that the Hb tetramer requires more than two states with respect to HPT binding. If ligand binding and dye binding were two independent processes, then the two-state model could still serve for the ligand reactions. However, the dye binding shifts the R–T equilibrium and therefore is strongly coupled to ligand binding. If the binding of effectors to Hb within the erythrocytes (for example, DPG) behaves similarly, then an extension of the two-state model is needed to represent the behavior of Hb under physiological conditions.

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Computer Simulations of Cyclic Enkephalin Analogues†

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ABSTRACT: Molecular dynamics simulations and energy minimization studies of cyclic enkephalin analogues incorporating retro-inverso modifications have been carried out. The dynamic trajectories are analyzed in terms of the relative mobiiity of the 14-membered rings, conformational transitions among equilibrium states, and hydrogen-bonding patterns. The cyclization of the molecules reduces the motion of the ring structures substantially. Time-correlated conformational transitions resulting in the reorientation of peptide units are observed. Hydrogen bonds form principally C_7 structures. Because of the incorporation of retro-inverso residues, C_6 and C_8 structures are also formed. Starting conformations for energy minimizations were obtained from the molecular dynamics simulations and from a systematic search of the conformational space available to the molecules. Several minimum energy backbone and side-chain conformations were found for each analogue. The effect of retro-inverso residues on hydrogen-bonding patterns and backbone conformations is discussed.

Opioid peptides have been of interest as possible substitutes for alkaloid opiate drugs and for their biological importance as natural analgesics. To understand 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. The importance of residues 1, 3, and 4, Tyr, Gly, and Phe, respectively, for biological activity is now well established (Morley, 1980).

The next stage in these studies is the determination of the conformations responsible for the interaction with the receptor. Much work has been carried out on the basis of the assumption that the conformations obtained from the application of experimental or theoretical techniques are relevant to biological activity, and in many cases the preferred conformation resulting from these studies has been directly related to the "active conformtions". Although this is certainly possible, there is no a priori reason why this should be so. The high flexibility of these linear peptides makes the interpretation of the conformational analysis difficult; experimental results yield

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Table I: Inhibitory Potencies of Partially Modified Enkephalin Analogues, [Leu^5]Enkephalin and [D-Ala^2,Leu^5]Enkephalinamide, in the Guinea Pig Ileum and Mouse Vas Deferens Assay[a]

compd	GPI rel potency[b]	MVD rel potency[b]
H-Tyr-*c*-(D-Glu-Gly-Phe-gLeu) (I)[d]	11.4 ± 1.6	0.035 ± 0.003
H-Tyr-*c*-(D-A_2bu-Gly-gPhe-L-mLeu) (*l*-II)[d]	163 ± 21	1.47 ± 0.60
H-Tyr-*c*-(D-A_2bu-Gly-gPhe-D-mLeu) (*d*-II)[d]	9.64 ± 0.74	0.767 ± 0.258
H-Tyr-*c*-(D-A_2bu-gGly-L-mPhe-Leu) (*l*-III)[e]	0.144 ± 0.022	0.00137 ± 0.00014
H-Tyr-*c*-(D-A_2bu-gGly-D-mPhe-Leu) (*d*-III)[e]	0.0638 ± 0.0075	<0.00000250
H-Tyr-*c*-(D-A_2bu-Gly-Phe-Leu)[c]	17.5 ± 3.6	0.140 ± 0.010
H-Tyr-D-Ala-Gly-Phe-Leu-NH_2[c]	32.2 ± 0.152	1.38 ± 0.27
[Leu^5]enkephalin	1	1

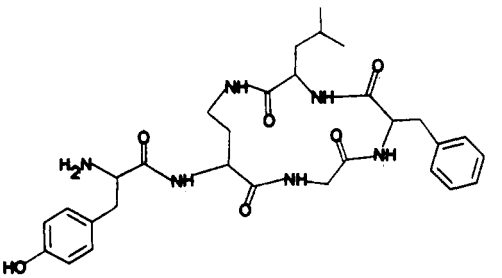
[a] Means of three determinations ± SEM. [b] Potency relative to [Leu^5]enkephalin (=1). [c] Data from Di Maio et al. (1982). [d] Data from Berman et al. (1983). [e] Data from Richman et al. (1985).

time-averaged values while theoretical techniques reveal the existence of numerous local energy minima.

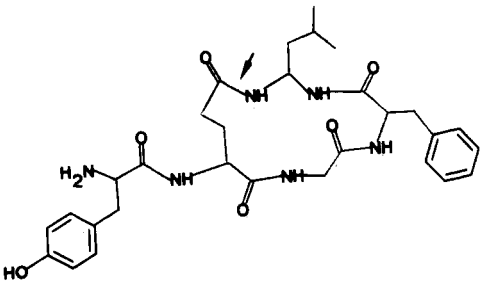
These difficulties can be alleviated by the design of conformationally constrained analogues, which, if active can provide valuable information concerning the conformation that interacts with the receptor. Such a compound has been recently reported (DiMaio & Schiller, 1980). This cyclic enkephalin with a highly constrained 14-membered ring greatly reduces the flexibility and the conformational space available to the molecule. It has been found to be more active than the natural linear enkephalin.

The importance of this constrained peptide prompted us to design a series of analogues with changes in the backbone structure, introduced by the incorporation of partial retro-inverso modifications (Goodman & Chorev, 1979, 1980; Chorev et al., 1979), consisting of the reversal of peptide bonds to give a *gem*-diaminoalkane residue followed by a 2-alkylmalonate or a retro-amide. The compounds used in these studies are shown in Figure 1. All of these analogues show high activity and selectivity for the μ-receptors, with the exception of analogues *l*-III and *d*-III, which are almost inactive. As a result of the backbone modifications, analogues I–III are more resistant to enzymatic degradation than the parent cyclic compound H-Tyr-*c*-(D-A_2bu-Gly-Phe-Leu) (Berman & Goodman, 1984). Table I presents the biological activity of these cyclic analogues.

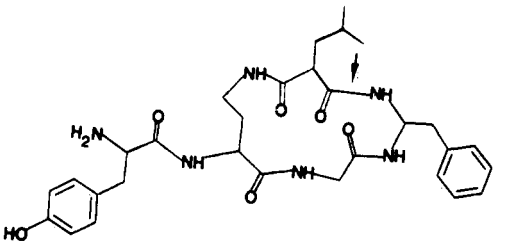
In this paper we report the computer simulation studies of the dynamic and conformational properties of these cyclic enkephalin analogues on the basis of molecular dynamics and energy minimizations. Molecular dynamics simulations provide information about the relative mobility of the molecules, by determining the individual trajectories followed by the atoms under the influence of the molecular potential field. Such simulations have been carried out in peptides and protein systems, both in vacuo and including solvent molecules (Levitt, 1983; Rossky & Karplus, 1978; Hagler et al., 1985; Struthers et al., 1984a,b; Hagler, 1985). They have revealed that these systems are not static but undergo constant fluctuations about the equilibrium positions, showing transitions among conformational states. These motions have been shown to play an important role in the biological activity of these molecules (Citri, 1973). To investigate the flexibility of the cyclic enkephalin analogues, molecular dynamics simulations were carried out for each compound, and the resulting trajectories were analyzed in terms of conformational transitions, side-



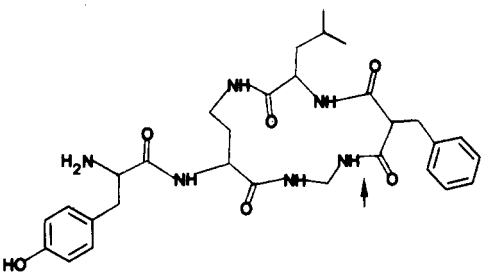
H-Tyr-c[-D-A_2bu-Gly-Phe-Leu]



I H-Tyr-c[-D-Glu-Gly-Phe-*g*Leu-]



II H-Tyr-c[-D-A_2bu-Gly-*g*Phe-L,D-*m*Leu-]



III H-Tyr-c[-D-A_2bu-*g*Gly-L,D-*m*-Phe-Leu-]

FIGURE 1: Structure of cyclic enkephalin analogues used in this study. *gem*-Diaminoalkane residues are represented by the standard three-letter notation corresponding to the specific amino acid preceded by the prefix g. Prefix m represents 2-alkylmalonate residues. The chirality at the α-carbon in these residues is the same as in the corresponding L-amino acids, unless they are preceded by a letter D, in which case the chirality is as in the corresponding D-amino acid.

chain and ring atom mobilities, intramolecular hydrogen-bond patterns, and average conformations and standard deviations.

Energy minimizations provide information concerning the preferred conformations of these molecules. Two methods were used to explore the conformational space available to the molecules. Initial structures were obtained from the molecular dynamics simulations in which the molecules visited different regions along the trajectories. To examine the conformational space, a second method was used in which each residue was systematically forced to different starting conformations. Previous studies have shown that the conformational preferences of *gem*-diaminoalkyl and 2-alkylmalonate residues differ from those shown by normal amino acid residues. For re-

tro-inverso residues, α-helical regions are more stable than C_7^{eq} and extended regions, which are favored in normal peptide residues (Stern et al. 1983a,b). In this paper, we investigate the conformational effect of retro-inverso modifications in more complex structures by comparing the preferred conformations of partial retro-inverso analogues with the conformations of the parent compound H-Tyr-*c*-(D-A_2bu-Gly-Phe-Leu).

A conformational analysis of the parent compound H-Tyr-*c*-(D-A_2bu-Gly-Phe-Leu) using computer simulations and nuclear magnetic resonance has already appeared (Mammi et al., 1985).

Experimental Procedures

The potential energy of the molecular system is expressed by a valence force field, including potential functions representing nonbonded interactions developed by Hagler and coworkers (Dauber et al., 1981; Hagler et al., 1979b–d). The molecular dynamics of the system was simulated by numerical integration of Newton's equations of motion with a fourth-order Gear algorithm (Gear, 1971) with a time step of 0.5 fs. The simulations, covering 10 ps, were preceeded by an equilibration period in which the temperature was adjusted to the desired level, around 300 K, by incrementing the atomic velocities in small steps, keeping the total momentum equal to zero to prevent translation or rotation of the molecule; the length of this period was 2000 time steps (1 ps). All the simulations were in vacuo, i.e., not including solvent molecules. The same starting conformation was used for the simulation of each of the analogues; the structure was partially minimized to eliminate possible strains, using 200 steps of steepest descent minimization. All the atoms in the molecule were allowed to move without any restriction. The calculations were carried out on a VAX 11/780, each picosecond of the simulations taking approximately 2 h of CPU time. An Evans & Sutherland Picture System was used to display the atomic trajectories, and motion pictures of selected periods of the simulations were obtained.

Minimizations of the energy with respect to all the Cartesian coordinates of the molecule were carried out with a steepest descent routine followed by a modified Newton–Raphson method (Lifson, 1983) until all the derivatives are smaller than 0.01 kcal/mol.

Initial conformations of the cyclic enkephalin analogues for energy minimizations were generated by three different procedures:

(1) The molecular dynamics simulations were used as a source of initial conformations by taking the coordinates of the molecules at regular intervals, e.g., every picosecond. In this manner, the minimum energy conformations examined by the molecules along the dynamic trajectories were obtained.

(2) Since the molecular dynamics simulations do not cover all the conformational space available to the molecules, a second procedure was used to explore the conformational space. For each cyclic analogue, each residue was systematically forced to five different starting conformations, namely, α_L (−60,−60), α_R (60,60), C_7^{eq} (−80,80), C_7^{ax} (80,80), and extended (180,180), where the numbers in parentheses refer to pairs of ϕ,ψ torsion angles. This was achieved by assigning a high value to the ϕ and ψ torsion potential parameters, 100 kcal/mol, of the corresponding residue and minimizing the energy, allowing all the other degrees of freedom to accommodate to the new conformation. Because of the large potential parameters, the selected torsion angles converge to the desired values upon minimization. After the molecules have 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 will reach it in the relaxation step. If the forced conformation does not correspond to a true minimum, the molecule will undergo major conformational changes upon relaxing. This procedure leads to several minima with different backbone conformations. The conformations of the side chains, classified as g^-, t, and g^+ for χ_1 equal to −60° ± 60°, 180° ± 60°, and 60° ± 60°, respectively, were not investigated by the above procedure. The χ_1 torsion angles of Tyr, Phe, and Leu have values corresponding to the g^- conformation in all the initial and final structures.

Table II: Standard Deviations of Torsion Angles (deg)

residue	torsion	PC	I	*l*-II	*d*-II	*l*-III	*d*-III
1	ψ	49.1	15.3	90.5	66.6	13.5	44.3
	χ_1	14.5	16.5	15.0	46.9	11.8	48.8
	χ_2	70.5	24.9	26.7	31.2	17.6	31.0
2	ϕ	31.4	9.5	43.1	45.3	13.7	20.8
	ψ	18.3	18.3	33.9	17.9	21.1	63.6
	χ_1	12.8	15.6	29.2	14.5	9.0	21.6
	χ_2	14.1	15.5	18.0	12.1	9.9	28.1
	χ_3	33.8	16.5	21.9	35.7	19.8	99.4
3	ϕ	15.9	25.7	37.6	21.0	15.8	65.5
	ψ	16.0	14.6	27.5	16.4	16.4	76.1
4	ϕ	9.7	10.5	11.7	17.0	10.3	65.3
	ψ	12.3	12.6	13.2	11.9	9.6	21.0
	χ_1	19.7	11.5	13.4	49.1	9.5	53.3
	χ_2	17.1	14.3	17.3	28.1	14.7	90.0
5	ϕ	16.5	14.2	17.3	23.2	14.3	30.1
	ψ	34.4	18.1	25.8	39.3	13.0	99.8
	χ_1	11.2	10.2	11.5	17.1	10.0	79.7
	χ_2	12.3	11.7	11.6	31.0	10.1	33.7

[a] For *gem*-diamine residues, the torsion angles ϕ,ψ correspond to ϕ,ϕ'. For alkylmalonate residues, ϕ,ψ correspond to ψ',ψ.

(3) To investigate the preferred conformation of the side chains, the side chains of residues 1, 4, and 5, Tyr, Phe, and Leu, respectively, were systematically forced to the g^-, t, and g^+ conformations for the low-energy minima of all the cyclic analogues. Several combinations of side-chain conformations were found for each particular backbone structure.

The minimum energy conformations obtained from the molecular dynamics simulations form a subset of those obtained by the application of the other two procedures, which is to be expected in view of the limited time covered by the simulations.

Results

Molecular Dynamics

Analysis of the molecular dynamics simulations show that the rms deviations of bond lengths and bond angles are very small; bond lengths fluctuate 0.01–0.03 Å around the equilibrium positions, and bond angles show deviations of 2°–5°. To compare the mobility of the 14-membered ring structure relative to the rest of the molecule, the rms deviations of the atomic coordinates were calculated by the expression drms = $(x^2\text{rms} + y^2\text{rms} + z^2\text{rms})^{1/2}$. The atoms were classified as belonging to the side chains, to the backbone within the ring, or to the backbone outside the ring. The average deviations of the three types of atoms shown by the seven cyclic analogues are 0.94, 1.37, and 1.55 Å for atoms within the ring, outside the ring, and in the side chains, respectively. Atoms within the ring exhibit smaller deviations than those in the backbone outside the ring or in the side chains. Also, side-chain atoms move more than atoms in the backbone outside the ring.

Conformational Transitions. Fluctuations of the torsion angles around the equilibrium values are large and in many cases lead to dynamic transitions between different confor-

Table III: Backbone Conformations during Molecular Dynamics Simulations[a]

compd	residue	time (min) 1	2	3	4	5	6	7	8	9	10
PC	D-A_2bu	A	C*	C*	C*	C*	C*	C*	C*	C*	C*
	Gly	A*	A*	A*	A*	A*	A*	A*	A*	A*	A*
	Phe	C*	C*	C*	C*	C*	C*	C*	C*	C*	C*
	Leu	C	A	A	A	A	A	A	A	A	A
I	D-Glu	A	C	C	C	C	C	C	C	C	C
	Gly	A*	C	C	C	C	C	C	C	C	C
	Phe	C*	C*	C*	C*	C*	C*	C*	C*	C*	C*
	gLeu	C	D	D	D	D	D	D	D	D	D
l-II	D-A_2bu	A	C*	C*	F*	F*	C*	C*	C*	F*	F*
	Gly	A*	A*	A*	G*	G*	A*	A*	A*	G*	G*
	gPhe	C*	C*	C*	C*	C*	C*	C*	C*	C*	C*
	L-mLeu	C	A	A	A	A	A	A	A	A	A
d-II	D-A_2bu	A	C	C	C	C	C	C	C	C	C
	Gly	A*	A*	A*	A*	A*	A*	A*	A*	A*	A*
	gPhe	C*	C*	C*	C*	C*	C*	C*	C*	C*	C*
	D-mLeu	C	A	A	A	A	A	A	A	A	A
l-III	D-A_2bu	A	A	A	A	A	A	A	A	A	A
	gGly	A*	A*	A*	A*	A*	A*	A*	A*	A*	A*
	L-mPhe	C*	C*	C*	C*	C*	C*	C*	C*	C*	C*
	Leu	C	D	D	D	D	D	D	D	D	D
d-III	D-A_2bu	A	F	C	C	C	C	C	C	C	C
	gGly	A*	A*	C	C	C	C	C	A	A	A
	D-mPhe	C*	C*	C*	C*	C*	C*	C*	A	A	A
	Leu	C	A	A	A	A	C	C	C	C	C

[a] Each letter represents a specific region of the ϕ,ψ map. Specifically, A represents the α_L region, A* represents the α_R region, and C and C* represent the C_7^{eq} and C_7^{ax} regions, respectively.

mational states. Table II shows the rms deviations for all the torsion angles in the six analogues, except for the ω's, which were all trans and show small fluctuations. Deviations larger than 25° generally indicate the occurrence of transitions between two or more states. These transitions involve torsions within the ring structure as well as in the side chains. The frequent transitions indicate that there are many local minima in the regions examined by the dynamic trajectories and that these minima are not very deep since the molecules moved readily among them with the energy available during the simulations.

Backbone Conformations. The torsion angles of the backbone were monitored as the molecules moved along the trajectories. Table III shows the conformation of residues 2–5 for the different analogues at selected times during the simulations. The one-letter code (Zimmerman et al., 1977) was used to classify the residue conformations. Each letter represents a specific region of the ϕ,ψ map. Specifically, A represents the α_L region, A* represents the α_R region, and C and C* represent the C_7^{eq} and C_7^{ax} regions, respectively. Residue 1, tyrosine, was not included since the torsion ϕ is not significant, corresponding to rotation of the terminal NH_2 group. Almost all the observed transitions of tortion angles within the backbone structure occurred in pairs involving ψ_i and ϕ_{i+1} angles or corresponding angles for partial retro-inverso analogues, i.e., ϕ_i and ψ_{i+1} for the unit formed by joining a *gem*-diaminoalkane residue followed by a 2-alkylmalonate. The time variation of these angles seems to be highly correlated; one of the torsions rotates clockwise while the other rotates simultaneously counterclockwise. The net effect of this coupled transition is the reorientation of the peptide unit connecting the two angles, without altering the overall conformation of the molecule. These transitions were observed for the pair of angles Tyr ψ,D-A_2bu ϕ of compound PC, Tyr ψ,D-A_2bu and D-A_2bu ψ,Gly ϕ of compound *l*-II; Tyr ψ,D-A_2bu ϕ of compound *d*-II, and D-A_2bu ψ,gGly ϕ and gGly ϕ',D-mPhe ψ' of compound *d*-III. Very similar transitions were observed involving ψ of residue 5 and χ_3 of residue 2; these two angles are now connected by an amide bond as a result of the cy-

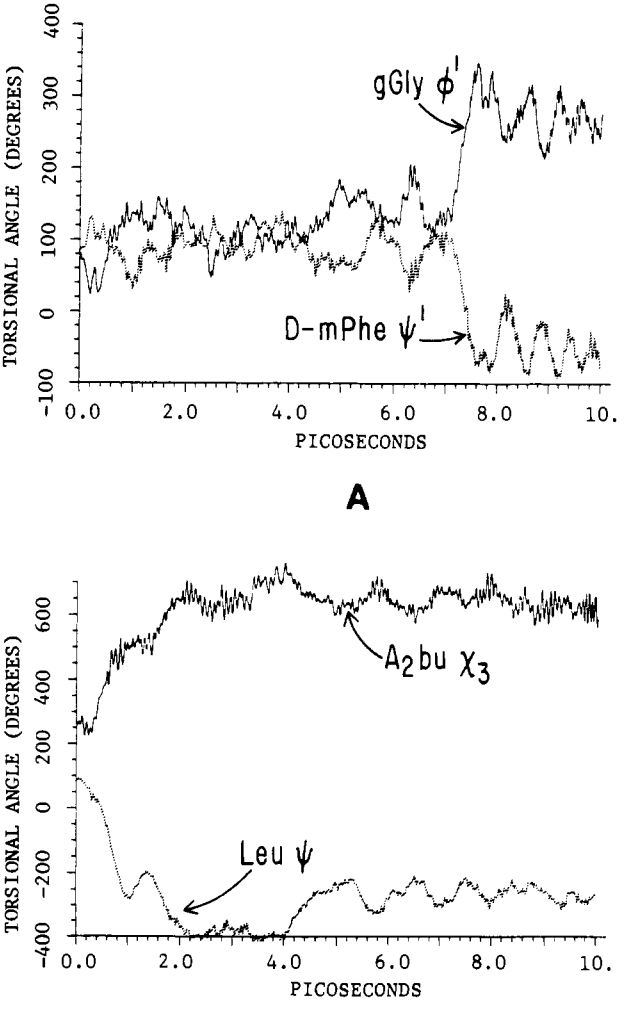


FIGURE 2: Time profiles of selected backbone torsion angles for analogue *d*-III: (A) gGly ϕ' (—) and D-mPhe ψ' (···); (B) D-A_2bu χ_3 (—) and Leu ψ (···).

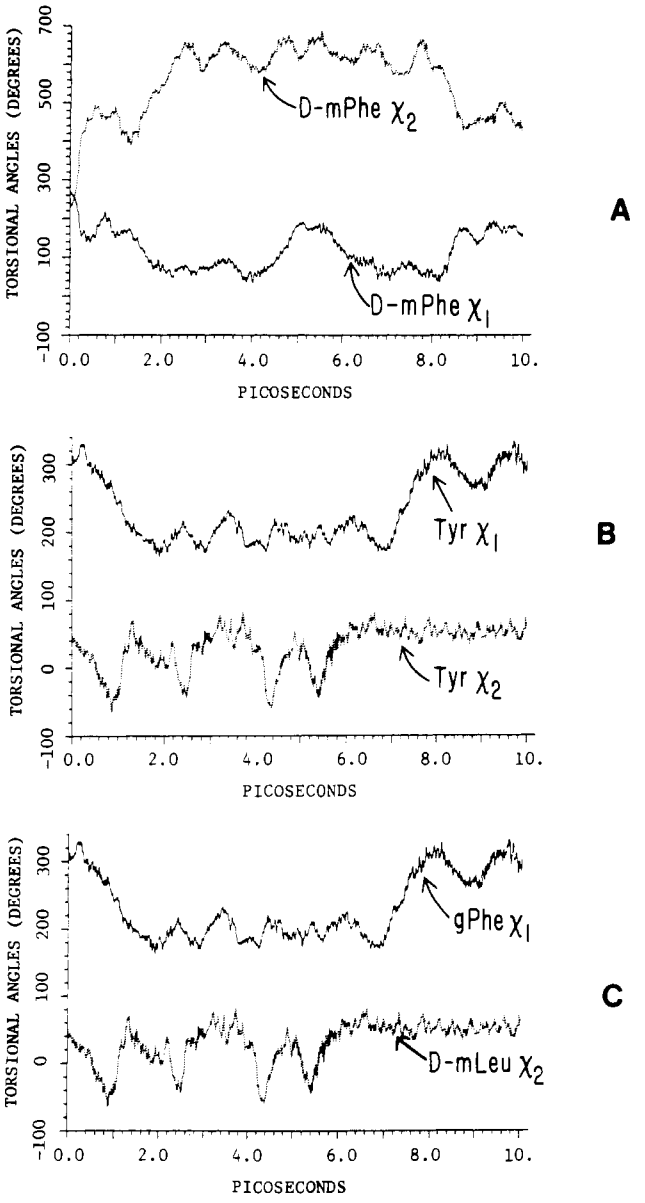


FIGURE 3: Time profiles of selected side-chain torsion angles: (A) D-mPhe χ_1 (—) and χ_2 (···) for analogue *d*-III; (B) Tyr χ_1 (—) and χ_2 (···) for analogue *d*-II; (C) gPhe χ_1 (—) and D-mLeu χ_2 (···) for analogue *d*-II.

clization. Correlated transitions of these two angles occurred for compounds PC, *l*-II, and *d*-II, see Figure 2.

Side-Chain Conformations. The side-chain torsion angles were also monitored during the simulations. As can be seen in Table II, many of these angles have high rms deviations, indicating conformational transitions. These transitions occur among the three equilibrium states designated by g^+, t, and g^-. The equilibrium positions in the three classes are near 60°, 180°, and −60°, respectively, except for χ_2 of Tyr and Phe, for which the equilibrium values are near 90° and −90°. Transitions among these conformations were observed for Tyr in PC, Tyr, gPhe, and D-mLeu in *d*-II, Tyr in I, and Tyr, D-mPhe, and Leu in *d*-III (Figure 3).

The tyrosine residue has considerable mobility since it is outside the ring structure. In the simulation of analogue *d*-II, the side chain of this residue folded from its initial extended conformation and came very close to the 14-membered ring. Figure 4 shows the time evolution of the distance between the phenolic OH group in Tyr and the CO group in Gly for compound *d*-II. Originally 15 Å apart, these two groups came very close and even formed an hydrogen bond at 9 ps. The

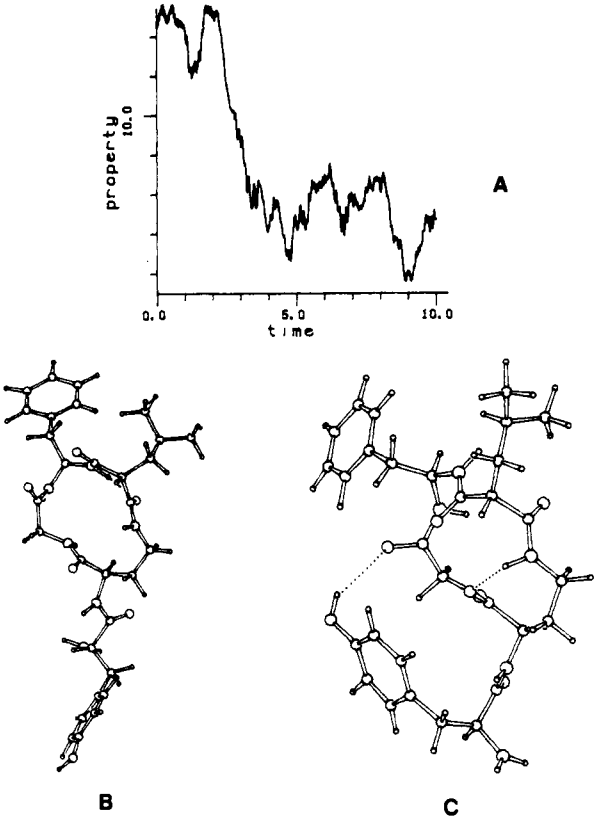


FIGURE 4: Folding of tyrosine residue in analogue *d*-II: (A) time variation of the distance between the phenolic OH group in Tyr and the CO group in Gly; (B) conformation of *d*-II at 1.05 ps; (C) conformation of *d*-II at 9.25 ps, now the Tyr residue being folded over the ring structure.

conformations corresponding to 1.05 and 9.25 ps are also shown in the figure, clearly illustrating the folding of the molecule.

Hydrogen-Bonding Patterns. Several hydrogen bonds are formed during the simulations. They were classified as stable if they were present more than 50% of the time or unstable if they were not. Table IV shows all the hydrogen bonds found for each analogue, the particular structure that is formed, and the type of hydrogen bond, either stable or unstable. The analogue PC exhibits five hydrogen bonds, two of them very stable, forming mainly C_7 structures. The other analogues show hydrogen bonds forming C_6 and C_8 rings. None of these hydrogen bonds were stable. In general, these analogues show fewer hydrogen bonds than the parent compound PC. These bonds were not static; different patterns were found as time evolved.

Energy Minimizations

Minimum Energy Conformations of Cyclic Enkephalin Analogues. (A) H-Tyr-c-(D-A_2bu-Gly-Phe-Leu). The backbone conformations of the local energy minima obtained for the parent compound PC are shown in Table V. The hydrogen-bond patterns observed for these minima are presented in Table VI. The lowest energy minimum, 1, contains two transannular hydrogen bonds, one involving D-A_2bu γ-NH and CO and another between Leu NH and Gly CO. There is a third hydrogen bond involving Gly NH and Tyr CO, which is outside the 14-membered ring. A small conformational change leads from minimum 1, with backbone structure C*A*C*A, to minimum 3, with a C*A*C*C conformation. Minimum 3 shows the same number of hydrogen bonds as minimum 1, but the NH of D-A_2bu is associated with the Phe CO instead of the D-A_2bu CO. Minimum 2, with backbone

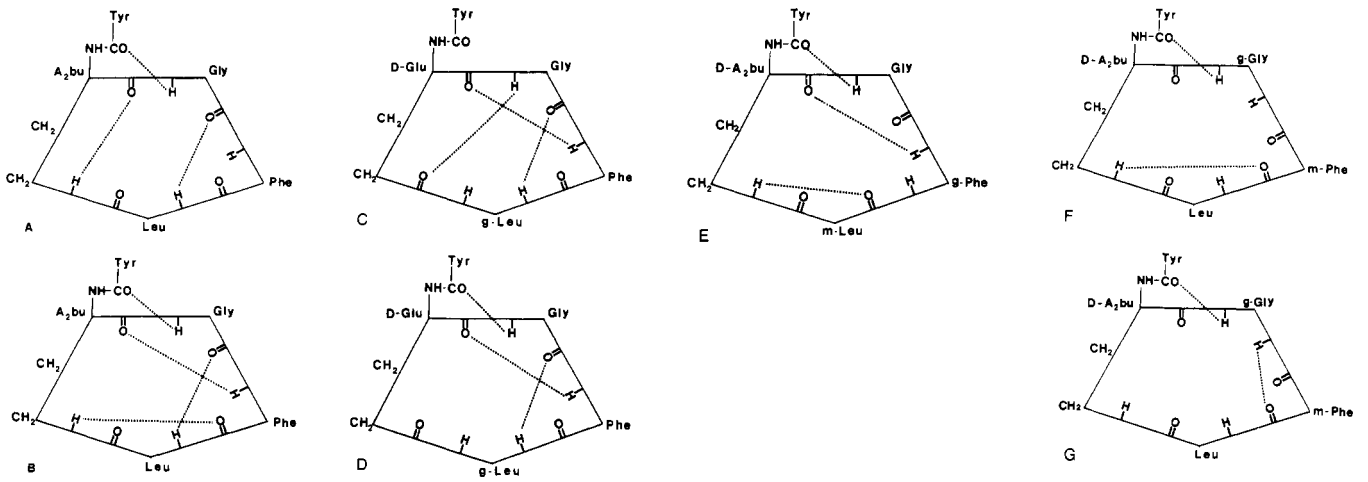


FIGURE 5: Hydrogen-bond patterns present in the cyclic enkephalin analogues: (A and B) H-Tyr-*c*-(D-A_2bu-Gly-Phe-Leu); (C and D) H-Tyr-*c*-(D-Glu-Gly-Gly-Phe-gLeu); (E) Tyr-*c*-(D-A_2bu-Gly-gPhe-LD-mLeu); (F and G) H-Tyr-*c*-(D-A_2bu-gGly-LD-mPhe-Leu).

Table IV: Hydrogen Bonds Observed during Molecular Dynamic Simulations

compd	hydrogen bonds	structure[a]	type[b]
PC	D-A_2bu CO---D-A_2bu NH (side chain)	C_7	stable
	Gly CO---Leu NH	C_7	stable
	Phe CO---D-A_2bu NH (side chain)	C_7	unstable
	Tyr CO---Gly NH	C_7	unstable
	Gly CO---D-A_2bu NH (side chain)	C_{10}	unstable
I	Tyr CO---Gly NH	C_7	stable
	Gly CO---gLeu NH (normal)	C_7	stable
	D-Glu CO (side chain)---Gly NH	C_8	unstable
	D-Glu CO---Phe NH	C_7	unstable
l-II	L-mLeu CO (new)---D-A_2bu NH (side chain)	C_6	stable
	Tyr CO---Gly NH	C_7	unstable
	D-A_2bu CO---D-A_2bu NH (side chain)	C_7	unstable
d-II	Tyr CO---Gly NH	C_7	stable
	D-mLeu CO (normal)---gPhe NH (new)	C_6	unstable
	D-mLeu CO (new)---D-A_2bu NH (side chain)	C_6	unstable
	D-A_2bu CO---D-A_2bu NH (side chain)	C_7	unstable
	Gly CO---Tyr OH		unstable
l-III	D-A_2Bu CO---gGly NH (new)	C_6	unstable
	L-mPhe CO (normal)---D-A_2bu NH (side chain)	C_7	unstable
d-III	Tyr CO---gGly NH (normal)	C_7	stable
	D-mPhe CO (normal)---gGly NH (new)	C_6	unstable
	D-mPhe CO (normal)---D-A_2bu NH (side chain)	C_7	unstable
	D-A_2bu CO---gGly NH (new)	C_6	unstable
	D-A_2bu CO---D-A_2bu NH (side chain)	C_7	unstable

[a] Ring structure formed by the hydrogen bond. [b] Hydrogen bonds are classified as stable when present over 50% of the time and unstable otherwise.

structure CCC*C, differs substantially from minima 1 and 3. Four hydrogen bonds are present in this minimum, between Gly CO and Leu NH, Phe CO and D-A_2bu NH in the side chain, Tyr CO and Gly NH, and D-A_2bu CO and Phe NH. All the energy minimum conformations exhibit hydrogen-bond patterns corresponding to C_7 structures, or γ-turns. No β-turn structures, in which a hydrogen bond closes a 10-membered ring, are observed. The hydrogen-bond patterns are shown schematically in Figure 5, where the 14-membered ring is represented by a pentagon, with the α-carbons of residues 2–5 occupying four corners and the γ-carbon of residue 2, D-A_2bu, in the remaining corner. A and B of Figure 5 correspond to minima 1 and 2, respectively.

(*B*) *H-Tyr-c-*(D-*Glu-Gly-Phe-gLeu*) (*Analogue I*). The incorporation of gLeu at position 5 and the substitution of D-A_2bu for D-Glu at position 2 result in the reversal of the peptide bond that closes the 14-membered ring. This analogue retains the activity and selectivity shown by the other compounds. Table VII contains the conformation of the minimum energy structures for this analogue. Table VIII presents the observed hydrogen bonds. Minimum 1 exhibits two hydrogen bonds, one between the CO in the side chain of D-Glu and NH of Gly, which forms an unusual C_8 ring, and another between Gly CO and the normal NH in gLeu, forming a C_7 structure. Minimum 5 shows the same hydrogen bonds and an additional bond between D-Glu CO and Phe NH. A different pattern is observed in minima 2 and 4. The hydrogen bond between Gly CO and the normal NH of gLeu is maintained as well as the bond between D-Glu CO and Phe NH, in minimum 4, but the Gly NH is now bonded to the CO of Tyr and not to the side-chain CO of D-Glu. These two hydrogen-bond patterns are represented in Figure 5. The two patterns can be compared to those observed in the parent compound. The hydrogen bonds observed in minima 2 and 4 of analogue I, shown in Figure 5D, are also observed in minimum 2 of compound PC, shown in Figure 5B; the fourth bond present

Table V: Minimum Energy Conformations of H-Tyr-*c*-(D-A_2bu-Gly-Phe-Leu)

			D-A_2bu		Gly		Phe		Leu	
minimum	energy[a]	Tyr,[b] ψ	ϕ	ψ	ϕ	ψ	ϕ	ψ	ϕ	ψ
1	0.0	104	84	−92	71	76	74	−67	−76	−56
2	1.4	−58	−73	73	−81	84	75	−71	−96	76
3	4.0	−59	87	−93	70	69	75	−73	−97	63
4	5.4	126	139	−176	−119	75	74	−72	−97	106
5	8.4	−58	−72	67	−86	−76	−73	−60	−131	92
6	8.8	−59	−73	72	−78	88	78	93	77	−67
7	9.3	−59	−59	−76	79	−80	−104	−73	−101	72

[a] Energy in kcal/mol, relative to the lowest energy minimum. [b] For residue 1, Tyr, only the angle ψ is reported because ϕ represents only rotation of the terminal NH_2 group. All the angles are reported in degrees.

Table VI: Hydrogen Bonds Present in H-Tyr-*c*-(D-A_2bu-Gly-Phe-Leu)

minimum	hydrogen bond	conformation
1	Gly CO---Leu NH	C_7
	D-A_2bu CO---D-A_2bu NH (side chain)	C_7
	Tyr CO---Gly NH	C_7
2	Gly CO---Leu NH	C_7
	Phe CO---D-A_2bu NH (side chain)	C_7
	Tyr CO---Gly NH	C_7
	D-A_2bu CO---Phe NH	C_7
3	Gly CO---Leu NH	C_7
	Phe CO---D-A_2bu NH (side chain)	C_7
	Tyr CO---Gly NH	C_7
4	Gly CO---Leu NH	C_7
5	Tyr CO---Gly NH	C_7
6	Phe CO---D-A_2bu NH (side chain)	C_7
	Tyr CO---Gly NH	C_7
	D-A_2bu CO---Phe NH	C_7
7	D-A_2bu CO---Phe NH	C_7

in the parent compound, between the γ-NH of D-A_2bu and the CO of Phe, is not possible for analogue I, because of the substitution of D-A_2bu for D-Glu. The patterns shown in Figure 5C,A are also similar. There is a bond between the CO of residue 3 and the NH of residue 5. In both analogues, the side chain of residue 2 is involved in hydrogen bonds: in PC, the side-chain NH is bonded to the CO of residue 2, forming a C_7 structure; in analogue I, the side-chain CO is bonded to the NH of residue 3, forming a C_8 structure.

(C) H-Tyr-c-(A2bu-Gly-gPhe-L-mLeu) and H-Tyr-c-(A2bu-Gly-gPhe-D-mLeu) (Analogues l-II and d-II). These two isomers incorporate retro-inverso residues at positions 4 and 5, giving rise to the reversal of the connecting peptide bond. Of the two compounds, isomer *l*-II shows the higher activity and μ-receptor selectivity. Tables IX and X present the backbone torsion angles of the minimum energy structures for both isomers. The only structural difference between the two isomers involves the configuration of residue 5, mLeu. Because of this, the two isomers exhibit minima with the same backbone conformations. Minima 1, 4, 5, 7, and 11 of isomer *l*-II show the same conformation as minima 2, 4, 5, 7, and 8 of isomer *d*-II.

Tables XI and XII show the hydrogen bonds present in the minimum energy conformations of both isomers. A single hydrogen-bond pattern is found for both compounds. Each minimum is stabilized by two or three hydrogen bonds, between Tyr CO and Gly NH, D-A_2bu CO and the normal NH of gPhe, and the new CO in mLeu and the γ-NH of D-A_2bu. These hydrogen bonds are shown schematically in Figure 5E. The pattern is similar to that found in minimum 2 of the parent compound, PC, with the exception that the bond between the CO of the third residue and the NH of the fifth residue, found in PC, is no longer possible in isomers *l*-II and *d*-II because of the incorporation of mLeu at position 5. Another difference is that the NH in the side chain of D-A_2bu, which interacts with the CO of residue 4 in the parent compound, now is hydrogen bonded to the new CO group in mLeu, forming a C_6 structure instead of the usual C_7 ring.

(D) H-Tyr-c-(D-A2bu-gGly-L-mPhe-Leu) and H-Tyr-c-(D-A2bu-gGly-D-mPhe-Leu) (Analogues l-III and d-III). The two isomers obtained by incorporating gGly at position 3 and mPhe at position 4 are less active than the other analogues in the series. Their minimum energy conformations are shown in Tables XIII and XIV. As with the pair of isomers *l*-II and *d*-II, several of the minima for compounds *l*-III and *d*-III share the same backbone conformations. Minima 1, 3, and 5 of isomer *l*-III correspond to minima 1, 4, and 6 of isomer *d*-III, respectively. Several other minima differ only in the conformation of a single residue, such as minima 2, 7, and 8 of isomer *l*-III, which correspond to minima 2, 8, and 9 of isomer *d*-III. The hydrogen bonds present in the energy minima are shown in Tables XV and XVI. Compared to other analogues in the series, these compounds have fewer hydrogen bonds stabilizing the structures. Most of the minima have only one bond, and many structures show no hydrogen bonds. Figure 5F,G represents the hydrogen-bond patterns observed in these minima. For isomer *l*-III, minima 1 and 3 have a hydrogen bond between Tyr CO and the normal NH in gGly, corresponding to a C_7 structure. Minima 5 and 6 have a hydrogen bond between the γ-NH of D-A_2bu and the normal CO of mPhe, also forming a C_7 structure. All the other minima of this isomer show no hydrogen bonds. Minimum 1 of isomer *d*-III is the only minimum that shows two hydrogen bonds, one between Tyr CO and the normal NH of gGly and the other between the normal CO of mPhe and the new NH of gGly, forming a C_6 ring. The other minimum energy conformations for isomers *l*-III and *d*-III show only one hydrogen bond. This single hydrogen bond involves either the Tyr CO and Gly normal NH or the normal CO of mPhe and the new NH of gGly. In some of the minima, the normal CO of mPhe is bonded to the side-chain NH of D-A_2bu.

Conformation of Side Chains. The residues at positions 1, 4, and 5, Tyr, Phe, and Leu, respectively, or the corresponding retro-inverso residues possess side chains that can adopt different conformations. The results of the energy minimizations indicate that for each one of the three side chains three conformations are possible, corresponding to χ_1 values of 180° ± 60°, 60° ± 60°, and −60° ± 60°. For Tyr and Phe, χ_2 was always found to be approximately ±90°. It was found that variation of the conformation of the side chain of residue 5, Leu, or the corresponding retro-inverso residue has a very small effect on the energy, usually less than 0.5 kcal/mol.

A similar effect was found for the side-chain conformation of residue 4. The differences in energy among the *t*, g^+, and g^- conformations are approximately 1.0 kcal/mol, with a slight preference for the trans conformation. The more significant

Table VII: Minimum Energy Conformations of H-Tyr-*c*-(D-Glu-Gly-Phe-gLeu)

			D-Glu		Gly		Phe		gLeu[a]	
minimum	energy	Tyr, ψ	φ	ψ	φ	ψ	φ	ψ	φ	φ′
1	0.0	128	150	−145	114	101	76	−67	−133	80
2	1.2	−59	−74	73	−84	96	79	−60	−116	88
3	1.4	−77	86	−96	87	82	77	−68	−97	−60
4	2.6	−59	−73	81	−79	88	78	−65	−82	−60
5	2.9	−60	−78	−121	86	−77	−87	78	57	78
6	4.4	−59	−70	−91	79	−83	−82	−53	−137	84
7	7.3	−62	−74	−155	139	94	76	−67	−130	81
8	7.6	−59	−72	71	−80	86	75	89	64	92
9	9.6	−59	−73	72	−103	−76	−71	−46	−136	85

[a] The torsion angles of gLeu are named φ and φ′, corresponding to the torsion angles φ and ψ in normal residues.

Table VIII: Hydrogen Bonds Present in H-Tyr-*c*-(D-Glu-Gly-Phe-gLeu)

minimum	hydrogen bond	conformation
1	D-Glu CO (side chain)---Gly NH	C_8
	Gly CO---gLeu NH (normal)	C_7
2	Tyr CO---Gly NH	C_7
	Gly CO---gLeu NH (normal)	C_7
3	Gly CO---gLeu NH (normal)	C_7
4	Tyr CO--Gly NH	C_7
	Gly CO---gLeu NH (normal)	C_7
	D-Glu CO---Phe NH	C_7
5	D-Glu CO (side chain)---Gly NH	C_8
	Gly CO---gLeu NH (normal)	C_7
	D-Glu CO---Phe NH	C_7
6	D-Glu CO---Phe NH	C_7
7	D-Glu CO (side chain)---Gly NH	C_8
	Gly CO---gLeu NH (normal)	C_7
8	Tyr CO---Gly NH	C_7
	D-Glu CO---Phe NH	C_7
9	Tyr CO---Gly NH	C_7
	Gly CO---gLeu NH (normal)	C_7

effect was found for the side chain of residue 1, Tyr. Energy differences of 3.0 kcal/mol or more were observed for minima with the same backbone conformation but different orientation of the Tyr side chain. This is illustrated in Table XVII for the parent compound, PC. This table shows the variation in energy upon changes in the side-chain conformations of Tyr and Phe, for several backbone structures. In all cases, the trans orientation of the Tyr χ_1 angle is favored.

Discussion

Simulation of the molecular dynamics of cyclic enkephalins incorporating *gem*-diaminoalkane and 2-alkylmalonate residues has been carried out. Because of the short time of the simulations and the probable existence of high-energy barriers between different minimum energy structures, these studies do not intend to completely characterize the conformational space available to the molecules, as this would require much longer simulations starting from many initial conformations. Our principal objectives are to investigate the relative mobility of these constrained molecules, with emphasis principally on the 14-membered ring structure, the motion of the side chains, and the effect of the incorporation of retro-inverso modifications.

As may be expected, cyclization of the molecules has a significant effect on their mobility; the 14-membered rings show rms deviations that are 30–40% smaller than those shown by the backbone outside the ring, indicating the presence of conformational constraints. This effect was found in all the analogues studied. With respect to the relative mobility of *gem*-diaminoalkane and 2-alkylmalonate residues as compared to those of normal amino acids, no significant differences were detected; the rms deviations show no correlation with the type of residue. Side-chain groups are the most flexible part of the molecules, as observed in reported simulations (Levitt, 1983).

The torsion angles are the softest degrees of freedom of the molecules. They exhibit the largest fluctuations. Analysis of these fluctuations reveals that, although the motion of the ring structure is restricted, it is still substantial. Many of the dynamic transitions observed in the simulations, in which particular torsion angles go from one equilibrium value to a new one, involve angles within the ring. Thus, the cyclization does not freeze the molecules in a unique conformation. The molecule can still move among different conformational states, although the overall shape of the ring structure does not change very much. The principal result of these transitions is the rotation of peptide groups within the ring, arising from the correlated movement of pairs of ψ_i-ϕ_{i+1} angles. This type of motion has been previously observed (McCammon et al., 1979; Levitt, 1983). The same correlated transitions were observed for nonnormal residues, i.e., involving the pair of angles ϕ'_i-ψ_{i+1} in the unit formed by joining a *gem*-diaminoalkane and a 2-alkylmalonate residue, and for ψ_5-χ_3, which are now connected by an amide bond as a result of the cyclization. Thus, the overall shape of the 14-membered structure is rather well-defined, but the relative orientation of CO and NH groups is not fixed.

In a previous study of the conformational properties of *N*-acetylglycine *N*-methylamide, *N*-acetylalanine *N*-methylamide, and their retro-inverso analogues bis(acetamido)methane, 1,1-bis(acetamido)ethane, *N*,*N*-dimethylmalonamide, and *N*,*N*-dimethyl-2-methylmalonamide (Stern et al., 1983a), it was found that whereas the normal glycine and alanine derivatives exhibit their lowest energy minima in the C_7^{eq} region, the *gem*-diaminoalkyl and malonyl residues show their lowest minima in the α-helical regions. It was suggested then

Table IX: Minimum Energy Conformations of H-Tyr-*c*-(D-A_2bu-Gly-gPhe-L-mLeu)

			D-A_2bu		Gly		gPhe[a]		L-mLeu[b]	
minimum	energy	Tyr, ψ	ϕ	ψ	ϕ	ψ	ϕ	ϕ'	ψ'	ψ
1	0.0	−59	−74	69	−83	89	68	85	77	77
2	1.3	−59	−73	69	−84	85	63	111	100	−65
3	1.9	−59	−64	−82	84	−89	−101	−59	−84	−58
4	4.1	−60	−56	−70	100	−72	−128	90	76	82
5	4.3	−58	−72	67	−89	−78	−73	−60	−132	81
6	5.4	−59	−74	72	−80	89	95	−74	−94	−59
7	6.8	100	86	−93	66	65	93	−70	−80	−62
8	7.3	116	75	52	−83	90	96	−72	−89	−53
9	8.2	122	84	−149	−115	77	100	−66	−98	−68

[a] The torsion angles of gPhe are named ϕ and ϕ'. These angles correspond to ϕ and ψ in normal residues. [b] The torsion angles of L-mLeu are named ψ' and ψ. These angles correspond to ϕ and ψ in normal residues.

Table X: Minimum Energy Conformations of H-Tyr-*c*-(D-A_2bu-Gly-gPhe-D-mLeu)[a]

			D-A_2bu		Gly		gPhe		D-mLeu	
minimum	energy	Tyr, ψ	ϕ	ψ	ϕ	ψ	ϕ	ϕ'	ψ'	ψ
1	0.0	−58	−73	72	−84	87	61	89	124	−79
2	0.3	−59	−74	69	−85	85	63	81	99	43
3	5.8	148	−71	71	−92	−82	−77	−64	−112	67
4	5.8	−59	−61	−78	79	−81	−129	89	101	38
5	9.0	102	85	−91	67	61	85	−79	−49	−84

[a] See footnotes of Table IX.

Table XI: Hydrogen Bonds Present in H-Tyr-*c*-(D-A_2bu-Gly-gPhe-L-mLeu)

minimum	hydrogen bond	conformation
1	Tyr CO---Gly NH	C_7
	A_2bu CO---gPhe NH (normal)	C_7
2	Tyr CO---Gly NH	C_7
	A_2bu CO---gPeh NH (normal)	C_7
3	mLeu CO (normal)---A_2bu NH (side chain)	C_6
	A_2bu CO---gPhe NH (normal)	C_7
4	A_2bu CO---gPhe NH (normal)	C_7
5	Tyr CO---Gly NH	C_7
6	Tyr CO---Gly NH	C_7
	mLeu CO (normal)---A_2Bu NH (side chain)	C_6
	A_2bu CO---gPhe NH (normal)	C_7
7	Tyr CO---Gly NH	C_7
8	mLeu CO (normal)---A_2bu NH (side chain)	C_6
	A_2bu CO---gPhe NH (normal)	C_7
9	Tyr CO---gPhe NH (normal)	C_{10}

Table XII: Hydrogen Bonds Present in H-Tyr-*c*-(D-A_2bu-Gly-gPhe-D-mLeu)

minimum	hydrogen bond	conformation
1	Tyr CO---Gly NH	C_7
	A_2bu CO---gPhe NH (normal)	C_7
2	Tyr CO---Gly NH	C_7
	A_2bu CO---gPhe NH (normal)	C_7
	mLeu CO (new)---A_2bu NH (side chain)	C_6
3	Tyr CO---Gly NH	C_7
4	A_2bu CO---gPhe NH (normal)	C_7
	mLeu CO (new)---A_2bu NH (side chain)	C_6
5	Tyr CO---Gly NH	C_7
	A_2bu CO---A_2bu NH (side chain)	C_7
	mLeu CO (normal)---gPhe NH (new)	C_6

that the different conformational properties of retro-inverso residues may have an effect upon their incorporation in peptide chains.

A close look at the minimum energy conformations of the cyclic enkephalin analogue PC and the analogues incorporating retro-inverso modifications shows the presence of a conformational effect produced by the substitution of normal residues by *gem*-diaminoalkyl or malonyl residues. There is a marked tendency for retro-inverso residues to adopt α-helical conformations in the lowest energy minima of the analogues in which they are incorporated. This effect is illustrated in Table XVIII, where the conformations of the lowest three energy minima of each analogue are shown, with retro-inverso residues enclosed in parentheses. In most cases, retro-inverso analogues are in the A or A* conformations, corresponding to the α_L and α_R conformations, respectively. Normal residues show a preference for the C and C* conformations, corresponding to C_7^{eq} and C_7^{ax}, respectively. Thus, the effect found in the model compounds is maintained in these cyclic molecules.

The 14-membered ring is stabilized by the formation of hydrogen bonds, which, because of the molecular vibration and frequent transitions, break and re-form several times during the simulations, giving rise to different patterns. The most stable hydrogen bonds were found to form C_7 structures. The parent compound, PC, shows primarily this type of hydrogen bond, with the exception of a C_{10}, which was present only a small fraction of time. Analysis of the hydrogen bonds present in cyclic pentapeptides by NMR and theoretical techniques have been reported (Kessler, 1982; Ramakrishnan & Rao, 1980), indicating the existence of two main patterns, one containing a C_{10} (β-turn) and a C_7 (γ-turn) and a second containing two C_7 structures. Although these cyclic enkephalin analogues are not really cyclic pentapeptides, the two structures are very similar. The results reported in this paper seem to favor the latter model, as C_7 structures were more commonly found than C_{10} rings. Because of the incorporation of retro-inverso residues, some of the hydrogen bonds found in the parent compound PC, which form C_7 ring structures, cannot be formed in analogues with *gem*-diaminoalkyl or malonyl residues, because such an arrangement would bring together two chemically equivalent groups, namely, two carbonyl oxygen atoms or two amino hydrogen atoms. In some cases, C_7 structures are substituted by C_6 or C_8 rings, which are not possible in normal peptides but are made possible by the reversal of peptide bonds. In general, the C_6 and C_8 hydrogen-bonding ring structures of the retro-inverso analogues occur less frequently in the calculations than in the C_7 structures of the parent compound.

Side-chain groups show frequent transitions between the g^-, t, and g^+ conformations, indicating the low-energy barriers that separate the different rotamers. The most interesting transitions involve the tyrosine side chain, which is considered one of the most important residues for the biological activity of the molecules. In two of the analogues, *d*-II and *d*-III, χ_1

Table XIII: Minimum Energy Conformations of H-Tyr-*c*-(D-A_2bu-gGly-L-mPhe-Leu)

			D-A_2bu		gGly[a]		L-mPhe[b]		Leu	
minimum	energy	Tyr, ψ	ϕ	ψ	ϕ	ϕ'	ψ'	ψ	ϕ	ψ
1	0.0	−59	−74	72	−73	−87	−81	−66	−98	84
2	2.0	−59	91	−99	65	92	84	−88	−120	78
3	3.9	−59	−73	63	−95	154	67	−92	−134	107
4	6.1	−59	77	46	−88	160	63	−92	−135	111
5	7.1	−61	−78	−128	72	99	72	86	78	−71
6	10.0	−60	−61	−75	82	−109	−81	−67	−96	70

[a] The torsion angles of gGly are named ϕ and ϕ'. They correspond to the torsion angles ϕ and ψ in normal residues. [b] The torsion angles of L-mPhe are named ψ' and ψ. They correspond to the torsion anlges ϕ and ψ in normal residues.

Table XIV: Minimum Energy Conformations of H-Tyr-*c*-(D-A_2bu-gGly-D-mPhe-Leu)[a]

			D-A_2bu		gGly		D-mPhe		Leu	
minimum	energy	Tyr, ψ	ϕ	ψ	ϕ	ϕ'	ψ'	ψ	ϕ	ψ
1	0.0	−59	−74	68	−79	−90	−58	−88	−101	92
2	0.8	114	78	51	−78	−84	−54	−89	−105	95
3	2.6	113	91	−99	63	91	84	−121	−92	77
4	5.4	−59	−74	61	−93	153	72	−111	−126	102
5	5.6	−59	−60	−76	90	−149	−89	41	−128	−69
6	7.0	−61	−78	−128	69	99	84	65	79	−67

[a] See footnotes of Table XIII.

Table XV: Hydrogen Bonds Present in H-Tyr-*c*-(D-A_2bu-gGly-L-mPhe-Leu)

minimum	hydrogen bond	conformation
1	Tyr CO---gGly NH (normal)	C_7
2	none	
3	Tyr CO---gGly NH (normal)	C_7
4	none	
5	mPhe CO (normal)---A_2bu NH (side chain)	C_7
6	mPhe CO (normal)---A_2bu NH (side chain)	C_7

Table XVI: Hydrogen Bonds Present in H-Tyr-*c*-(D-A_2bu-gGly-D-mPhe-Leu)

minimum	hydrogen bond	conformation
1	Tyr CO---gGly NH (normal)	C_7
	mPhe CO (normal)---gGly NH (new)	C_6
2	mPhe CO (normal)---gGly NH (new)	C_6
3	mPhe CO (normal)---D-A_2bu NH (side chain)	C_7
4	Tyr CO---gGly NH (normal)	C_7
5	D-A_2bu CO---D-A_2bu NH (side chain)	C_7
6	mPhe CO (normal)---D-A_2bu NH (side chain)	C_7

Table XVII: Effect of Variation of Side-Chain Conformations of Analogue H-Tyr-*c*-(D-A_2bu-Gly-Phe-Leu)

		side-chain conformation		
minimum	backbone conformation[a]	Tyr	Phe	energy[b]
1	C*A*C*A	*t*	g^-	0.0
		t	*t*	1.4
		g^-	*t*	4.4
2	CCC*C	*t*	g^-	0.0
		g^-	g^-	3.1
3	C*A*C*C	*t*	g^-	0.0
		t	*t*	0.2
		g^-	*t*	3.7
		g^-	g^-	4.7
4	CAAD	*t*	g^-	0.0
		g^-	g^-	4.7
5	CCA*C*	*t*	g^-	0.0
		g^-	g^-	3.0
6	AC*AC	*t*	g^-	0.0
		g^-	g^-	2.0
7	AA*C*C	*t*	g^-	0.0
		g^-	g^-	3.1

[a] Conformation of residues 2–5. The one-letter code introduced by Zimmerman et al. is used. [b] Energy in kcal/mol relative to the lowest minimum with the same backbone conformation but different side-chain conformation.

Table XVIII: Conformation of Lowest Energy Minima of Cyclic Enkephalin Analogues[a]

	minimum energy conformation		
analogue	1	2	3
PC	C*A*C*A	CCC*C	C*A*CC
I	E*H*C*(D)	CCC*(D)	CCC*(A)
l-II	CC(A*A*)	CC(H*C*)	AC*(AA)
d-II	CC(A*D*)	CC(A*A*)	CA(AD)
l-III	C(AA)C	C*(A*C*)D	C(FC*)E
d-III	C(AA)C	A*(AA)C	C*(A*C*)C

[a] Backbone conformations of the lowest energy minima of the cyclic enkephalin analogues. The one-letter code introduced by Zimmerman et al. is used to represent the conformation of residues 2–5. Letters in parentheses correspond to retro-inverso residues.

of this residue underwent a transition from g^- to *t*, producing more compact structures, and in the case of analogue *d*-II, the resulting conformation has the Tyr residue folded over the 14-membered ring and forming a hydrogen bond involving the phenolic OH and the CO of Gly. Such an interaction has been proposed previously on the basis of NMR studies (Khaled et al., 1977). Although not all the analogues show this transition, the fact that one of them does, and that the energy barriers involved are not very high, reveals that it is possible for the molecules to adopt that conformation. Flipping of the aromatic rings, i.e., transitions involving χ_2 of Tyr or Phe, were also observed, in some cases correlated with transitions of the corresponding χ_1 angle. This correlation has not been found in reported simulations of protein dynamics (Levitt, 1983; McCammon et al., 1979).

The conformation of the side chains should play an important role in the interaction with the receptor. We have found that the side chains of residues 4 and 5, Phe and Leu, or the corresponding retro-inverso analogues are very flexible. The difference in energy among the g^- and g^+ conformations is less than 1 kcal/mol, indicating that all these conformational states will be populated at room temperature. It is possible, particularly for the Phe side chain, that a specific conformation is required for the right interaction at the receptor site. Because of the small energy difference among the possible conformations, the molecules can readily undergo interconversions to adopt the right orientation of the side chain, as shown by the molecular dynamics simulations. Experimental studies have also indicated the relative flexibility of the Phe and Leu side chains (Bleich et al., 1976).

The side chain of Tyr shows a different behavior. In this case, the trans conformation, with $\chi_1 \approx 180°$, is favored by more than 3.0 kcal/mol over gauche conformations. In the trans orientation, the side chain of Tyr is situated close to the 14-membered ring, and for some backbone conformations, the phenolic OH group of Tyr interacts with CO groups in the backbone, forming hydrogen bonds, which contribute to the stabilization of these structures. For the parent compound, H-Tyr-*c*-(D-A_2bu-Gly-Phe-Leu), two backbone conformations were found for which the Tyr OH group interacts with CO groups in the backbone, corresponding to minima 1 and 5. In minimum 1, the Tyr OH interacts with the Gly CO, while in minimum 5 it interacts with the Leu CO. The same kind of interaction was found for analogues incorporating retro-inverso modifications. The reduced flexibility of the Tyr residue in enkephalin analogues has been previously reported on the basis of NMR results (Khaled et al., 1977; Bleich et al., 1976) and empirical energy calculations (Isogai et al., 1977; Loew & Burt, 1978).

The pair of isomers *l*-II and *d*-II, for which the only difference is the configuration of residue 5, mLeu, shows differences in biological activities. In the guinea pig ileum assay, which measures potencies toward the μ-receptors, isomer *l*-II is 17 times more potent than isomer *d*-II. The minimum energy conformations of the two isomers show similar backbone conformations, with the same intramolecular hydrogen bonds. The difference in activity may be attributed to the different orientation of the Leu side chain. Isomers *l*-III and *d*-III also show different potencies. These two isomers differ in the configuration of residue 4, mPhe. Isomer *l*-III, which topographically resembles the normal L-amino acid, is more active than isomer *d*-III, which resembles a D-Phe amino acid. As with the analogues *l*-II and *d*-II, these two isomers share most of the low-energy conformations, leading to the conclusion that the difference in activity is produced by the different orientation of the mPhe side chain. The importance of the configuration of residue 4 in enkephalin analogues is well established (Morley, 1980). Both isomers are less active than the other analogues in the series. This has been attributed to

the substitution of Gly at position 3 by gGly, which modifies the peptide bond between residues 3 and 4. This bond is apparently important for binding to the receptor (Richman et al., 1985).

Extensive ^{1}H NMR studies of these cyclic enkephalin analogues have been carried out (Mammi & Goodman, 1986). The experimental results obtained in these studies support the results reported in this paper. Hydrogen bonds observed in minimum energy conformations were also detected experimentally for the different analogues.

The cyclic enkephalin analogues, based on a 14-membered ring, are relatively constrained, but significant motion involving both the backbone and the side chains remains. These results should be taken into account in the interpretation of experimental data from NMR and other techniques that reflect average rather than absolute values. Further studies on the flexibility of cyclic peptides containing 12–16-membered rings have been initiated.

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Registry No. I, 87619-78-1; II (L-mLeu), 104372-38-5; II (D-mLeu), 104372-37-4; III (L-mPhe), 101854-38-0; III (D-mPhe), 101854-39-1; H-Tyr-*c*-(D-A_2bu-Gly-Phe-Leu), 77171-72-3; [D-Ala^2,Leu^5]enkephalinamide, 65189-64-2; [Leu^5]enkephalin, 58822-25-6.

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