Conformational Analysis of a Cyclic Enkephalin Analogue by ^{1}H NMR and Computer Simulations

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Abstract: An investigation of the conformations of a highly active and selective cyclic enkephalin analogue was carried out to gain further understanding of the structure–activity relationships of opioid peptides. Cyclization of the enkephalin incorporates constraints which limit the number of possible conformations. The conformation of the cyclic enkephalin analogue, H-Tyr-c[-D-A_2bu-Gly-Phe-Leu-] (where A_2bu represents α,γ-diaminobutyric acid), was probed by computer simulations including molecular dynamics and energy minimizations and by proton NMR studies. Molecular dynamics shows the reduced mobility of the ring structure resulting from the cyclization and the kind of conformational transitions that can be expected. Energy minimizations show the existence of a small number of backbone structures. The conformations derived from the computer calculations and experimental NMR data are in good agreement. Two transannular hydrogen bonds are present in Me_2SO-d_6 solution, Leu NH to Gly CO and D-A_2bu side chain NH to either D-A_2bu CO or Phe CO. The hydrogen bond involving the Leu NH is disrupted with the addition of H_2O, but the overall ring conformation does not undergo major changes.

Opioid peptides have been of interest as possible substitutes for alkaloid opiate drugs and for their biological importance as natural analgesics. For an understanding of the physiological response triggered by the binding of these molecules, knowledge of the structure of the receptor and the conformation of the opioid is needed. By specifically modifying peptide opiates and monitoring binding, biological activity, and receptor selectivity, the important pharmacophores of the enkephalins have been elucidated.[1]

Because of the tremendous flexibility of linear enkephalins, it has been impossible to ascertain the spatial array of the functionalities necessary for biological activity. Constraints have been incorporated via cyclization to limit conformational mobility.[2-4] From the decreased number of possible conformations available to an active constrained peptide, the approximate receptor shape may be surmised.

A very active cyclic enkephalin was first synthesized by DiMaio and Schiller.[5] This compound shows high μ(alkaloid) receptor selectivity arising from the constraints of the 14-membered ring.[2] The importance of this enkephalin analogue prompted us to study this compound and several isomers.[6-9] Here we report the conformational analysis of the parent compound, H-Tyr-c[-D-A_2bu-Gly-Phe-Leu-],[10] shown in Figure 1, using two complementary physical techniques, computer simulation of dynamic and energetic properties, and proton magnetic resonance.

Materials and Methods

Computational Methods. The molecular dynamics of the enkephalin analogue (in vacuo) was simulated by numerical integration of Newton's equations of motion. The forces acting on each atom were calculated from a valence force field including van der Waals and electrostatic terms for nonbonded interactions, developed by Hagler and co-workers.[11] The integration was carried out with a fourth-order Gear algorithm[12] with a time step of 0.5 fs. The simulation, lasting 10 ps, was preceeded by an equilibration period of 2000 time steps (1 ps). During equilibration, the temperature was adjusted to approximately 300 K by increasing the atomic velocities in small steps, keeping the total momentum equal to zero to prevent translation or rotation of the molecule. The starting conformation was partially minimized with 200 steps of steepest descent minimization. All of the atoms in the molecule, 82 including hydrogens, were allowed to move without restriction. The calculations were carried out on a VAX 11/780, each picosecond of the simulation taking approximately 2 h of CPU time. An Evans and Sutherland Picture System was used to display the atomic trajectories.

The molecular dynamics simulation was used as a source of initial conformations for energy minimization studies, by taking the coordinates of the molecule at regular intervals of 0.5 ps and minimizing the energy with respect to all the Cartesian coordinates of the molecule by using a steepest descent method followed by a modified Newton–Raphson method.[13]

Although the molecular dynamics provides insights into the motional properties of the cyclic enkephalin, it does not cover all the conformational space available to the molecule. Because of this limitation, a second procedure was used to generate minimum energy conformations. Each residue of the molecule was systematically forced to five different combinations of ϕ and ψ by assigning large values to the corresponding torsional parameters (100 kcal/(mol·deg)). These starting conformations were α helix (−60, −60), reversed α helix (60, 60), C_7 equatorial (−80, 80), C_7 axial (80, −80), and extended (180, 180), where the numbers in parentheses refer to pairs of ϕ, ψ torsion angles. The energy was minimized thus allowing all the other degrees of freedom to accommodate the new conformation. Because of the large potential parameters, the selected torsion angles converge to the specified values upon minimization. After the molecule has reached the minimum under these constraints, a second minimization is carried out, with normal potential parameters, allowing the molecule to relax. If the forced conformation is near a true minimum, the molecule reaches it in the relaxation step. If the forced conformation does not correspond to a true minimum, the molecule undergoes major conformational changes upon relaxation. This procedure led to several minima with different backbone conformations. The preferred conformations of the side chains were investigated by systematically forcing the side-chain groups of Tyr, Phe, and Leu to the g^+, t, and g^- conformations, corresponding to χ_1 in the three intervals (0° to 120°), (120° to 240°), and (240° to 360°), respectively. Several combinations of side-chain conformations were found for each particular backbone structure.

Experimental Methods. The cyclic enkephalin analogue, originally prepared by Schiller, was resynthesized in our laboratory. Proton NMR spectra were obtained on a 360-MHz NMR spectrometer built in-house from a Varian instrument equipped with an Oxford magnet and a Nicolet 1280 computer. Water suppression was achieved by the symmetric 1331 pulse sequence, found to be more effective than the antisymmetric version

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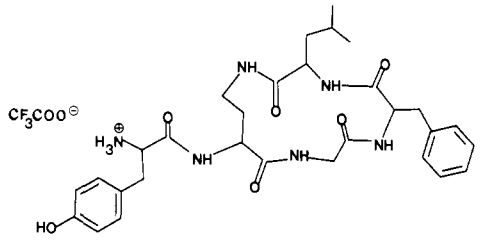

Figure 1. Structure of H-Tyr-c[-D-A_2bu-Gly-Phe-Leu-].

Table I. Minimum Energy Conformations Obtained from the Molecular Dynamics Simulation

	minimum[a]				
	VIII		III		I
time[b]	0.5		1.0		1.5–10.0
energy[c]	17.7		4.0		0.0
tyr					
ψ	−59	→	104		104
χ_1	−68		−72		−72
χ_2	101		101	→	−81
D-A_2bu					
ϕ	−60	→	87		84
ψ	−79		−95		−92
χ_1	147		158		164
χ_2	66		68		73
χ_3	−110		−118	→	−7
Gly					
ϕ	67		71		71
ψ	67		70		76
Phe					
ϕ	75		73		74
ψ	−73		−75		−67
χ_1	−61	→	−169		−167
χ_2	98		70		82
Leu					
ϕ	−97		−94		−76
ψ	62		66	→	−56
χ_1	−64		−65		−61
χ_2	54		54		57
χ_3	−178		−178		−178

[a] Energy minima are classified according to their energies. Minima obtained from all the different methods are included in the classification. [b] Time during the molecular dynamics simulation at which the energy was minimized, in picoseconds. [c] Energy relative to the absolute minimum, in kcal/mol. The arrows indicate torsion angles which undergo conformational transitions.

on our spectrometer.[14] Assignments were made based on two-dimensional shift correlation[15] and relayed coherence transfer spectroscopy.[16] A 31-mM solution was prepared in Me_2SO-d_6 (MSD Isotopes). The titration/temperature studies were carried out by adding H_2O to the Me_2SO-d_6 solution and obtaining spectra at 5–7 temperatures over a range of 20–65 °C for several solvent compositions.

Results

Molecular Dynamics and Energy Minimizations. The average temperature of the molecular dynamics simulation was 293 K, corresponding to an average kinetic energy of 71.6 kcal/mol. The average potential energy during the simulation was 203.7 kcal/mol; this value is about 70 kcal/mol higher than the potential energy surface obtained by minimization of the energy during the simulation. This shows that the kinetic energy added to the system in the equilibration period was equipartitioned between kinetic and potential energy.

To compare the motional freedom of the ring atoms relative to the other atoms of the molecule, the root mean square deviations

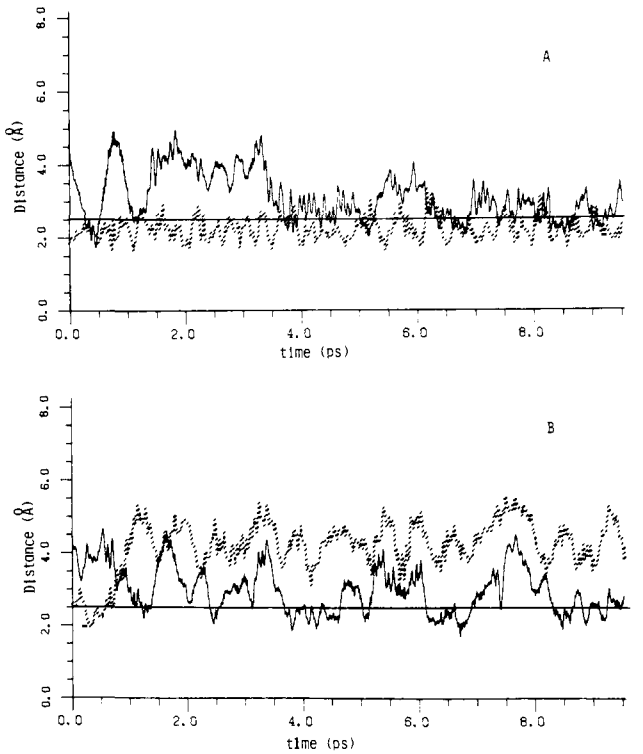

Figure 2. Time variation of the distance between amide protons and carbonyl oxygens involved in hydrogen bonds in the molecular dynamics simulation: A, (—) Tyr CO to Gly NH, (···) Gly CO to Leu NH; B, (—) D-A_2bu CO to D-A_2bu NH in the side chain, (···) Phe CO to D-A_2bu NH in the side chain.

of the atomic coordinates were calculated. The atoms were classified as belonging to the backbone within the ring, to the backbone outside the ring, or to the side chains. The root mean square deviations, 0.77, 1.22, and 1.23 Å, respectively, show the constraint imposed by the 14-membered-ring structure.

Fluctuations of the torsion angles about equilibrium values are large and in some cases lead to dynamic transitions between different conformational states. Energy minimizations revealed that three local minima were visited during the simulation. Table I shows the torsion angles defining these structures. Minima VIII and III, obtained at 0.5 and 1.0 ps, respectively, differ in the torsion angles Tyr ψ, D-A_2bu ϕ, and Phe χ_1. Minimum I is a result of transitions involving Tyr χ_2, Leu ψ, and D-A_2bu χ_3. This minimum was obtained from all the minimizations after 1.5 ps. Examination of the time variation of the torsion angles during the dynamics revealed that the conformational transitions were highly correlated in time, particularly those involving ring atoms. Pairs of torsion angles underwent simultaneous changes from one equilibrium state to another.

Hydrogen bonds were continuously breaking and reforming during the simulation. They were classified as stable when present more than 50% of the time and unstable otherwise. Figure 2 presents time profiles of the distance between donor and acceptor atoms in hydrogen bonds, showing their dynamic character.

The local minima obtained from the molecular dynamics simulation were among the minima generated from the systematic forcing of the molecule. Table II shows the different backbone conformations and hydrogen bond patterns present in the structures within 20 kcal/mol of the absolute minimum. Variation of the side-chain conformations showed that the three equilibrium positions t, g^-, and g^+ were allowed for the side chains of Tyr, Phe, and Leu, but with different stabilities. For Leu and Phe, the three positions are almost equally stable, within 1.0 kcal/mol. For Tyr, the t position is more stable, by more than 3.0 kcal/mol. This property for the side-chain populations was found for several backbone conformations.

Nuclear Magnetic Resonance. The assigned one-dimensional spectrum of the enkephalin analogue in Me_2SO-d_6 is shown in

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Table II, Ring Conformations and Hydrogen Bonds Observed in the Minimum Energy Conformations

minimum[a]	energy[b]	ring conform.[c]	hydrogen bonds
I	0.0	C*A*C*A	Gly C=O --- Leu N—H D-A_2bu C=O --- D-A_2bu N—H (side chain) Tyr C=O --- Gly N—H
II	1.4	C C C*C	Gly C=O --- Leu N—H Phe C=O --- D-A_2bu N—H (side chain) Tyr C=O --- Gly N—H D-A_2bu C=O --- Phe N—H
III	4.0	C*A*C*C	Gly C=O --- Leu N—H Phe C=O --- D-A_2bu N—H (side chain) Tyr C=O --- Gly N—H
IV	5.4	E*C C*C	Gly C=O --- Leu N—H
V	8.4	C A A D	Tyr C=O --- Gly N—H
VI	8.8	C C A*C*	Phe C=O --- D-A_2bu N—H (side chain) Tyr C=O --- Gly N—H D-A_2bu C=O --- Phe N—H
VII	9.3	A C*A C	D-A_2bu C=O --- Phe N—H
VIII	17.7	A A*C*C	Gly C=O --- Leu N—H Phe C=O --- D-A_2bu N—H (side chain)

[a] Only minima with different backbone conformations are included. Several combinations of side chain orientations are allowed for each minimum. The relative energies remain the same. [b] Energies relative to the absolute minimum, in kcal/mol. [c] The letter code developed by Zimmerman et al.[19] is used. Each letter corresponds to a specific region of the ϕ–ψ map, in particular A = α helix, A* = reversed α helix, C = C_7 equatorial, C* = C_7 axial.

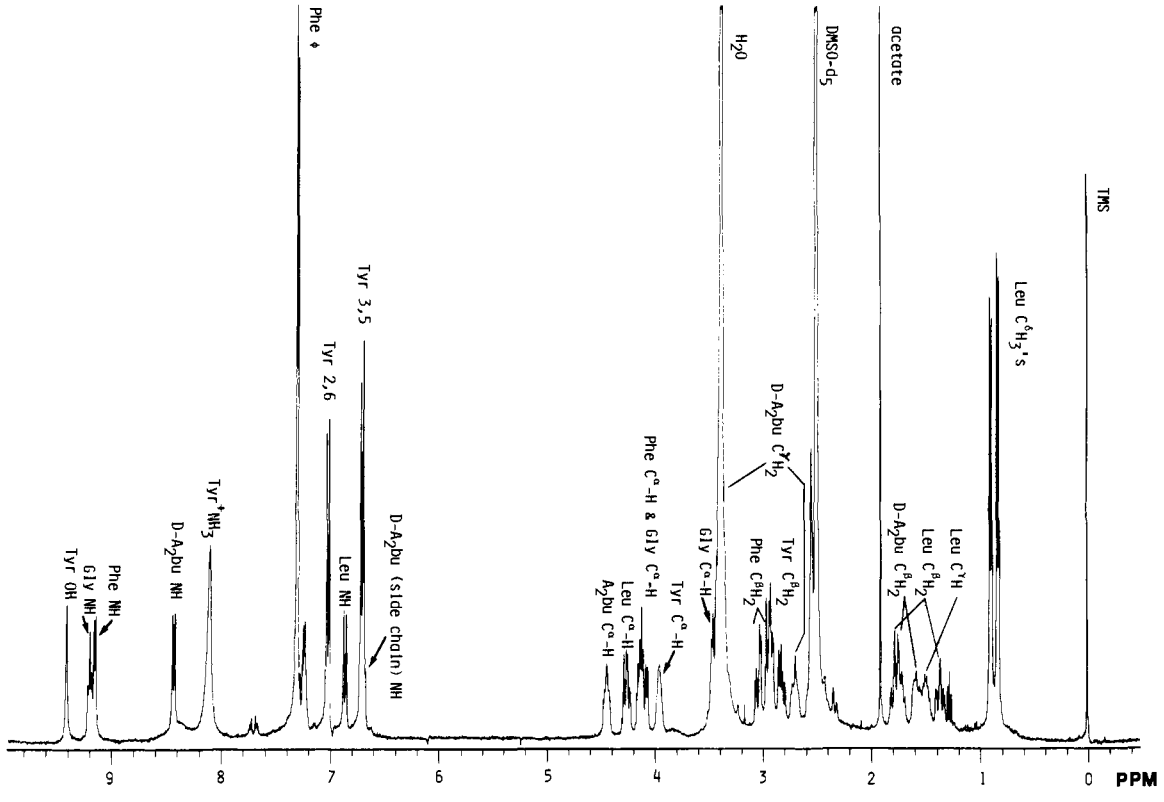

Figure 3, Assigned ^{1}H NMR spectrum of H-Tyr-c[-D-A_2bu-Gly-Phe-Leu-] in Me_2SO-d_6.

Figure 3. Unambiguous assignment of D-A_2bu and Leu resonances was possible with shift correlation and relayed coherence spectra. The latter is shown in Figure 4 with D-A_2bu and Leu coupling patterns indicated.

The results of the H_2O titration are shown in Figures 5 and 6. At each solvent composition, the least-squares line was calculated for chemical shift vs. temperature for every NH resonance. In Figure 5, chemical shifts are plotted vs. solvent composition for three temperatures as calculated from the least-squares lines. The spread between the curves for each resonance reflects temperature coefficients. The error bars on the 20 °C points indicate the 95% confidence interval for all three points for a given resonance and solvent composition. Temperature coefficients vs. solvent composition are shown in Figure 6. Three types of behavior are apparent; three protons maintain high temperature coefficients, one maintains a comparatively low temperature coefficient, and the last amide changes from the low to the high region with the addition of H_2O.

The $^3J_{NH-C^\alpha H}$ coupling constants of D-A_2bu, Phe, and Gly amide protons show small (0.0–1.1 Hz) changes between the pure Me_2SO-d_6 and 50% H_2O in Me_2SO-d_6 solution which correspond to changes in ϕ torsions of less than 12°. Coupling constants and calculated ϕ torsions are shown in Table III. For D-A_2bu, Leu, and Gly, there are torsions that are within 10° of those found in

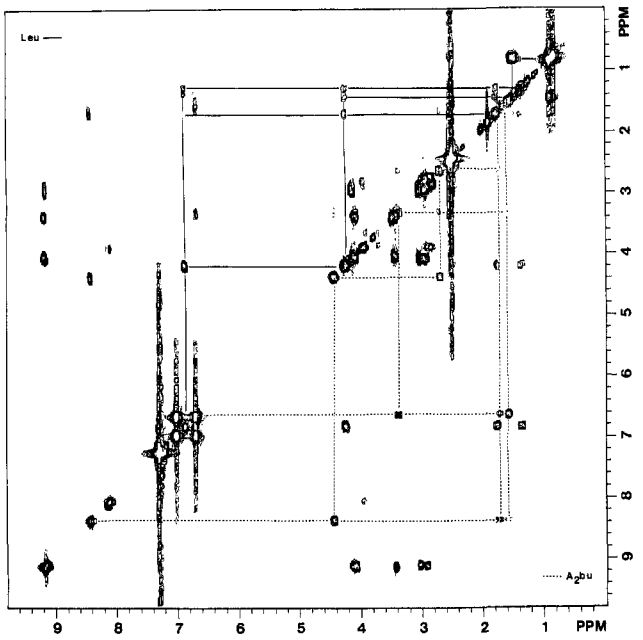

Figure 4. Two-dimensional relayed coherence transfer spectrum of H-Tyr-c[-D-A_2bu-Gly-Phe-Leu-]. The ^3J and ^4J crosspeaks were used to assign resonances in conjunction with the shift correlation spectrum. The coupling patterns of D-A_2bu and Leu are indicated.

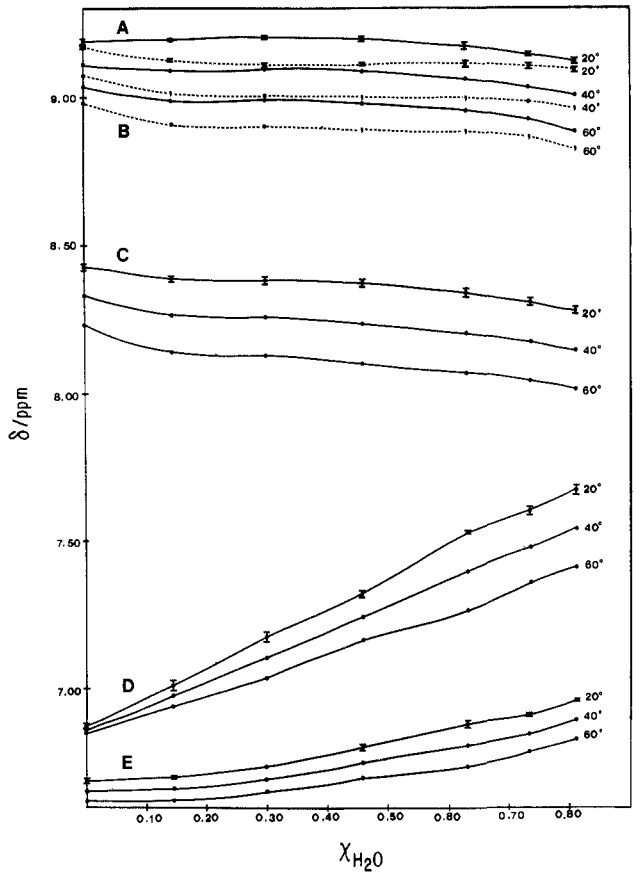

Figure 5. Chemical shifts of amide resonances vs. mole fraction of H_2O in Me_2SO-d_6 obtained from temperature studies. The results shown are at three interpolated temperatures: A, Gly; B, Phe, C, D-A_2bu; D, Leu; E, D-A_2bu (side chain). No error bar is shown for E (χ = 0.30) where two points were used to calculate this point because of overlapping peaks.

minima I and III from the computer minimizations. One calculated Phe torsion is within 30° of the ϕ's found in minima I and III.

A control study was carried out to detect aggregation. A range of concentrations was examined with a constant solvent composition of 50% (v/v) H_2O in Me_2SO-d_6. This composition was chosen as a control solution because it has the highest water concentration employed in the titration study and as such is the most likely to encourage aggregation. Spectra were obtained at concentrations of 11 to 31 mM with no appreciable change in peak widths or positions. We conclude that aggregation does not occur under our experimental conditions.

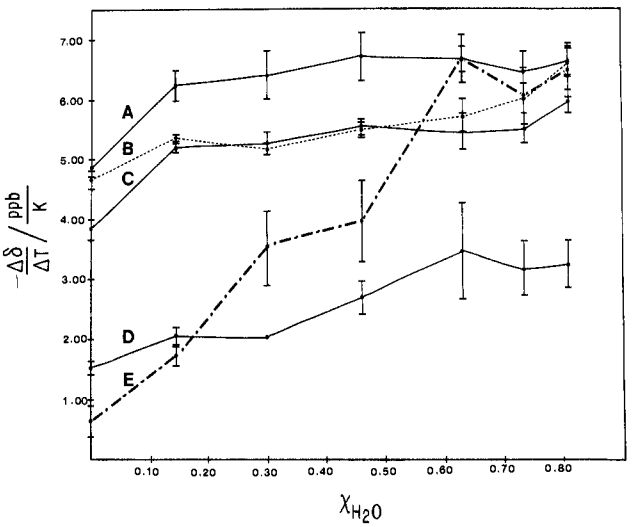

Figure 6. Temperature coefficients of amide resonances vs. mole fraction of H_2O in Me_2SO-d_6: A, D-A_2bu; B, Phe; C, Gly; D, D-A_2bu (side chain); E, Leu. No error bar is shown where two points were used to calculate this point because of overlapping peaks.

Table III. $^3J_{NH\text{-}C^\alpha H}$ Coupling Constants from NMR Spectra and Corresponding Torsions[a] for Pure Me_2SO-d_6 Solution and a 0.81 Mole Fraction of H_2O in Me_2SO-d_6 Solution

	100% Me_2SO-d_6		0.81 mole fraction H_2O	
residue	J	ϕ	J	ϕ
		−77[b]		−87
D-A_2bu	7.93	−43	6.84	−32
		87		81
		153		160
		−127		−125
Gly	12.20[c]	−61	12.50[c]	−63
		61[b]		63
		127		125
		−168		−174
Phe	5.40	−72	4.32	−66
		98[b]		105
		22		15
Leu	9.37	−143	9.37	−143
		−97[b]		−97

[a] Torsions were calculated after Bystrov.[27] [b] These values are in agreement with energy minima I and III. [c] The Gly coupling constants are $\Sigma(^3J_{NH\text{-}C^\alpha H})$.

Discussion

The ultimate goal of our enkephalin research is an understanding of peptide opioid binding to the receptor(s) and subsequent signal transduction. Linear enkephalin analogues have only elucidated the functional groups necessary for activity. Useful peptide probes of receptor shape are now being synthesized in the form of cyclic analogues. We are closer to a rigid peptide model of the receptor with the 14-membered-ring analogue under investigation. It is important to recognize, however, that this compound is not rigid. Constraints have been imposed which have reduced the range of conformational space available so that there remain few equilibrium conformations about which the molecule fluctuates.

The molecular dynamics simulation shows the enkephalin analogue undergoing continuous motion. Transitions occur in

which the molecule surmounts energy barriers to arrive in new conformational states on a picosecond time scale. The torsion angles exhibit the largest fluctuations as the softest degrees of freedom. The transitions are typically rotations of peptide groups, i.e., the correlated movement of the ψ torsion of one residue and the ϕ torsion of the next. They also involve side-chain reorientations among the t, g^+, and g^- conformations and flipping of the aromatic rings.

In the dynamics simulation, the 14-membered ring is stabilized by the formation of hydrogen bonds which, because of molecular vibration and transitions, break and reform several times to give rise to different patterns. The most stable hydrogen bonds were found to give C_7 structures, much like the γ-turn model of cyclic pentapeptides though the ring is one atom smaller in this case.[17,18]

The conformation calculated by the minimizations to have the lowest energy, minimum I, contains two transannular hydrogen bonds, between D-A_2bu side chain NH and carbonyl and Leu NH and Gly carbonyl. There is a third hydrogen bond involving Gly NH and Tyr CO, which is outside the ring. This conformation dominates the molecular dynamics simulation in which the transannular hydrogen bonds are more stable than the hydrogen bond outside the ring structure. A small conformational change leads from minimum I, with backbone structure C*A*C*A to minimum III, with a C*A*C*C conformation, where the letter code developed by Zimmerman et al.[19] is used to represent the conformation of residues 2 to 5, D-A_2bu, Gly, Phe, and Leu, respectively. Residue 1, Tyr, is not included because the corresponding ϕ angle represents only rotation of the terminal amino group. Minimum III shows the same hydrogen bonds as minimum I, but the NH of D-A_2bu is associated with the Phe carbonyl instead of the D-A_2bu carbonyl. The amide proton of Leu remains hydrogen bonded to the Gly carbonyl in this structure. Minimum II, with backbone conformation CCC*C and a hydrogen bond between Phe NH and D-A_2bu CO (among others), differs substantially from minima I and III. It was not observed in the dynamics and is not supported by the NMR data.

Experimental NMR evidence confirms the presence of the intramolecular hydrogen bonds consistent with the computer simulations. Temperature coefficients of the cyclic enkephalin analogue in 100% Me_2SO-d_6 indicate a stable hydrogen bond involving the Leu NH and a somewhat less stable hydrogen bond involving the side-chain NH of D-A_2bu. We deduce from the computer calculations that the acceptor of the Leu NH is the Gly carbonyl and the acceptor for the D-A_2bu NH may be either the D-A_2bu or Phe carbonyl. The two possible conformations of the cyclic enkephalin analogue in Me_2SO-d_6 consistent with the NMR data and the computer simulations are shown in Figure 7. They correspond to energy minima I and III.

The hydrogen bond involving the Leu amide proton is disrupted upon the addition of H_2O as shown by the large change in temperature coefficient, −0.66 in pure Me_2SO-d_6 to −6.5 ppb/K in a 0.81 mole fraction of H_2O. Thus, the backbone conformation of this cyclic peptide changes from the double hydrogen bonded structure found in the Me_2SO-d_6 solution to a conformation with a single hydrogen bond in solutions with substantial H_2O concentrations. The two conformations are not very different as evidenced by the small changes in coupling constants.

It is interesting to note that accompanying the disruption of the hydrogen bond involving the Leu amide proton, there is a large (0.40 ppm) downfield shift of the NH resonance. This indicates that the intramolecular hydrogen bond to a carbonyl is less deshielding of the amide proton than hydrogen bonding to H_2O or Me_2SO-d_6. The effect is not limited to this compound; we have seen the amide resonances shifted downfield when a hydrogen bond is disrupted upon addition of H_2O in other peptides. For example, two retro-inverso enkephalin analogues, the diastereomers, H-Tyr-c[-D-A_2bu-gemGly-R,S-malonylPhe-Leu-], show similar

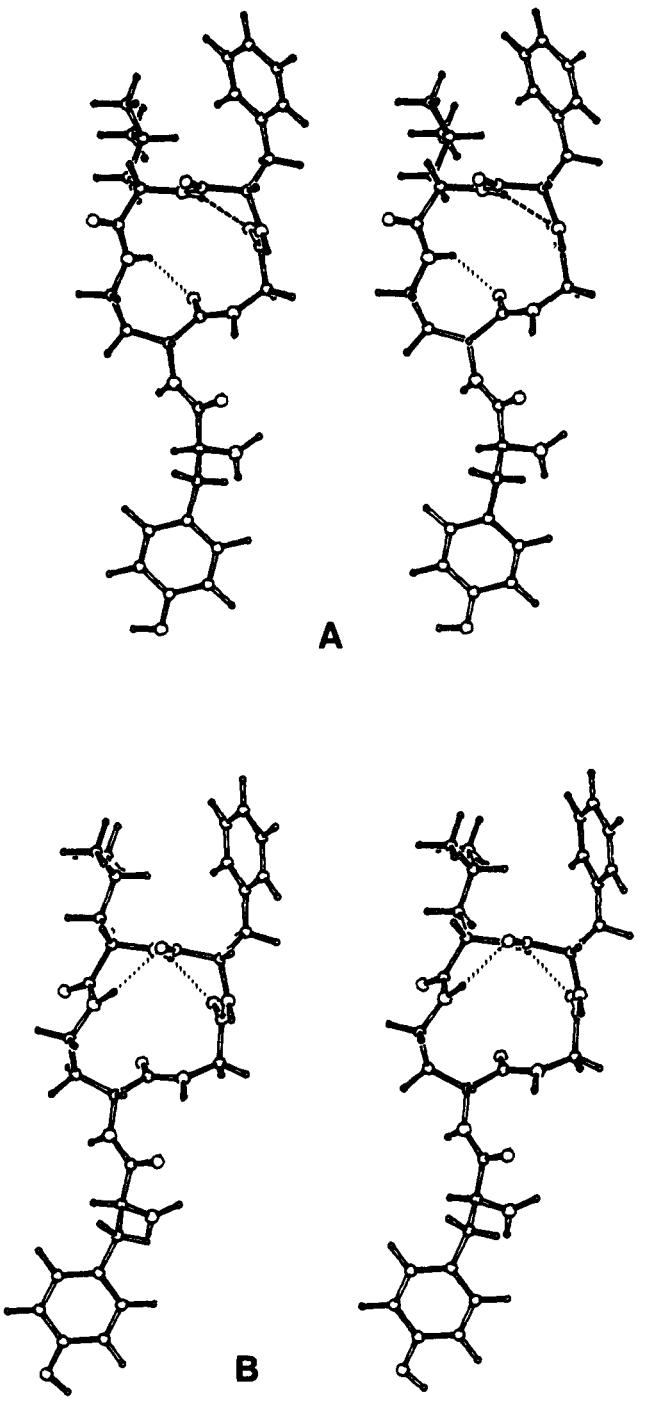

Figure 7. ORTEP stereodrawings of minimum energy conformations of H-Tyr-c[-D-A_2bu-Gly-Phe-Leu-]: A, minimum I; B, minimum III. Both structures are in agreement with the structure postulated from NMR (Me_2SO-d_6) data.

behavior (unpublished results). Upfield shifting of intramolecularly hydrogen bonded amide resonances has been reported by Kopple et al. for two cyclic pentapeptides in Me_2SO-d_6 solution.[20]

Other amide protons experience more subtle changes in chemical shift with the change in solvent composition. Ring current shifts from aromatic side chains or proximate carbonyls, as well as magnetic anisotropy of neighboring atoms, are conformationally dependent effects.[18] Thus, it is difficult to explain small changes in chemical shift with solvent perturbation when simultaneous conformational changes occur.[21] We expect that amide protons undergo a change in shielding as hydrogen bond donors become

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available in the titration of Me_2SO-d_6 with H_2O.[22] As more H_2O is added, H_2O and Me_2SO-d_6 compete for NH hydrogen bond donors. These effects are under investigation in our laboratories with compounds containing stable hydrogen bonds and linear peptides that do not form stable intramolecular hydrogen bonds.

The temperature dependence of chemical shifts arises from an increase in molecular motion which disrupts peptide hydrogen bonds to solvent as the temperature is increased. Hydrogen bonding deshields protons; thus, a reduction in the time the proton is hydrogen bonded causes an upfield shift of its resonance. The magnitude of temperature coefficients increases as H_2O is added to Me_2SO-d_6 (Figure 6). This implies that hydrogen bonds from the cyclic peptide to solvent molecules in solutions of Me_2SO-d_6 plus H_2O are disrupted more easily than those in pure Me_2SO-d_6.

Several of the proposed important conformations suggested for enkephalins cannot be obtained with this constrained cyclic analogue as determined by CPK model studies.[5] The bend models described by Roques et al.[23] and Jones et al.[24] and the antiparallel Phe to Tyr hydrogen bonding pattern observed by crystallography[25] cannot be formed by this analogue. We further find that the β-bend structure proposed by Bradbury et al.[26] containing a hydrogen bond between the Phe NH and Tyr CO is not observed.

The computer simulations suggest some interesting conformational features involving side chains and backbone atoms external to the ring. A hydrogen bond between Tyr CO and Gly NH is seen in low-energy conformations although it is not substantiated by NMR solution data. The effect of solvation of the Tyr side chain and the charged amino terminus prevents the formation of this hydrogen bond in Me_2SO-d_6 and mixed H_2O/Me_2SO-d_6 solutions. Hydrogen bonding between the Tyr OH and Gly CO has also been observed in similar isomers (unpublished data). Although the biological significance of such structures cannot easily be probed experimentally, their importance may lie in defining conformations of the enkephalin at a receptor site.

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Conclusions

We have investigated the dynamics and conformations of a 14-membered cyclic enkephalin analogue by proton NMR and computer simulations. Elucidation of hydrogen bonds has provided a framework for the three-dimensional shape of the ring without the ambiguity of torsions calculated from coupling constants. The approximate conformations of the compound in Me_2SO-d_6 and mixed H_2O/Me_2SO-d_6 solutions have been deduced by combining experimental NMR results and theoretical computer calculations. A double hydrogen bond structure is found in pure Me_2SO-d_6 with Leu NH bound to Gly CO and D-A_2bu side-chain NH bound to either D-A_2bu CO or Phe CO or a combination thereof. The preferred conformation in mixed H_2O/Me_2SO-d_6 solutions is similar, but the hydrogen bond involving Leu NH is disrupted. The severe constraints on this cyclic peptide make it an important compound. Novel peptide opiates are being synthesized in our laboratories which incorporate additional constraints to obtain an essentially rigid biologically active enkephalin.

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Electronic Structures of Active Site Models for Compounds I and II of Peroxidase

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Abstract: Xα multiple scattering calculations are reported for the d^4 oxo complexes $Fe(P)(py)(O)^{0,1+}$ (P = porphine, py = pyridine), models for the active sites of intermediates I and II of horseradish peroxidase. Orbital energy trends for the addition of pyridine and then oxygen to Fe(II) porphine are used to rationalize spin and oxidation states of these clusters. Particular attention is paid to comparisons with ENDOR spectral data on intermediate I. Our results suggest that both σ and π spin transfer to the ligands is important and that the spins localized at the FeO and porphyrin sites interact strongly.

Peroxidases are hemoprotein enzymes which carry out a variety of biological functions calling for the use of peroxide as an oxidant.[2] Two spectrally distinct intermediates (HRP-I and HRP-II) have been observed on treatment of horseradish peroxidase with peroxide. A model for these intermediates involving a low-spin ferryl (FeO^{2+}) moiety is supported by electron nuclear double resonance (ENDOR),[3] Mossbauer,[4] and NMR[5] experiments, as well as by

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