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Theoretical Study of the Flexibility and Solution Conformation of the Cyclic Opioid Peptides [D-Pen^2,D-Pen^5]Enkephalin and [D-Pen^2,L-Pen^5]Enkephalin

CLIFFORD CHEW, HUGO O. VILLAR, and GILDA H. LOEW
Molecular Research Institute, Palo Alto, California 94304
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SUMMARY

An investigation of the conformational profiles of two cyclic δ-selective opioid peptides, [D-Pen^2,D-Pen^5]-enkephalin and [D-Pen^2,L-Pen^5]-enkephalin, has been made. The methods and procedures used are more extensive and systematic than those previously reported, involving a combination of nested grid rotations, cyclic ring-closing algorithms, molecular dynamic simulations at high and low temperature, and total geometry optimizations. The reexamination is a necessary first step in further characterization of the bioactive form of δ-selective peptides. This study also addresses the question of how rigid such cyclic analogs actually are. Finally, the effect of solvent environment on the low energy conformers obtained from the extensive search strategy has been determined. Simulation of the effect of water as a solvent by a continuum dielectric constant of 80 results in the breaking of internal hydrogen bonds and rearrangement of the rank order of energy of the conformers. The lowest energy solution conformation for [D-Pen^2,D-Pen^5]-enkephalin, obtained without utilizing any experimental data, is in excellent agreement with the geometric properties deduced from its solution NMR spectra.

Since the characterization of enkephalins as one type of endogenous ligand for the opioid receptor (1), numerous analogs have been synthesized and pharmacologically evaluated. These analogs, in general, bind with varying affinities and selectivities to the μ and δ receptors, while exhibiting low affinity for the κ receptor (2–4). In the search for highly selective ligands, many cyclic enkephalins have been synthesized and evaluated. The rationale behind their synthesis was that cyclization could result in highly conformational constrained peptides, which would be suitable for recognition at only one or the other of the opioid receptor subtypes (5, 6). Another perceived advantage of cyclization is that, if it actually limited the number of accessible conformers, it would make the formidable task of identifying the bioactive forms of peptides, i.e., the forms in which they bind to receptors, more tractable. The determination of the bioactive form is complicated by the fact that, except for totally rigid analogs, the conformation in which a drug interacts with receptors does not need to be either the minimum energy structure or the conformation that it adopts in a given solvent.

The resulting structure-activity relationships, although leading to high affinity, δ-selective peptides such as DPDPE and its steroisomer DPLPE, revealed that cyclization was neither necessary nor sufficient to produce highly μ- or δ-selective peptides. In particular, a large number of closely related cyclic enkephalins of the form [X^2, Y^5]enkephalin, where X and Y are all combinations of D-Cys, L-Cys, D-Pen, and L-Pen and cyclization is by side chain disulfide bridges between the second and fifth residues, have been synthesized (7). The resulting analogs spanned the range of nonselective to highly δ-selective for the peptides.

Previous conformational studies of seven of these cyclic peptides and four linear peptides with high affinity and selectivity at the δ receptor also revealed that cyclization does not necessarily lead to rigid analogs, i.e., conformational profiles with only a few low energy conformers (4). In that study, the search strategy adopted for the cyclic peptides was a combination of a systematic backbone dihedral variation in conjunction with a ring-closing algorithm and minimization of the structures that were within 10 kcal/mol of the lowest energy one. The result was a significant number of low energy conformers for DPLPE, which, as the highest δ-affinity analog studied, was used as a template for all the other cyclic peptides studied. Unfortunately then, cyclization, at least in cases where the resulting rings contain 14–16 atoms, did not necessarily make

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ABBREVIATIONS: DPDPE, [D-Pen^2,D-Pen^5]-enkephalin; DPLPE, [D-Pen^2,L-Pen^5]-enkephalin; DCDCE, [D-Cys^2,D-Cys^5]enkephalin; ECEPP, empirical conformational energy program for peptides; AMBER, assisted model builder and energy refinement; CHARMm, chemistry at Harvard molecular mechanics; NOE, nuclear Overhauser effect.

the task of identifying favorable conformations that lead to selective recognition appreciably easier. In general, it is not possible to identify a bioactive form from the study of a single peptide. Rather, to accomplish this aim, the steric commonalities and dissimilarities among analogs with varied affinities at the δ receptor, including linear and cyclic analogs, were analyzed. A low energy structure common to all analogs, both cyclic and linear, was identified as a possible bioactive form, with a highly compact conformation (4).

Recently, two studies by Hruby and co-workers (8, 9) have been reported. One of these studies focuses on the identification of the solution conformation of DPDPE (8), whereas the second one is an attempt to characterize the conformational flexibility and plausible bioactive forms of DPDPE and DCDCE (9).

In the first study, different initial structures generated by inspection of space-filing models were minimized and checked for consistency with the NMR data. A solution conformation was identified by the combined use of NMR information, including NOE spectroscopy experiments, and molecular mechanics minimization, using the CHARMm potential. This conformer was also selected as the candidate bioactive form, with no attempt being made to verify that it was also accessible to other high affinity peptides and was absent in low affinity ones. In the second study performed, 40 different sets of dihedral angles compatible with ring-closing constraints were generated, considering only the backbone atoms of the ring, which were subsequently minimized using the AMBER (10) potential. No specific efforts were made to reconcile the results obtained in the search with the NMR data. This study clearly illustrates the flexibility of these cyclic peptides and the difficulties in identifying their bioactive form.

Mosberg and co-workers (11) more recently performed an ^{1}H NMR study of DPDPE. In this study, they assigned all resonances and used the resulting assignments for the development of a structural model that would explain the observed spectrum. The NMR data were used in a different fashion than in the studies by Hruby and co-workers. Instead of optimization of initial structures chosen for compatibility with the NMR results, a combination of two distance-geometry embedded algorithms were used to locate conformations consistent with the NMR-derived distance constraints (11). Again, many such conformers were found. The resulting conformations were then subjected to energy minimizations, because, although gross atomic overlaps were avoided, the structures produced by the distance-geometry algorithms were not optimal. Three low energy conformers were reported. Modifications of one of the originally proposed conformers, to be consistent with the experimentally observed populations of rotamers associated with both aromatic rings, led to a lower energy conformer that was selected as a candidate conformer in solution. This conformation, before energy minimization, was consistent, with a certain error, with the values of the geometric parameters deduced from the NMR spectra. Although the final unconstrained optimized conformer was reported, its consistency with the observed NMR spectra was not explicitly discussed.

In the work reported here, we describe the results of an extensive and systematic reexamination of the conformational profile of the two seminal δ-selective cyclic enkephalins,

TABLE 1
Torsion angles and relative energies for the lowest energy conformers of DPLPE

Dihedral angle	Torsion angle												
	1[a]	2	3	4	5	6	7	8	9	10	11	12	13
	degrees												
Tyr[1]													
Ψ	−44	159	−75	−62	−54	−68	176	−70	160	−64	−70	−65	177
ω	180	175	−174	−177	−178	−171	−179	180	177	−177	−176	173	−179
χ^1	69	−172	162	169	−63	180	−64	−176	−173	168	167	−172	55
χ^2	−64	−120	−118	−117	−64	−160	−64	−170	−120	−128	−124	−119	−110
Pen[2]													
ϕ	65	49	67	68	65	61	66	65	49	61	56	63	63
Ψ	−159	−91	−77	−76	−83	−149	−83	−80	−91	−150	−147	−157	−145
ω	177	180	174	−177	−171	−179	−171	−167	180	179	−174	173	−174
χ^1	174	−38	170	−49	−175	−37	−175	−38	−36	−41	−171	−49	−173
χ^2	75	−27	47	−50	−94	−48	−95	−34	−24	−54	−164	−40	−162
Gly[3]													
ϕ	68	−50	46	9	66	179	66	−29	−52	55	73	38	73
Ψ	−76	−48	−113	−99	−71	53	−71	−57	−43	−130	−67	−84	−68
ω	172	−179	−171	179	178	−132	179	179	179	−177	−178	−175	−177
Phe[4]													
ϕ	−76	−59	−55	−57	−52	−179	−52	−132	−71	−76	−51	−57	−52
Ψ	59	−27	−21	−47	−39	−76	−39	51	−13	60	−55	−17	−54
ω	−174	−177	178	−179	179	58	180	176	−170	170	−177	175	−177
χ^1	−174	−70	−76	−178	−177	−62	−178	−64	−64	−69	−169	−57	180
χ^2	59	113	120	64	65	115	65	112	114	174	108	115	64
Pen[5]													
ϕ	−70	−63	−69	−67	−67	−63	−53	−60	−61	−71	−69	−50	−68
χ^1	−47	−33	56	41	−59	−68	−59	−64	−49	−59	71	−46	70
χ^2	−98	161	−179	107	65	168	65	167	164	176	−45	172	−46
Cβ-S-S Cβ	96	−107	107	−104	103	−113	103	−112	−109	−116	−106	−103	−106
ΔE (kcal/mol)	0.0	1.08	1.70	1.70	1.91	2.11	2.91	3.61	3.71	4.11	4.16	4.34	4.35

[a] Conformers.

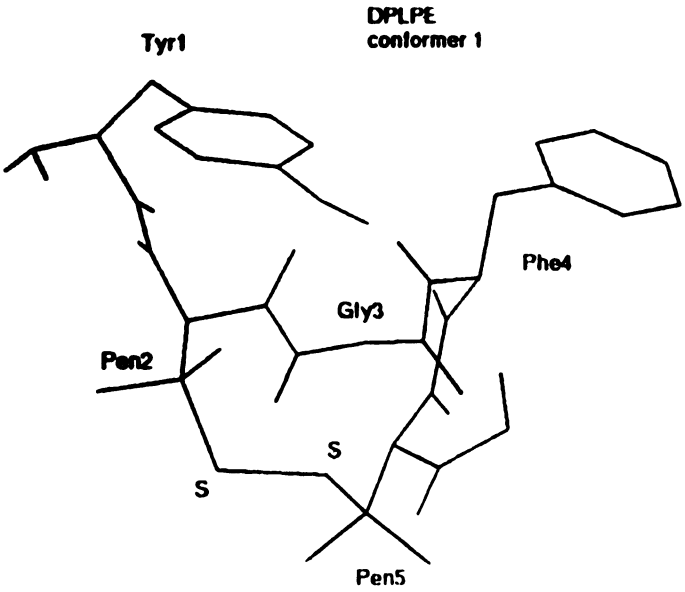

Fig. 1. Lowest energy conformer of DPLPE, considering a distance-dependent dielectric constant.

DPDPE and DPLPE. The reexamination was deemed necessary because of the limited search strategies utilized in each of the previous studies reported for these peptides. Previous work also failed to convincingly identify the solution conformation of DPDPE, even with the use of experimental NMR data in conjunction with these limited searches. An extensive and systematic conformational search is a required first step in identifying both solution conformations and the bioactive form of these peptides that is favorable for recognition at the δ receptor. Thus, in this study, we have made such an investigation of the conformational profile of cyclic peptides, using a variety of procedures. Specifically, we have used an algorithm to scan the conformational space of the backbone of cyclic peptides, together with energy minimizations of selected conformers. The resulting structures were then used as initial conformers in high and low temperature molecular dynamics simulations of the compounds. The purpose of the molecular dynamics study is 2-fold. It serves, first, to further scan the conformational space of the peptides and, second, to analyze their putative rigidity. For the first purpose, molecular dynamics at high temperature offer an efficient manner to overcome energy barriers and, thus, sample a large portion of the conformational space. For the second goal, low temperature molecular dynamics simulations are an efficient means to study the flexibility of different parts of the molecule. Knowledge of the flexibility of the different parts of the peptide should guide the selection for synthesis of some rigid analogs that could serve as more useful probes for the characterization of the ligand-receptor interactions.

Another goal of the current study was to determine whether a low energy solution conformer of DPDPE could be identified, consistent with its NMR spectra, from the results of a conformational analysis alone, without the explicit use of the experimental data.

TABLE 2
Torsion angles for the conformers reoptimized using a dielectric constant of 81

Dihedral angle	Torsion angle							
	DPLPE				DPDPE			
	11[a]	13	3	5	4	1	3	7
	degrees							
Tyr[1]								
Ψ	−69	177	−61	−55	−53	−62	−62	172
ω	−178	180	−175	−178	179	−173	180	−175
χ^1	171	56	173	−63	176	174	178	−167
χ^2	59	−110	−116	−64	−117	−118	−118	−120
Pen[2]								
ϕ	58	65	69	66	65	68	58	67
Ψ	−159	−156	−88	−96	−153	−82	−142	−138
ω	−174	−173	166	−176	−172	−178	−178	−176
χ^1	−177	−179	169	−176	−177	−175	−169	−31
χ^2	−161	−159	48	−96	−152	−92	−162	−50
Gly[3]								
ϕ	83	84	56	79	84	77	87	65
Ψ	−55	−56	−90	−62	−55	−75	−71	−95
ω	178	178	−177	171	180	175	−178	173
Phe[4]								
ϕ	−60	−61	−57	−58	−66	−65	−71	−71
Ψ	−57	−57	−38	−37	−65	−35	−56	−66
ω	178	179	180	179	−176	178	−175	−179
χ^1	−170	180	−63	−177	−57	61	64	179
χ^2	107	64	124	65	116	−77	112	61
Pen[5]								
ϕ	−68	−68	−56	−60	57	73	62	54
χ^1	75	74	57	−57	68	−70	69	61
χ^2	−43	−43	−178	65	−48	64	−56	74
Cβ-S-S Cβ	−103	−103	110	102	−100	106	−108	−114
ΔE (kcal/mol)	0.0	0.90	3.30	3.55	0.00	0.51	1.70	3.80

[a] Conformers.

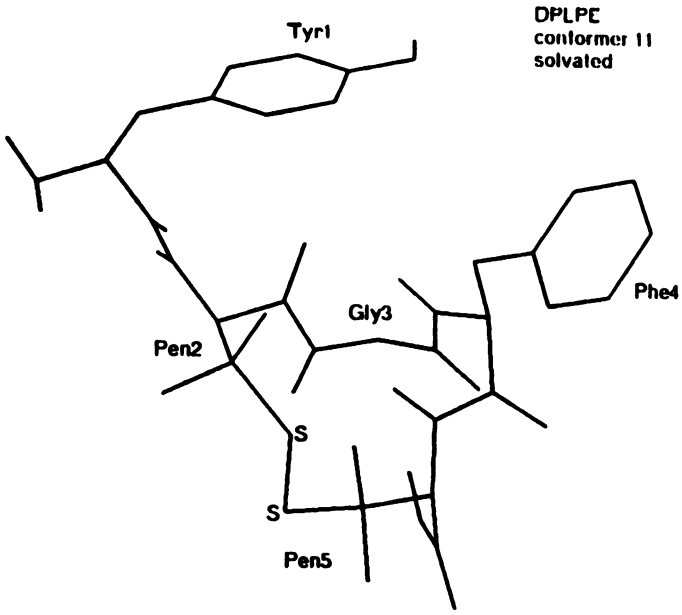

Fig. 2. Lowest energy conformer of DPLPE when the molecule is embedded in a continuum dielectric of 80.

Finally, even though this paper does not aim in itself to characterize a bioactive form for δ-selective peptides, a study of the flexibility of these two analogs should be regarded as an important milestone towards the characterization of molecular determinants for receptor recognition by these ligands.

The characterization of a bioactive form results from the systematic identification of structural and electronic commonalities and dissimilarities, considering all the accessible conformers of a series of high and low affinity analogs. The more flexible the analogs, the more complex this process, because the number of possible conformations that should be considered for each compound is larger. In the particular case of peptides, the task of simultaneously considering all possible low energy conformers for many analogs can be overwhelming, due to the large number of low energy conformational domains that are present. This study represents the first step in a procedure designed to overcome this difficulty. It is a careful conformational analysis of two high affinity peptides that could serve as templates for all the other peptides, with varied pharmacological profiles, that will be subsequently studied in order to characterize the bioactive form. For these additional analogs, only those conformations that resemble the conformations accessible to the template peptides, using objective criteria, will be considered. In this way, a significant reduction in the overall number of conformations to be considered can result. Among the δ-selective opioid peptides, both DPDPE and DPLPE have similar affinity and selectivity, which makes them well suited as templates for the characterization of structural and electronic effects that modulate the interaction. The present study should point out which of these two peptides is less flexible and, hence, more useful as a template for the interaction of other peptides.

Methods

The purpose of the first step in the search strategy used was to determine an initial set of low energy conformers, using a variation of the ring cyclization algorithm developed by Go and Scheraga (12). This procedure involved a systematic nested grid search of internal torsion angles for DPLPE, with standard bond distances and angles. The neutral peptide was considered for the entire study, unless otherwise noted. In this search, the ring is divided into three fragments, and the torsion angles of each segment are varied systematically, using user input increments, with $\Delta\tau = 60°$ being selected here. The values of the torsion angles adjacent to the vertex atoms are set by the ring closure requirement. Each structure generated was subsequently checked for close contacts. If the distance between any two atoms was less than 1.5 Å, the conformer was discarded. Otherwise, a single-point energy calculation was performed, using Scheraga's ECEPP potential (13, 14). At this level, only electrostatic repulsion, dispersion, hydrogen bonding, and torsional angles were considered. The conformers obtained were then ranked in order of increasing energy, and those within 60 kcal/mol of the lowest energy conformation were stored. One hundred and ten conformations resulted from this procedure and constitute an initial set of conformations from which to scan the potential surface of the peptides.

Each of the 110 conformations was minimized using the CHARMm (15, 16) potential. The internal energy in this potential includes bond distance, bond angle, torsional angles, improper torsional potentials, and electrostatic dispersion and hydrogen bonding interactions (15). The torsions, as well as the bond distances and angles, were allowed to relax using the Powell algorithm (15). Only the polar hydrogens were explicitly considered, and a distance-dependent dielectric constant was used during the first study. Eight unique conformations that were within 7.5 kcal/mol of the lowest energy structure were found and saved.

From these eight minimized structures for DPLPE, eight corresponding conformers of DPDPE were generated by inversion of the chiral center in the Pen[5] residue. The resulting diasteromers were minimized to remove all stress in the molecule, using the same method as for DPLPE.

Each of the eight minimized structures for each analog was used as a starting point for molecular dynamics studies to further scan the conformational space. The molecular dynamics study was performed by thermalization to 900 K, followed by an equilibration step and subsequent simulation. A high temperature was chosen because it allows high energy barriers to be overcome, resulting in a sampling of a large portion of the conformational space. The thermalization was carried out by increasing the temperature in 9° steps every 0.1 psec. The thermalization step was followed by 10 psec of equilibration and 25 psec of simulation. From the simulation, 500 structures were generated, by storing one set of coordinates every 0.05 psec. All structures were rank ordered, and the 30 lowest energy ones were fully optimized. This procedure was repeated for each of the eight conformers, which resulted in a total of 248 conformations for each compound. Of these, the lowest energy one was used as a starting point for a similar molecular dynamics study. Because no lower energy conformer was found by further molecular dynamics searching using the lowest energy conformer for each analog, the search was terminated.

In an additional effort, we characterized the flexibility of different fragments of these peptides, as determined by the variation in the torsion angles of their lowest energy form, during a molecular dynamic simulation at physiologically relevant temperatures. To analyze the conformational flexibility of both diastereomers, we performed molecular dynamics simulations using their CHARMm potential at 312 K, the physiological temperature for humans. The thermalization was performed by increasing the temperature 1.5° every 0.01 psec and equilibrating for 10 psec. The simulation step was performed for 75 psec, with data collection every 0.05 psec. A distance-dependent dielectric constant was used in each case.

A distance-dependent dielectric constant is a commonly used approach aimed at simulating a macromolecular environment without explicitly including water and counterions. The rationale for this approach is that two atoms inside a protein and close to one another have an interaction that is largely unaffected by the solvent, whereas inter-

TABLE 3

Torsion angles and relative energies for the lowest energy conformers of DPDPE

The torsional values from Refs. 8 and 11 are also reported.

Dihedral angle	Torsion angle									
	1[a]	2	3	4	5	6	7	8	Mosberg et al.	Hruby et al.
	degrees									
Tyr^1										
Ψ	−60	13	−59	−52	176	−43	176	−59	163	164
ω	−174	179	−172	−179	178	178	−176	−178	−177	−173
χ^1	174	57	170	−175	55	−175	−176	−178	−173	−163
χ^2	63	−104	−117	−116	−109	−116	−120	−118	−115	51
Pen^2										
ϕ	66	70	63	65	67	71	66	58	149	111
Ψ	−75	−157	−90	−142	−158	−155	−136	−127	−153	14
ω	−175	176	−171	−173	173	174	177	−175	−175	173
χ^1	−175	−53	−172	−174	−44	−52	−25	−167	−78	180
χ^2	−92	−67	−179	−157	−38	−68	−43	−166	178	143
Gly^3										
ϕ	66	42	50	75	41	43	54	69	78	−98
Ψ	−78	−98	−85	−63	−86	−93	−90	−76	−111	−18
ω	−179	−173	−173	−178	−176	−174	176	−172	−164	177
Phe^4										
ϕ	−63	−68	−73	−59	−61	−64	−73	−69	−85	−72
Ψ	−33	−24	−51	−60	−20	−32	−57	−49	38	−46
ω	174	−177	−169	−176	179	−173	−174	−175	172	−175
χ^1	62	−60	67	−59	54	−178	180	68	−64	179
χ^2	−80	115	116	118	132	65	61	113	105	68
Pen^5										
ϕ	67	63	54	51	68	63	42	53	61	63
χ^1	−64	60	77	67	−51	61	60	69	−87	−70
χ^2	61	−170	−52	−48	171	−168	70	−54	60	119
$C\beta$-S-S $C\beta$	106	130	−114	−103	−105	131	−113	−110	110	−110
ΔE (kcal/mol)	0.0	2.28	3.37	3.43	3.52	4.28	4.37	4.52		

[a] Conformers.

actions between atoms separated by longer distances are more affected. Hence, the use of a distance-dependent dielectric constant should be more appropriate for the description of the environment in which the peptide assumes its bioactive form.

In addition, we decided to study the effects of a larger dielectric constant of 80, for two reasons. The first was to compare the results with the experimental structural data for DPDPE in water. This comparison serves as a validation of the completeness of the search strategy and an assessment of the ability of the method to correctly depict solution conformations without the use of experimentally derived distance constraints. It is also possible that, because the peptides are transported to the receptor in an aqueous environment, their low energy conformers in solution will be the most available at the receptor site, even if the peptide is dehydrated just before binding to the site. In this case, the bioactive form would be one that is energetically accessible from the solution conformation of the peptide.

Thus, the four lowest energy conformers obtained for DPDPE and DPLPE were reoptimized, using a dielectric constant of 80 to simulate the effects of water as a solvent (16).

To further investigate the effect of solvent, the most favorable conformations found for DPDPE and DPLPE were subjected to molecular dynamics at 312 K, with a dielectric constant of 80 and a duration of 75 psec, using heating and equilibration protocols similar to those described for the distance-dependent dielectric constant approach. All the structures collected were minimized as described above. No lower energy structure resulted from this procedure.

Results and Discussion

From the extensive searching procedure described in the previous section, only those conformations within 4.5 kcal/mol of the lowest energy one are reported.

For DPLPE, the number of structures found is 13. Their torsional angles and relative energies are given in Table 1.

For this analog, all the structures found are compact, due to the formation of internal hydrogen bonds. The *p*-OH group of the Tyr as well as the OH on the carboxyl terminus form internal hydrogen bonds in several of these conformations. The lowest energy conformer forms three internal hydrogen bonds, NH(Pen^5) to CO-(Gly^3), NH(Phe^4) to CO(Pen^2), and the *p*-OH of the Tyr^1 to the CO(Phe^4). This conformer is shown in Fig. 1. All conformers found within this range of energies have a minimum of two hydrogen bonds, except for conformer 11 with only one hydrogen bond between NH(Tyr^1) and CO(Pen^2).

When the conformers of DPLPE are reoptimized using a dielectric constant of 80 to simulate the effect of water as a solvent, instead of the distance-dependent dielectric constant, the internal hydrogen bonds are all broken and the rank of order of energy of the optimized conformers is changed. Conformer 11 becomes the lowest energy one. In Table 2, we present the torsion angles for the four lowest energy conformers found when the conformers obtained using a distance-dependent dielectric constant were reoptimized using a dielectric constant of 80, to simulate the effects of the solvent as a continuum. The changes observed in geometry, compared with the results with a distance-dependent dielectric constant, are due largely to a rearrangement that breaks all internal hydrogen bonds. Fig. 2 shows conformer 11, the lowest energy structure after reoptimization.

For DPDPE, the other analog studied, a total of eight con-

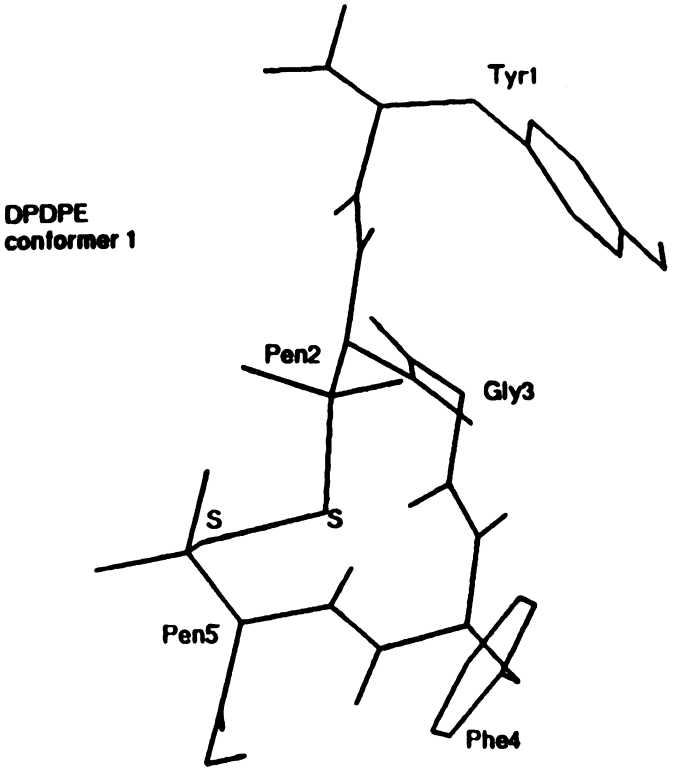

Fig. 3. Lowest energy conformer of DPDPE, considering a distance-dependent dielectric constant.

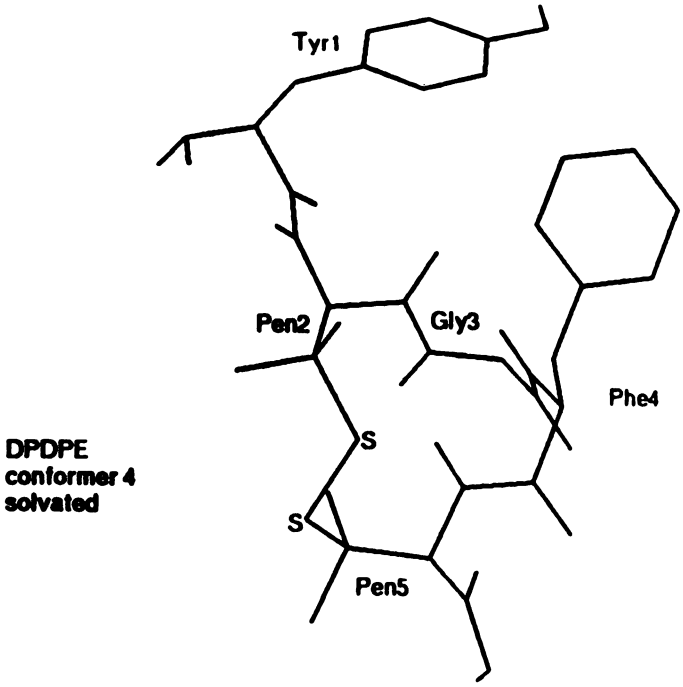

Fig. 4. Lowest energy conformer of DPDPE, considering a dielectric constant of 80 for the environment.

formers within 4.5 kcal/mol of the lowest energy structure were found, using the procedure outlined in the previous section. The torsional angles and related energies for these eight conformers are presented in Table 3. This number of conformers is considerably smaller than the number found for DPLPE for the same energy range. Another difference is less internal hydrogen bonding. The total number of internal hydrogen bonds formed in these conformations does not exceed two and for several of these conformations is only one. For example, the lowest energy conformer of DPDPE, shown in Fig. 3, has two hydrogen bonds, NH(Gly^3) to OC(Tyr^1) and NH(Phe^4) to OC(Pen^2). For those structures with only one hydrogen bond, additional stabilization is gained by van der Waal interaction between the phenyl rings of the Tyr^1 and Phe^4.

The most significant effect of embedding the peptide in a continuum dielectric constant of 80 is that reoptimization breaks all internal hydrogen bonds and affects the energy order of the structures. In Table 2, the relative energies of the four lowest conformers in the field of the dielectric are given.

In general, the structures tend to remain within the same conformational domain, as reflected by the small changes in the values of the torsional angles, relative to those found by optimization with a distance-dependent dielectric constant. However, some important differences can be found. For instance, conformers 3 and 8 of DPDPE, which were distinct in the presence of a distance-dependent dielectric constant (Table 3), collapse to the same minimum energy structure 3 shown in Table 2 when the larger dielectric constant is considered. Fig. 4 shows the lowest energy conformer found under this condition.

A striking result of the present study is that conformer 4, which is predicted to be the lowest energy structure for DPDPE using a dielectric constant of 80 to simulate water as a solvent, shows most of the characteristics derived from the experimental solution NMR. This conformer was obtained from a systematic search procedure without use of any experimental data.

The inclusion of the dielectric constant of 80 breaks the internal hydrogen bonds found between NH(Phe^4) and OC(Pen^5) in vacuum. The resulting structure has only the NH(Pen^5) pointing inwards to the center of the ring and no internal hydrogen bonds. Hence, only the NH(Pen^5) is inaccessible to the molecular surface for interaction with the solvent, consistent with the observed low temperature dependence of this amide proton chemical shift (11). In addition, the phenyl rings of Tyr^1 and Phe^4 in these structures form a van der Waals complex, and Phe^4 is less than 6 Å from the sulfur atoms, as suggested by Hruby *et al.* (8). The conformer also gives consistent values for χ^1 to Tyr^1 and Phe^4, in agreement with the values for the most populated rotamers, which are approximately 180° and −60°.

In Table 4, the interproton distances derived from NOE experiments (11) are given, together with those calculated for our candidate solution conformer 4, deduced without use of any experimental information. As seen in this table, calculation of interproton distances for this conformer led to values consistent with the range inferred from the NMR studies (11).

Shown in Table 3 are data for the two previously proposed candidates (8, 11) for the solution conformation of DPDPE. Comparison of these conformers with conformer 4, our calculated lowest energy conformer in a simulated solution environment, shows significant differences in conformation. In Table 4 are given the interproton distances calculated using conformer 4 and the two previously reported conformers. In order to calculate these distances, it was necessary to generate complete three-dimensional structures from the torsion angles reported for the two previous candidates. This was done by using these torsion angles together with standard values of bond lengths and angles taken from CHARMm. Comparison of the

TABLE 4

Interproton distances from NOE experiments. The values for conformer 4, and the candidate structures by Hruby and coworkers and Mosberg and coworkers are also reported. Limits to the validity of these comparisons are indicated throughout the text. All distances are given in Å.

Proton[a]	Proton	Experiment[b]	4 this work	Hruby et al.[c]	Mosberg et al.[d]
αH (1)	NH (2)	2.76	3.60	2.47	2.37
βH (1)	Me-pro S (2)	3.15	3.56	4.96	2.60
βH (1)	Phe-meta (1)	2.64	2.10	2.19	2.28
NH (2)	Me-pro S (2)	3.61	2.69	2.44	2.84
αH (2)	Me-pro R (2)	2.52	3.06	3.01	2.48
αH (2)	Me-pro S (2)	2.76	3.55	3.15	2.75
αH (2)	NH (3)	2.28	2.19	3.42	2.25
αH (2)	αH-pro R (3)	3.46	4.13	5.00	4.44
Me-pro R (3)	NH (3)	2.66	4.26	5.03	2.29
NH (3)	αH-pro R (3)	2.83	2.78	2.89	2.33
αH-pro R (3)	NH (4)	3.40	3.34	3.63	3.41
αH-pro S (3)	NH (4)	2.28	3.40	2.53	2.17
NH (4)	βH (4)	2.76	1.96	2.36	2.41
NH (4)	NH (5)	2.71	3.03	2.66	3.04
αH (4)	NH (5)	2.43	3.62	3.60	2.88
βH (4)	Me-pro S (5)	2.92	3.15	5.62	5.73
NH (5)	Me-pro R (5)	2.54	2.10	4.37	4.18
αH (5)	Me-pro R (5)	2.96	3.37	3.17	2.45

[a] The numbers in parentheses indicate the residue to which the hydrogen atom belongs.
[b] From Reference 11.
[c] Derived from the torsional angles provided in Reference 8. See limitations of the comparison in text.
[d] Derived from the torsional angles provided in Reference 11. See limitations of the comparison in text.

TABLE 5

Variation in the torsion angles for the lowest energy analogs of each stereoisomer, for 75 psec of molecular dynamics simulation at 312°K

Dihedral angle	Torsion angle					
	DPLPE			DPDPE		
	Minimum	Maximum	Δ	Minimum	Maximum	Δ
	degrees					
Tyr^1						
Ψ	−73	−15	58	−101	−12	89
ω	−197	−159	38	−201	−154	47
χ^1	47	103	56	−219	−142	77
χ^2	−88	−39	49	35	92	57
Pen^2						
ϕ	36	94	58	39	92	61
Ψ	−182	−73	109	−114	−52	62
ω	156	205	49	148	209	51
χ^1	153	191	38	−195	−152	43
χ^2	44	96	52	−128	−64	64
Gly^3						
ϕ	−20	89	109	29	103	74
Ψ	−123	−57	66	−105	−42	63
ω	152	188	36	156	203	47
Phe^4						
ϕ	−94	−54	40	−95	−34	61
Ψ	31	86	55	−61	−8	53
ω	−196	−154	42	154	198	44
χ^1	155	236	81	107	256	149
χ^2	−154	−86	68	69	145	76
Pen^5						
ϕ	−102	−40	62	37	93	56
χ^1	−70	−25	45	−85	−42	43
χ^2	−139	−67	72	35	81	46
Cβ-S-S Cβ	75	119	44	87	131	44

experimental values obtained from conformer 4 and from the two previous candidates that were developed using distance constraints indicates that our conformer has better overall agreement with the experimental information. When these two conformers were reoptimized using the CHARMm potential, the resulting conformers were significantly higher in energy than our lowest energy conformer, and the disagreement with the NOE data was even greater. Although the conformers described by Mosberg *et al.* (11) were originally developed using the AMBER potential, this high energy is most likely due to

TABLE 6

Variation in the torsion angles for each of the lowest energy conformers using a distance-dependent dielectric constant, after 75 psec of simulation at 312°K

The RMS/residue is also reported. The RMS/residue of the lowest energy conformers at a dielectric constant of 80, after 75 psec of simulation at 312°K, are also reported, in parentheses.

Dihedral angle	DPLPE				DPDPE			
	Torsion angle			RMS	Torsion angle			RMS
	Minimum	Maximum	Δ		Minimum	Maximum	Δ	
	degrees				degrees			
Tyr[1]								
Ψ	−73	−15	58	0.448 (0.800)	−101	−12	89	1.650 (1.108)
ω	−197	−159	38		−201	−154	47	
χ^1	47	103	56		−219	−142	77	
χ^2	−88	−39	49		35	92	57	
Pen[2]								
ϕ	36	94	58	0.314 (0.335)	39	92	61	0.724 (0.596)
Ψ	−182	−73	109		−114	−52	62	
ω	156	205	49		148	209	51	
χ^1	153	191	38		−195	−152	43	
χ^2	44	96	52		−128	−64	64	
Gly[3]								
ϕ	−20	89	109	0.520 (0.475)	29	103	74	0.883 (0.576)
Ψ	−123	−57	66		−105	−42	63	
ω	152	188	36		156	203	47	
Phe[4]								
ϕ	−94	−54	40	0.478 (0.465)	−95	−34	61	0.548 (0.503)
Ψ	31	86	55		−61	−8	53	
ω	−196	−154	42		154	198	44	
χ^1	155	236	81		107	256	149	
χ^2	−154	−86	68		69	145	76	
Pen[5]								
ϕ	−102	−40	62		37	93	56	
χ^1	−70	−25	45		−85	−42	43	
χ^2	−139	−67	72		35	81	46	
Cβ-S-S Cβ	75	119	44		87	131	44	

the fact that an extensive search of conformational space was not made but, instead, distance-dependent constraints were incorporated in a limited search strategy to identify conformers consistent with the NMR data.

One other experimental condition of the solution NMR spectra should be noted. All spectra were taken at a pH of 3 (11), presumably to prevent zwitterion formation by protonation of both the amino-terminal and carboxyl-terminal ends. To further check the relevance of conformer 4 as the solution NMR-compatible structure, we reoptimized the eight lowest energy conformers of DPDPE with the amino-terminal amine being protonated. In the resulting rank order of energy, conformer 4 is still the lowest energy structure for DPDPE, without any significant structural modification.

When we examine the flexibility of the lowest energy conformers of each analog obtained using the distance-dependent dielectric constant, significant structural changes are observed when thermal fluctuations are considered. In Table 5, we report the variations observed for each torsion angle during 75 psec of the molecular dynamics simulations at 312 K. The results indicate that the ϕ Gly[2] angle shows considerable dispersion during the dynamics simulation for both analogs. Another portion of these peptides with considerable flexibility is the χ^1 Phe[4] torsion. For DPDPE, this apparent flexibility masks the existence of two clearly differentiated populations of rotamers for this torsion, centered around +60° and −60°, that are interconvertible at physiological temperature.

The analysis of the root mean square fluctuations/residue for the two lowest energy conformers found using the distance-dependent dielectric constant and a dielectric constant of 80 is given in Table 6. The larger fluctuations observed during the simulation for DPDPE are a direct consequence of less internal hydrogen bonding being observed for this peptide. In DPLPE, the backbone angles of Gly[3] show considerable dispersion, which is larger than that observed for Phe[4]; this is a surprising result because this rotatable phenyl group is not part of the ring. The large flexibility of the Tyr[1] side chain in DPDPE is due to the lack of internal hydrogen bonds that preferentially stabilize a conformation of this residue in DPLPE. In the cyclic portion, the most flexible region is again associated with the Gly[3] residue.

With a dielectric constant of 80, both compounds have increased flexibility, because under this condition internal hydrogen bonds are not as strong.

Conclusion

In this paper, we have systematically and extensively reexamined the conformational profile of DPDPE and DPLPE, studying both the static and the dynamic aspects of the conformational space of these molecules. We have found that most of the structural features of the different minima are preserved independently of the use of a dielectric constant to simulate the environment, but the rank order as well as the energy differences among conformers are greatly influenced by the environment.

The use of molecular dynamics techniques reveals that such

cyclic peptides are not as rigid as originally assumed (15). A significant number of low energy conformers of both DPLPE and DPDPE are obtained and, even in the lowest energy one, large conformational flexibility is still present in the cyclic structure, associated mainly with the Gly^3 residue. Residues 1 and 4 also have some flexibility, due to the free rotation of their aromatic rings. These qualitative results clearly identify the regions of greatest flexibility of the peptide and suggest that new analogs with side chains or backbones constrained in those areas of the peptide would be more rigid. Synthesis and evaluation of such analogs can lead to some that may be valuable probes in the characterization of the interaction of these peptides with the different opiate receptor subtypes, increasing the chances of enhancing selectivity and determining the bioactive form of these compounds.

In these studies, we have obtained a lowest energy conformer of DPDPE in a simulated aqueous environment that is in excellent agreement with all the observed NMR spectra features and geometric parameters, using a search strategy that does not involve experimental data. NMR spectroscopic information obtained for a particular analog does not lead to the identification of a unique and energy-accessible conformation in solution. The full characterization of a three-dimensional structure in solution requires additional information attainable only by theoretical analysis. The results obtained indicate that the straightforward approach to finding candidate solution conformers used here, which combines an extensive conformational search with reoptimizations of the lowest energy conformations in a solvent environment, is a promising one, leading to conformers that are both low in energy and compatible with NMR data.

The selection of conformer 4 as a plausible candidate for the description of the structure of DPDPE in polar solvents, nevertheless, requires further refinements. Chief among them is the explicit inclusion of the solvent. The presence of direct interactions between the solvent and the peptides can alter both the minimum energy structure and the putative flexibility of this analog. Solvent effects are likely to be different for DPDPE and DPLPE, due to the tendency of the latter to maximize the number of intramolecular hydrogen bonds in its lower energy structures.

Even though the environmental effects at the binding site of the receptor will be considerably different from those of a highly polar solvent, the conformers with internal hydrogen bonds, which were found to be the low energy forms of DPDPE and DPLPE with a distance-dependent dielectric constant, are unlikely to be the conformers that interact with the receptor site, for two reasons. The first is that the peptide is most likely transported to the receptor site in a solvated form and is desolvated at the binding site. Thus, the form it has upon desolvation would more closely resemble the lowest energy conformer found in a simulated solvent environment. Second, in this non-hydrogen-bonded form, polar groups in the peptide are available for recognition by the receptor via long range electrostatic interactions. Third, there is the possibility that the opiate peptides dynamically accommodate the conformational requirements of the binding site and that these analogs are binding by an induced fit, a mechanism that resembles the concept of a dynamic pharmacophore or a zipper mechanism, as proposed in the past.

The determination of the bioactive forms of peptides requires the study of more than one analog. Such studies are the focus of current effort in our laboratory.

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Send reprint requests to: Hugo O. Villar, Molecular Research Institute, 845 Page Mill Road, Palo Alto, CA 94304.