pen-Gly-Acc[a]-D-Pen-OH	3.67, 4.51	0.84	25
H-Tyr-D-Pen-Gly-Phe-D-Cys-NH_2	3.53, 3.98	0.45	24
Met-Gly-Met	*b*	0.00	26
Phe-Gly-Phe-Gly	*b*	0.10	26
[Pen¹,Leu²,Cys⁶,Gly⁹]oxytocin[27]	3.87, 3.96	0.09	27
[Pen¹,Tyr²,Cys⁶,Gly⁹]oxytocin[27]	3.85, 3.93	0.08	27

[a] Acc: α-aminocyclohexane carboxylate. [b] Not given in the reference.

observed. This small nonequivalence of the Gly Hαs (Δδ = 0.0–0.10) in most peptides (a few examples are given in Table IV) previously had been explained[26-28] by suggesting that one of the protons (*pro*-R) was shielded more than the other (*pro*-S) as a result of the slight difference in orientation of these hydrogens with respect to the carbonyl group of the preceding peptide bond. In the case of DPDPE, it might be suggested a priori that the side chain rotomer population of the aromatic residue, especially Phe⁴, could play a role in this nonequivalence. However, an almost identical Δδ value (0.8 ppm) in DPDPE and in [D-Pen²,Acc⁴,D-Pen⁵]enkephalin (Table IV) (Acc = α-aminocyclohexane carboxylate) indicates that the ring current effect from the Phe⁴ aromatic ring is not a major contributor to the nonequivalence of the Gly³ α protons. This is consistent with the rotamer analysis for Phe⁴ in DPDPE (vide infra) in which the most populated χ^1 conformation places the Phe⁴ aromatic ring more than 4 Å from the Gly³-Hα. Flexible peptide backbones, such as found in linear peptides and in the neighborhood of Gly⁹ of the oxytocin analogues (i.e., Gly⁹ is in the acyclic tripeptide side chain of OT), tend to average out anisotropic effects of the carboxyl groups and thus lead to small Δδ values (less than 0.1 ppm in Table IV). The very large chemical shift difference for the two Gly H_α in DPDPE can be accounted for by the more rigid conformation of the peptide backbone in the 14-membered ring. Comparison of the measured Δδ values for DPDPE, [D-Cys²,D-Pen⁵]enkephalin, and [D-Pen²,D-Cys⁵]enkephalin indicates that the C-terminal penicillamine⁵ contributes to the rigidity of the 14-membered ring. The bulky disulfide bond and geminal dimethyl groups of the Pen² and Pen⁵ residues tend to limit backbone flexibility and force the carboxyl groups away from the 14-membered ring in DPDPE. As a consequence of this, one of the Gly H_α is fixed in the shielding zone and the other in the deshielding zone of the carbonyl group of residues 3 and 4, leading to a strong nonequivalence.

Temperature Dependence and NOE Studies. The temperature dependencies of the amide protons indicate that though the NH protons are somewhat solvent shielded there does not appear to be any strong intramolecular hydrogen bonds for DPDPE in aqueous solution. Values of 2.6–5 × 10^{-3} ppm/K are obtained, which generally are higher than those seen for intramolecular hydrogen bonds in model cyclic peptides[21,22b] which generally are on the order of 0–2.5 × 10^{-3} ppm/K. It should be noted that the value of 2.6 × 10^{-3} ppm/K for the D-Pen⁵ amide proton is somewhat higher than the value of 0.9 × 10^{-3} recently reported for DPDPE.[24] However, it should be further noted that this proton is buried in the Phe⁴ aromatic protons. Thus we determined its temperature dependence from the cross peaks in 2D DEFT-COSY experiments at several temperatures, which should make the value somewhat more accurate than the number obtained previously with 1D experiments. Experimental[29-31] and theoretical[20,32,33] studies of solvent effects on peptides have shown that hydration of the peptide backbone in aqueous solution tends to weaken intramolecular hydrogen bonds and promote hydrophobic clustering of nonpolar side chains. This is consistent with the results that indicated that the carbonyl groups in the cyclic, 14-membered ring of DPDPE are pointing out toward the solvent. All the amide bonds in DPDPE appear to be trans, and the amide protons in the 14-membered ring are partially shielded from the solvent by their relative proximity to the hydrophobic side chains (Tyr¹, Phe⁴ and Pen²-CH_3) and the disulfide moiety. With regard to hydrophobic clustering of side chains, we have consistently observed NOEs between the β-methyl groups of Pen² and the Tyr¹ and Phe⁴ aromatic protons (Figure 5a,b) (in some NOESY experiments at shorter mixing times Tyr¹ aromatic to Pen⁵ β CH_3 NOEs also were suggested).[34] These results indicate that DPDPE possesses

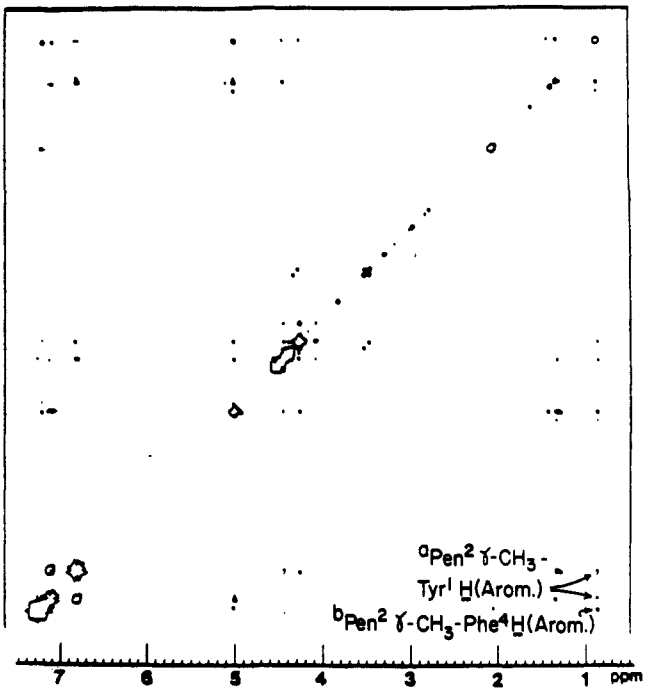

Figure 5. 250 MHz ^1H–^1H NOESY spectrum of DPDPE in D_2O. The pulse sequence II was applied with θ = 63.5°, D = 3 s, τ_s = 2.0 s, t_1 = 6 × 10^{-6} s, t_m = 0.9 s (varying by 15%) and 16 scans. A total of 256 FIDs with Δt_1 = 0.556 ms were accumulated. A 1024 × 1024 data matrix was acquired within a total of 11.4 h. Observed transannular NOEs are as follows: (a) Pen² γ-CH_3-Tyr¹ H (aromatic); (b) Pen² γ-CH_3-Phe⁴ H (aromatic).

Table V. Calculated Side Chain Rotamer Populations (%) about the C_α–C_β Bond (χ_1) for Tyr¹ and Phe⁴ in DPDPE[a]

residue	coupling constants $^3J_{\alpha CH\text{-}\beta CH}$	gauche (−) (−60°)	trans (±180°)	gauche (+) (+60°)
Tyr¹	6.2, 9.2	27.0 (73.0)	73.0 (27.0)	0.0
Phe⁴	5.5, 9.0	30.0 (70.0)	70.0 (30.0)	0.0

[a] Calculated according to de Leeuw and Altona.[39]

(25) Hruby, J. J.; Kao, L.-F.; Hirning, L. D.; Burks, T. F. *Peptides: Structure and Function*; Deber, C. M., Hruby, V. J., Kopple, K. D., Eds.; Pierce Chem. Co.: Rockford, IL, 1985; pp 487–490.
(26) Anteunis, M. J.; Becu, C.; Kala, A. K.; Verhegge, G.; Narayan-Lala, K. *Bull. Soc. Chim. Belg.* **1977**, *86*, 161.
(27) Mosberg, H. I.; Hruby, V. J.; Meraldi, J. P. *Biochemistry* **1981**, *20*, 2822.
(28) Anteunis, M. J. *Tetrahedron Lett.* **1977**, *18*, 1535.
(29) Madison, V.; Kopple, K. D. *J. Am. Chem. Soc.* **1980**, *102*, 4855.
(30) Avignon, M.; Carrigon-Lagrange, C.; Bothorel, D. *Biopolymers* **1973**, *12*, 1651.
(31) Avignon, A.; Von Huang, P. *Biopolymers* **1970**, *9*, 427.
(32) Rossky, P.; Karplus, M. *J. Am. Chem. Soc.* **1979**, *101*, 1913.
(33) Mezei, M.; Mehrota, P. K.; Beveridge, D. L. *J. Am. Chem. Soc.* **1985**, *107*, 2239.
(34) We have investigated DEFT-NOESY experiments at a variety of mixing times in the range of 20–900 ms. It has been difficult to obtain a quantitative correlation because though peak intensities increase and decrease depending upon the time, both first-order and second-order NOE effects appear to be observed as has been previously observed in other peptides.[35-37] This is being further investigated at this time.

a compact topology with the Tyr[1]-aromatic, Phe[4]-aromatic, and the Pen β-methyl groups in close proximity. Observations from these NOESY experiments are in agreement with previous studies[38] involving the penicillamine- and cystine-containing analogues [D-Pen[2],D-Cys[5]]enkephalinamide and [D-Pen[2],Cys[5]]-enkephalinamide, which led to the suggestion that the Tyr[1] side chain is situated near the disulfide moiety.

Backbone and Side-Chain Conformations. The NMR studies discussed above provide a variety of parameters which can be used to help construct a conformational model for DPDPE in solution. Rotamer populations were calculated[39] by using the best-fit values of ^3J αβ protons. The calculated rotamer populations for trans and gauche (−) (Table V) can be reversed since the β and β′ protons in Tyr[1] and Phe[4] are not stereochemically assigned. In either case, there is little or no contribution from the gauche (+) conformation.

The backbone conformation of DPDPE was examined by correlating the coupling constants ^3J[HN–αCH] with the possible dihedral angles Φ by using the angular dependence of the coupling constant with the Bystrov parameterizations.[22a] Since several values of the angles are allowed as previously indicated (vide supra), a range of possible Φ values were utilized with the results from NOE and temperature studies to obtain the starting conformations for energy minimization.

In addition, the NOE results which indicate transannular interactions of the Tyr[1] and Phe[4] aromatic groups with the Pen[2] β-methyl groups are compatible restrictions in the overall conformation. Given the constrained nature of the cyclic 14-membered ring, it is possible to utilize the NMR results to construct a limited set of conformations that are consistent with the NMR results. However, the apparent lack of strong intramolecular hydrogen bonds, the fact that the $^3J_{NH-\alpha CH}$ coupling constants permit several possible backbone conformations consistent with ring closure, and recognition that even with careful model building one generally does not obtain a minimum energy conformation point to the necessity of using molecular mechanics in conjunction with model building and the NMR results to find one or more self-consistent, low-energy conformations for DPDPE. Since a 14-membered ring requires a reverse turn conformation to allow ring closure, we utilized as starting points for our energy studies standard β-turn type conformations and C_7 conformations as well as turn conformations that deviated from these but which were consistent with ring closure (see above). Energy minimization of these structures were carried out, and the resulting conformations were examined for their compatibility with the NMR parameters. The details of the approach and the results are outlined below.

Conformational Analysis–Energy Minimization. Initially, model building was used to construct starting conformations for energy minimization with minor input from the NMR parameters. A variety of possible C_5, C_7, and various β-turn conformations with intramolecular hydrogen bonds were examined; Table II shows a few (structures I–VI) starting conformations examined in these cases. None of the C_7 conformations nor the type IV β-turn conformations which resulted from these energy minimization studies led to conformations consistent with the NMR data, i.e., several of the final Φ angles were outside the range (±25°) consistent with ^3J NMR parameters (Table I). Also it is of interest that the overall energies of none of these minimized structures was as low as those subsequently obtained when other criteria were used for model building prior to energy minimization. From these studies, the lowest energy conformation obtained was about −8.5 kcal/mol and had no intramolecular hydrogen bond; this structure was inconsistent with the NMR results because the Phe[4] aromatic ring could not interact easily with the Pen[2] methyl groups. The most stable intramolecular hydrogen-bond-containing structure involved a C_7 conformation with the D-Pen[5]-NH to Gly[3]-CO intramolecular hydrogen bond (Phe[4] is the i + 1 residue) and an overall energy equal to −6.0 kcal/mol; however, the conformation is inconsistent with the NMR parameters.

Table VI. Low-Energy Backbone Conformations of DPDPE Satisfying NMR Parameters

residue	angle	1	1′	2	2′
	ψ	139	157	162	164
Tyr[1]	ω	−176	−173	−173	−173
	χ_1	−171	−164	−164	−163
	χ_2	58	51	50	51
D-Pen[2]	Φ	94	116	114	111
	Ψ	83	53	27	14
	ω	−179	169	175	173
	χ_1	−167	−166	179	−180
	χ_2	−97	−137	87	143
Gly[3]	Φ	−75	−108	−109	−98
	Φ	−41	12	−17	−18
	ω	−177	−176	177	177
Phe[4]	Φ	−94	−128	−74	−72
	Ψ	−50	−70	−63	−46
	ω	−177	−174	176	−175
	χ_1	−60	−56	180	179
	χ_2	102	103	62	68
D-Pen[5]	Φ	87	69	83	83
	χ_1	−56	68	45	−70
	χ_2	74	−51	−140	119
		106°[a]	−99°[a]	102°[a]	−110°[a]
		−12.4[b]	−10.0[b]	−11.2[b]	−13.7[b]

[a] ∠CSSC. [b] E(kcal).

To obtain low-energy structures consistent with the NMR data, a series of starting conformations were generated by model building subject to consistency with the NMR results. These included the coupling constant parameters (Table I). In Table I are shown the starting φ angles (±25°) which were examined during this phase of the energy minimization study. *All* possible φ angles were utilized, but not all possible combinations were energy minimized because they were not compatible with construction of a 14-membered ring. For example, though extended conformations (φ = ±180 ± 25°) of individual amino acid residues were examined while building a variety of possible conformations, it was found that during the systematic model building many such extended conformations could not be accommodated into a 14-membered ring within the criteria established for ring closure and/or such that other φ angles satisfied the NMR results (±25°). Nonetheless, every one of the possible φ angles in Table I were examined at least once in a ring-closed structure which was energy minimized. Some examples of starting conformations in these studies are given in Table II (structures VII–XI). These studies lead to a number of low-energy conformations with overall energies ranging from −6 to −13 kcal/mol. None contained intramolecular hydrogen bonds; most had type IV β-turn conformations as defined by Lewis, Momany, and Scheraga.[21] However, after energy minimization, none of these structures satisfied all of the NMR criteria. This led us to further model building which used constraints in addition to the allowed $J_{NH-\alpha CH}$ values (Table I) consistent with the large chemical shift nonequivalence (0.8 ppm, vide infra) of the Gly[3] diastereotopic α-hydrogens. Since the anisotropic effects that lead to this extraordinarily large nonequivalence does not appear to be due to the aromatic rings in DPDPE (vide supra), we postulated that this was the result of a rather constrained glycine residue with one of the α-protons in the shielding cone of a peptide bond and the other in a deshielding region. An additional conformational consideration was the observation from the NOESY NMR studies that the Tyr[1] and Phe[4] aromatic ring protons were probably within a distance (≤4.5 Å) which allowed interaction with the D-Pen[2] β-methyl groups. This led us to examine starting conformations consistent with these observations (for a few examples see structures XII–XV, Table II) and again both right-handed and left-handed disulfide conformations were examined. We noted that often the low-energy

(35) Kumar, A.; Wagner, G.; Ernst, R.; Wüthrich, K. *J. Am. Chem. Soc.* **1981**, *103*, 3654.
(36) Vander Elst, P.; Gondol, D.; Wynants, C.; Tourwe, D.; Van Binst, G. *Int. J. Peptide Protein Res.* **1987**, *29*, 331.
(37) Bothner-By, A. A.; Noggle, J. A. *J. Am. Chem. Soc.* **1979**, *101*, 5152.
(38) Schiller, P. W. *Biochem. Biophys. Res. Commun.* **1983**, *114*, 268.
(39) de Leeuw, F. A. A. M.; Altona, C. *Int. J. Peptide Protein Res.* **1982**, *20*, 120.

Table VII. Energy Partitioning in the Energy Minimized Conformations of [D-Pen²,D-Pen⁵]Enk

energy term[a] (Kcal)	conformer 1	conformer 1′	conformer 2	conformer 2′
total energy	−12.4	−10.0	−11.9	−13.6
van der Waals	−20.0	−21.5	−22.1	−24.2
H bonds	−0.16	−0.72	−0.67	−0.40
bonds	0.70	1.10	0.93	0.79
dihedral angles	2.6	6.3	4.7	5.7
bond angles	3.7	4.1	4.5	3.9
improper dihedrals	0.52	0.43	0.39	0.29
electrostatic[b]	0.19	0.24	0.30	0.30
constraints	0.00	0.00	0.00	0.00

[a] For a detailed description of the energy terms, see ref 17. [b] A dielectric constant of 80 was used to simulate solvent.

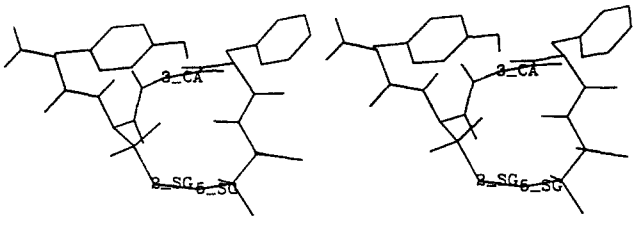

Figure 6. Stereostructure of low-energy conformation 2′ of [D-Pen²,D-Pen⁵]enkephalin (Table VII).

conformations come in pairs, which differ primarily in their disulfide helicity (right handed (+) or left handed (−)), but otherwise have only minor perturbations. Energy minimization of a number of initial models led to the four low-energy conformations shown in Table VI which were the only conformations which satisfied the NMR criteria. (In fact, conformer 1′ (Table V) does not satisfy the criteria for the Phe⁴ ϕ angle but is included because of its close conformational similarity to conformer 1.) It is interesting that for both the Phe⁴ and Tyr¹ side chains the gauche (+) (+60°) conformation appears to be excluded. We have investigated this question by building models of 2′ and 1 (Table II) with either or both χ_1 values changed from the 180 to −60° or vice versa and then doing energy minimizations. These changes had virtually no effect on the backbone conformations and only minor effects (±1 kcal) on the overall energy (data not shown). However, when conformations with χ_1 angles of +60° were used, less stable conformations were obtained.

The results from the evaluation of the individual energy terms in the selected low-energy conformations are shown in Table VII. It is of interest to consider the individual energy contributions. (The energy which results from specific conformational constraints can also be evaluated, but since none were imposed, this value, of course, is zero.) Apparently some distortion of bond angles and some dihedral angle strain is compatible with low-energy conformations as a result of the very significant stabilization provided by the intramolecular van der Waal interactions. The electrostatic energy is small in all cases because of the high dielectric constant used to represent the solvent effect. The importance of the van der Waals term was confirmed by evaluating the energy terms during the course of the energy minimization. While essentially all the energy terms were slightly reduced or showed little change during the energy minimization, the van der Waal energies decreased greatly as a result of elimination of bad contacts and the increase in the attractive interactions.

Examination of the properties of the energy-minimized structures of the conformers in Table VI indicated that the carbonyl groups of the amide bonds in the 14-membered ring (D-Pen² to Gly³, Gly³ to Phe⁴, and Phe⁴ to D-Pen⁵ amide bonds) are all pointing outward from the 14-membered ring on the side opposite of the two lipophilic aromatic rings and the β,β-dimethyl groups of D-Pen² to give an amphiphilic structure (Figure 6 shows a stereo view of the three dimensional structure of the lowest energy conformer 2′, and Figure 7 shows a space filling model of the same structure.) This structural feature led us to examine the possibility

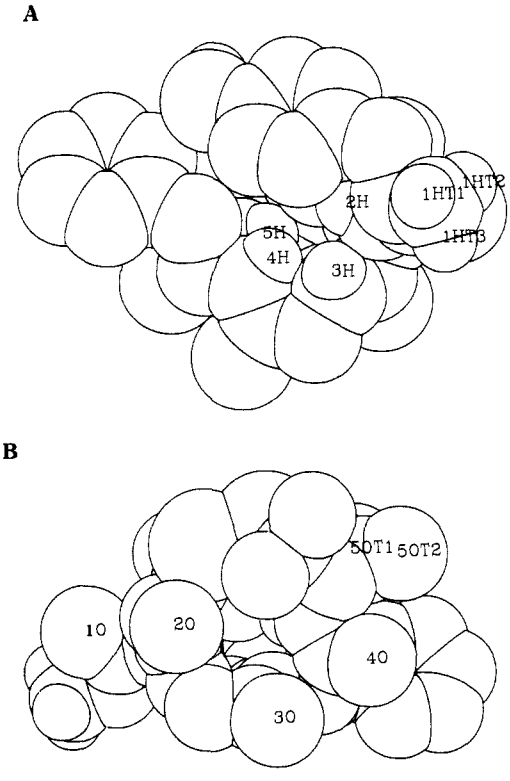

Figure 7. Structure of the low-energy conformation 2′ of DPDPE using van der Waals spheres with a view both of the lipophilic "top" view A (top) and of the more hydrophilic "bottom" view B (bottom). Note in view A how hidden the peptide NHs are for residues 2 and 5 and to a lesser extent 3 and 4 by the aromatic rings consistent with the temperature dependence data. From view B it is easily seen that all of the peptide carbonyl groups (10, 20, 30, and 40 corresponding to Tyr¹, Gly³, and Phe⁴ carbonyls, respectively) are exposed to the surrounding solvent and are located on that side of the molecule.

for conformational changes as a result of correlated motions about a peptide bond in which the ω angle was maintained, but in which the ψ_n and ϕ_{n+1} angles undergo correlated motion in a manner consistent with the same overall topology but with a different local conformation (this is similar to the conformational change that occurs in going from a type I to a type II β-turn) of the peptide bonds in the ring. A systematic study was performed by systematically modifying the three intraring amide groups one at a time: ψ D-Pen², ϕ Gly³ (14°, −98° to 175°, 87°); ψ Gly³, ϕ Phe⁴ (−18°, −72° to 157°, 68°); and ψ Phe⁴, ϕ D-Pen⁵ (−46°, 83° to 99°, −54°). The new conformations are consistent with the results from the NMR coupling constants as can be seen from Table I. The conformations generated in this way were then energy minimized. In each case the new energy-minimized conformations are of considerably higher energy than the reference conformation 2′, being respectively 3.3, 10.1, and 6.9 kcal/mol higher in energy (data not shown). We conclude that the amphiphilic conformation of 2′ (and the other low-energy conformations) is a highly favored conformation; each of the modified conformers has one or more of the carbonyl groups partly "buried" by the aromatic rings and thus no longer as accessible to the solvent. We also examined conformations with cis peptide bonds especially the Phe⁴-D-Pen⁵ bond, but in all cases these were found to be of higher energy.

Discussion

The results from combined use of NMR studies and energy minimization with a large number of starting conformations have provided us with a small family of low-energy conformations which are self-consistent in that they satisfy constraints imposed by NMR parameters and provide the lowest energy conformations. Conformer 1′ appears to be the least important because it is the highest energy conformer, but more importantly the Phe⁴ ϕ angle is not consistent with the NMR results. In conformer 1, the Phe⁴ aromatic ring is not interacting as favorably with the Pen² methyl

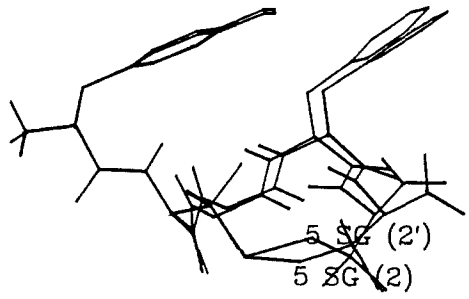

Figure 8. Superimposed structures of the low-energy conformations 2 and 2′ of [D-Pen^2,D-Pen^5]enkephalin from Table VII. Note difference in disulfide helicity.

group as in the other three low-energy conformations. Except for the screw sense of the disulfide bond, conformer 2′ and conformer 2 have very similar topologies (Figure 8). Though the helicity of the disulfide bonds in conformers 2′ and 1′ are the same (Table VI), the overall topologies differ somewhat, especially with respect to the 14-membered ring. Since conformer 2′ is the lowest energy conformation and is consistent with the NMR data, we favor it as a working model for the aqueous solution conformation of DPDPE. Also, of all the low-energy conformations, it best satisfies the NMR criteria, and there were no lower energy conformers found in this study. As with the deaminooxytocin conformation,[40] it may be that the disulfide dihedral angle can readily interconvert from a left-handed to right-handed helicity which is the essential conformational difference between 2 and 2′ (Table II). Preliminary molecular dynamics simulations indicate that interconversion from right-handed to left-handed disulfide conformations occur quite readily (unpublished results).

The conformation 2′ (and 2) has several interesting structural features. First, the 14-membered disulfide-containing ring has three trans amide bonds. Viewed from the side, this ring has a bent boatlike shape, with both of the aromatic rings of Tyr^1 and Phe^4 "above" the 14-membered ring (see Figure 6). There appear to be no strong intramolecular hydrogen bonds stabilizing the 14-membered ring conformation. Indeed, the conformation of 2′ (and the other low-energy conformations as well) approximates a type IV beta turn[21a] with some structural similarities to both type III and type I turns but with no intramolecular hydrogen bond. As noted previously, the ring amide NH protons are pointing toward this lipophilic side of the ring and thus are somewhat shielded from water. This is consistent with the small temperature dependence of the amide proton chemical shifts found for DPDPE. As a result, all of the amide carboxyls are pointing outward, away from the 14-membered ring on the side which otherwise is featureless and fully exposed to solvent (Figure 7). The Gly^3-αCH_2 protons are positioned quite differently with respect to the amide bonds which flank it, consistent with the NMR data. Stabilization of these conformational features comes from the interactions of the aromatic side chain groups with the β-methyl groups and sulfur of the D-Pen residue. This kind of conformation gives the peptide a decidedly amiphiphilic character with a lipophilic side and a more hydrophilic side.

Though the present study does not establish a relationship between the conformation of DPDPE in aqueous solution and the biologically active conformation, its properties can provide some insight into the features which may be important in binding to the δ opioid receptor. More importantly, it can suggest features that play a role in the high specificity of DPDPE for the δ opioid receptor. In particular, since so few opioid peptides, or for that matter other compounds, have selectivity for the δ receptor (most have no selectivity or favor the μ receptors[2,3,8-10]), the conformation properties found here may be significant. The exceptions to the above are the linear δ receptor selective compounds of Roques and co-workers such as Tyr-D-Ser-Gly-Phe-Leu-Thr (DSLET) and Tyr-D-Thr-Gly-Phe-Leu-Thr (DTLET) and their *O-tert*-butyl ethers.[41,42] There have been some efforts to determine their conformational properties.[43] These linear peptide analogues are quite conformationally flexible, and thus they appear to have a wide variety of possible conformations. In preliminary studies, Loew et al.[43] have used the empirical energy program ECEPP to compare DTLET conformations to low-energy conformations for [D-Pen^2,L-Pen^5]enkephalin. Their results suggest that the flexible DTLET can assume a conformation in which the aromatic rings in positions 1 and 4 have considerable overlap and are on the same face in fairly close proximity. Our studies suggest that indeed the aromatic rings are in close proximity in DPDPE. Though there is disagreement about the "biological active conformation" of opioids at the μ receptor,[3,9,37] there is some evidence that the peptide is folded with the two aromatic rings of Tyr^1 and Phe^4 separated from each other (the amount of separation depending on the investigator). The topological proximity of the Tyr^2 and Phe^4 aromatic rings and their mutual interaction with the Pen β,β-dimethyl groups of DPDPE suggests that this lipophilic, topological arrangement may be critical for binding of the peptide to the δ receptor in preference to the μ receptor. Since DPDPE has no significant interaction with the κ receptor, it would appear that such a topological feature is also not compatible with interaction with the κ receptor. In this regard, it is interesting to note that the NMR results indicated that only the trans (180°) and gauche (−) (−60°) conformations are highly populated. In fact, only these two conformations are consistent with the topographical features of conformation 2′ (Table II) in which the aromatic ring of Tyr^1 interacts with the D-Pen β-methyl groups and is in close proximity to the Phe^4 aromatic ring as well. Similarly, only the gauche (−) (−60°) and trans (180°) χ_1 conformations are compatible with the Phe^4 aromatic ring interacting with the D-Pen^2 β-methyl groups in the low-energy conformation. Stammer at al.[47] have reported that the leucine enkephalin analogue with an (*E*)-dehydrophenylalanine in position 4 has very low potency (with slight δ selectivity), whereas the analogue with a (*Z*)-dehydrophenylalanine in position 4 has high potency with some δ selectivity. Though the overall conformational properties of these analogues are unknown, it is interesting that in (*E*)-dehydrophenylalanine the aromatic ring is held rigidly in the trans conformation, whereas in (*Z*)-dehydrophenylalanine the aromatic ring is held rigidly between the gauche (−) and gauche (+) conformations due to the C_α–C_β double bond. Taken together, these results and the present conformational studies might suggest that the Phe^4 aromatic side chain would prefer the gauche (−) conformation for binding to the δ opioid receptor. A possible suggestion for the transduction step is that it involves changes in the peptide–receptor interaction in which the aromatic side chain groups move from the gauche (−) to the trans or other conformation about the χ_1 bond. The transduction step may also involve a disulfide conformational change from left-handed to right-handed (or vice versa). Similar suggestions have been made in

(40) Wood, S. P.; Tickle, I. J.; Treharne, A. M.; Pitts, J. E.; Mascarenhas, Y.; Li, J. Y.; Husain, J.; Cooper, S.; Blundell, T. L.; Hruby, V. J.; Wyssbrod, H. R.; Buku, A.; Fishman, A. J. *Science (Washington: D.C.)* **1986**, *232*, 633.

(41) Gacel, G.; Fournie-Zaluski, M.-C.; Roques, B. P. *FEBS Lett.* **1980**, *118*, 245.

(42) Zajac, J.-M.; Gacel, G.; Petit, F.; Dodey, P.; Rossignot, P.; Roques, B. P. *Biochem. Biophys. Res. Commun.* **1983**, *111*, 390.

(43) Loew, G. H.; Toll, L.; Uyens, E.; Cheng, A.; Judd, A.; Lawson, J.; Keys, C.; Amsterdam, P.; Polgar, W. In *Opioid Peptides: Medicinal Chemistry*; Rapaka, R. S., Barnett, G., Hawks, R. L., Eds.; NIDA Research Monograph 69, Rockville, MD, 1986; pp 231–256.

(44) Portoghese, P. S.; Alreja, B. D.; Larsen, D. L. *J. Med. Chem.* **1981**, *24*, 782.

(45) Pastori, A.; Tancredi, T.; Temussi, P. A.; Salvadori, S.; Tomatis, R. In *Peptides: Structure and Function*; Deber, C. M., Hruby, V. J., Kopple, K. D., Eds.; Pierce Chemical Co.: Rockford, IL, 1985; pp 529–532.

(46) (a) Schiller, P. W. In *The Peptides: Analysis, Structure, Biology. Vol. 6. Opioid Peptides: Biology, Chemistry, and Genetics*. pp 220–268. (b) Rapaka, R. S.; Renugopalakrishnan, V.; Bhatnagar, R. S. *Am. Biotech. Lab.* **1985**, *3*, 8.

(47) Shimohigashi, Y.; Costa, T.; Nitz, T. J.; Chen, H. C.; Stammer, C. H. *Biochem. Biophys. Res. Commun.* **1984**, *121*, 966. Nitz, T. J.; Shimohigashi, Y.; Costa, T.; Chen, H. C.; Stammer, C. H. *Int. J. Peptide Protein Res.* **1986**, *27*, 522.

a dynamic model of oxytocin-receptor interaction and transduction.[4a,40,48] Studies are in progress to test these models by use of additional conformational restrictions[1,14] and by molecular dynamics simulations, coupled with NMR constraints.[49]

(48) (a) Meraldi, J.-P.; Hruby, V. J.; Brewster, A. I. R. *Proc. Natl. Acad. Sci. U.S.A.* **1977**, *74*, 1373. (b) Hruby, V. J.; Mosberg, H. I.; Hadley, M. E.; Chan, W. Y.; Powell, A. M. *Int. J. Peptide Protein Res.* **1980**, *16*, 372. (c) Hruby, V. J.; Mosberg, H. I. In *Neurohypophyseal Peptide Hormones and Other Biologically Active Peptides*; Schlesinger, D. H., Ed.; Elsevier/North Holland: New York, 1981; pp 211-226. (d) Hruby, V. J. In *Topics in Molecular Pharmacology*; Burgen, A. S. V., Roberts, G. C. K., Eds.; Elsevier/North Holland: Amsterdam, 1981; pp 99-126.

Acknowledgment. This work was supported by grants from the U.S. Public Health Service Grants to V.J.H. (NS-19972) and M.K. and from the National Science Foundation (V.J.H.). V.J.H. thanks the Guggenheim Foundation for a Fellowship that supported in part his research done at Harvard University. R.M.P. thanks the Robert A. Welch Foundation and the Texas Advanced Technology Research Projects Coordination Board for support.

Registry No. DPDPE, 88373-73-3.

(49) Brünger, P. T.; Clore, G. M.; Gronenborn, A. M.; Karplus, M. *Proc. Natl. Acad. Sci. U.S.A.* **1986**, *83*, 3801.

Side Chain Dynamics of Crystalline L-[3,3-$^2H_2$]Methionine Studied by Deuterium NMR Spectroscopy

S. W. Sparks, N. Budhu,† P. E. Young,† and D. A. Torchia*

Contribution from the Bone Research Branch, National Institute of Dental Research, National Institutes of Health, Bethesda, Maryland 20892, and the Department of Chemistry, York College, City University of New York, Jamaica, New York 11451. *Received April 21, 1987*

Abstract: In order to better understand the dynamics of the side chain of methionine, we have measured and analyzed ^2H nuclear magnetic resonance line shapes and spin-lattice relaxation times in polycrystalline L-[3,3-$^2H_2$]methionine from -35 to +106 °C. At -35 °C, the C^β-^2H bond axes are nearly rigid and execute only small amplitude librations. However, at temperatures above 0 °C, two dynamically distinct types of methionine side chains, designated A and B, are observed and are assigned to the two crystallographically different methionine side chains found in the unit cell. The values of the generalized order parameters of the A and B side chains decrease from unity at -35 °C to 0.69 and 0.25, respectively, at 106 °C. At 38.45 MHz, we are able to analyze all the data obtained for both types of side chains using a two-site jump model. Using this model we find the respective A side chain and B side chain correlation times decrease from 0.45 to 0.019 ns and from 4.2 to 0.049 ns in the -19 to 77 °C temperature range. The apparent activation energy for the A side chain is 21.6 ± 8.3 kJ/mol, whereas the apparent activation energy of the B side chain is 30.9 ± 1.6 kJ/mol below 31 °C and 30.9 ± 4.9 kJ/mol above 50 °C. The Arrhenius plot of the B side chain correlation times is nonlinear in the 30-50 °C range, where crystalline methionine undergoes a phase transition. At temperatures above 50 °C, the analysis of the ^2H line shapes shows that the populations of the two sites are nearly equal and that the jump angle is ca. 90°. Using a modified two-site jump equation and a six-site computer calculation to analyze the field (at 38.45 and 76.77 MHz) and orientation dependence of T_1 at low temperature, we find that the jump angle remains fixed at ca. 90° but that the difference in relative populations of the two sites approaches unity as the temperature decreases from 24 to -35 °C. Because the 90° jump angle corresponds approximately to jumps on a tetrahedral lattice, we conclude (a) that the C^α-C^β bond of the B side chain undergoes rapid trans-gauche isomerization and (b) that the trans isomer is the predominate species below 0 °C, while the isomer populations are nearly equal above 50 °C.

The recent interest in understanding the internal dynamics of proteins[1,2] has stimulated studies of amino acid side chain dynamics in crystalline amino acids[3-8] and peptides.[9,10] At first sight, the stable crystals formed by these compounds would appear to offer unfavorable habitats for molecular motion. However, significant motions have been observed for a number of side chains of hydrophobic amino acids (e.g., Phe, Tyr, Pro, and Met) in the crystalline state. Motions of side chains of hydrophobic residues are of particular interest, because these residues are often buried within proteins. Therefore, studies of motions of hydrophobic side chains of amino acids in the crystalline state can provide information about possible internal motions within proteins.

Among the side chain motions studied so far, the motions of the side chain of L-methionine are the most complex and potentially interesting. Large changes in the line shapes of the methyl deuterons are observed in ^2H NMR spectra of crystalline L-[*methyl*-$^2H_3$]methionine in the 10-80 °C temperature range.[4] It has been shown[4,11] that these changes in line shape are not due to either rotation about the S-C^ϵ bond (the changes in line shape caused by methyl rotation occur at temperatures below -130 °C) or motions involving reorientation of the C^α-H bond. Therefore motions about the C^α-C^β, C^β-C^γ, and/or C^γ-S bonds are responsible for the observed changes in methyl deuteron line shapes.

* National Institutes of Health.
† York College.

(1) Gurd, F. R. N.; Rothgeb, T. M. *Adv. Protein Chem.* **1979**, *33*, 73-165.
(2) Karplus, M.; McCammon, J. A. *CRC Crit. Rev. Biochem.* **1981**, *9*, 293-349.
(3) Gall, C. M.; DiVerdi, J. A.; Opella, S. J. *J. Am. Chem. Soc.* **1981**, *103*, 5039-5043.
(4) Keniry, M. A.; Rothgeb, T. M.; Smith, R. L.; Gutowsky, H. S.; Oldfield, E. *Biochemistry* **1983**, *22*, 1917-1926.
(5) Torchia, D. A. *Annu. Rev. Biophys. Bioeng.* **1984**, *13*, 125-144.
(6) Shaefer, J.; Stejskal, E. O.; Mackay, R. A.; Dixon, W. T. *J. Magn. Reson.* **1984**, *57*, 85.
(7) Hiyama, Y.; Silverton, J. V.; Torchia, D. A.; Gerig, J. T.; Hammond, S. J. *J. Am. Chem. Soc.* **1986**, *108*, 2715-2723.
(8) Sarkar, S. K.; Young, P. E.; Torchia, D. A. *J. Am. Chem. Soc.* **1986**, *108*, 6459-6464.
(9) Rice, D. M.; Wittebort, R. J.; Griffin, R. G.; Meriovitch, E.; Meinwald, Y.; Freed, J. H.; Scherage, H. A. *J. Am. Chem. Soc.* **1981**, *103*, 7707-7710.
(10) Sarkar, S. K.; Torchia, D. A.; Kopple, K. D.; VanderHart, D. L. *J. Am. Chem. Soc.* **1984**, *106*, 3328-3331.
(11) Batchelder, L. S.; Niu, C. H.; Torchia, D. A. *J. Am. Chem. Soc.* **1983**, *105*, 2228-2231.

0002-7863/88/1510-3359$01.50/0