Journal of Computer-Aided Molecular Design, 6 (1992) 175–190
ESCOM

J-CAMD 153

Characterization of low-energy conformational domains for Met-enkephalin

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Received 18 March 1991
Accepted 5 November 1991

Key words: Met-enkephalin; Conformation; Solvent effects; Conformational search

SUMMARY

An extensive exploration of the conformational hypersurface of Met-enkephalin has been carried out, in order to characterize different low-energy conformational domains accessible to this pentapeptide. The search strategy used consisted of two steps. First, systematic nested rotations were performed using the ECEPP potential. Ninety-two low-energy structures were found and minimized using the CHARMm potential. High and low-temperature molecular dynamics trajectories were then computed for the lowest energy structures in an iterative fashion until no lower energy conformers could be found. The same search strategy was used in these studies simulating three different environments, a distance-dependent dielectric $\varepsilon = r$, and two constant dielectrics $\varepsilon = 10$ and $\varepsilon = 80$. The lowest energy structure found in a distance-dependent dielectric is a Gly-Gly β-II′-type turn. All other structures found for $\varepsilon = r$ within 10 kcal/mol of this lowest energy structure are also bends. In the more polar environments, the density of conformational states is significantly larger compared to the apolar media. Moreover, fewer hydrogen bonds are formed in the more polar environments, which increases the flexibility of the peptide and results in less structured conformers. Comparisons are made with previous calculations and experimental results.

INTRODUCTION

Met-enkephalin (Tyr-Gly-Gly-Phe-Met) is an endogenous opioid pentapeptide with a pharmacological profile similar to morphine. Since its discovery, a great deal of theoretical and experimental effort has been devoted to the characterization of its structure and conformational profile. A large number of synthetic peptides have also been synthesized and pharmacologically evaluated in attempts to understand the molecular requirements for peptide recognition and activation of

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the opioid receptor subtypes. In general, the vast majority of these analogs are agonists with varying affinity and selectivity at the μ- and δ-receptors. Despite these extensive studies, the bioactive form and molecular determinants of recognition of these two receptors by opioid peptides have not yet been definitively characterized. The lack of definitive resolution of these questions is due largely to the fact that previous theoretical studies lack one or another of the two components required for unambiguous conclusions. One such component is the use of search strategies that allow extensive sampling of the conformational space available to the peptides and hence ensures a relatively complete set of energy-ordered conformers, including the lowest energy ones. A complete sampling of the conformational space is required since the bioactive form need not be the lowest energy conformer that a single high-affinity peptide adopts in vacuo or condensed phase. Another essential component is the inclusion of a significant number of peptides with varying affinities in the study, in order to develop molecular criteria for recognition common to all high-affinity and absent in low-affinity analogs.

A majority of the theoretical studies reported have considered only Met-enkephalin itself and have recently focused particularly on the use of different search strategies to identify its absolute minimum-energy structure or 'global minimum'. A pioneering study dealing with the preferred conformations of the peptide was carried out by Isogai et al. [1]. The calculations were carried out with the ECEPP force field [2], which evaluates the conformational energy without considering bond distance or bond angle terms in the potential, thus constraining these structural parameters to fixed values during an optimization. From this study, Scheraga and co-workers concluded that the 'global minimum' was a β-II′-type turn structure with the turn formed between residues Gly^3-Phe^4, which is also labeled as a G-P β-II′ turn. Many additional low-energy bend structures were also reported. Theoretical calculations carried out by other investigators also reported a bend structure [3,4] as the most stable conformation. In more recent work, Scheraga [5–8] continued to study the performance of different searching methods in identifying the global minimum structure using, as a reference for the success of the search, the first candidate G-P β-II′ turn found.

Monte Carlo simulated annealing has been used to obtain a global minimum in the conformational space of Met-enkephalin [9–11]. Moskowitz et al. [9] were the first to test the ability of simulated annealing to find a global minimum for Met-enkephalin as a model peptide. Consistent with all previous results, they also found a G-P β-II′ turn conformation as the lowest energy form. However, the lowest energy structure found by Moskowitz and co-workers was somewhat different and 2.3 kcal/mol lower in energy, than the structure reported by Scheraga as a global minimum, when both conformations were evaluated using the same force field. The discrepancies in the minima found can be attributed to the different force fields used since the AMBER potential [12] was used by Moskowitz rather than ECEPP. Ascribing the differences in the force fields as being responsible for the different structures characterized appears justified, since a conformation similar to that reported by Scheraga is found when the ECEPP potential is used in combination with this procedure [11].

Recently, an efficient implementation of a modification of Scheraga's build-up procedure combined with a restrained energy minimization of the ECEPP energy function was reported [13]. Application of this method potential to Met-enkephalin using ECEPP resulted in a large number of minima. However, the lowest energy structure found by this procedure was approximately 2 kcal/mol higher in energy than the minima reported in the above-mentioned studies using the same potential.

Experimental studies have also attempted to characterize the preferred conformation for this peptide. Early proton NMR spectroscopy carried out in DMSO [14–17], supported a G-P β-I-type turn structure, reporting evidence of a hydrogen bond between residues Gly^2-Met^5. Later studies [18], corroborated the existence of this hydrogen bond and the bend nature of the structure found in solution. Moreover, further proton NMR studies at 500 MHz [19] demonstrated the absence of a single rigid conformation for this molecule. On the other hand, three independent X-ray diffraction studies [20–22] indicate that the structures observed in the solid phase correspond to extended conformations. However, the analysis of the diffraction patterns revealed the existence of dimers in the solid state in which the extended form was stabilized by a large number of intermolecular hydrogen bonds between the pairs.

As mentioned, identification of the global minimum of Met-enkephalin alone, or characterization of its structure in solution or condensed phase, is not enough to identify the bioactive form that binds to the opioid receptors. There have been a small number of studies that included a number of Met-enkephalin analogs in an attempt to characterize the bioactive form recognized by the μ-opioid receptor. However, these studies, described below, did not include systematic search strategies designed to ensure extensive sampling of possible low-energy conformers. They focused instead on comparisons of plausible forms of high-affinity peptides and nonpeptide opioids from different families, and in some cases used inactive peptides as a control for selection of the bioactive form.

Bradbury et al. [23] using CPK models argued that the bioactive conformation of Met-enkephalin was most likely to be a G-G β-bend formed between residues Tyr^1-Phe^4. They found that this type of conformation had most of the structural features found in the potent rigid opiate oripavine. Momany [24], using ECEPP, tried to explain the conformational changes that affected the peptide in its zwitterionic form, as well as to explain the higher affinity found in some derivatives. Simultaneously, our group [25] also carried out a conformational study, which complemented the work reported by Isogai et al. [1] to characterize conformations of Met-enkephalin analogs with significant overlap with the potent thebaine PET opiate: 7-(1-phenyl-3-hydroxybutyl-3-)endo-ethenotetrahydrothebaine. This study and a subsequent one for tetrapeptides [26] also supported a G-G β-II′ turn as the bioactive conformation for recognition at the μ-receptor.

More recently, Ishida et al. [27], in order to characterize 'the folding mechanism' of the molecule and to understand the type of conformation involved in the activity of Met-enkephalin, carried out a molecular dynamics study restricted to the trajectory of an extended conformation. They also concluded that the G-G β-II′ turn is the 'folded' conformation in equilibrium.

While these previous studies may have identified some of the correct structural features of the bioactive form of the peptide, the limited search strategies used weaken their credibility in two ways: (1) other low-energy conformers that could also consistently account for observed profiles may have been overlooked, and (2) after a more extensive conformational search, the conformer chosen could turn out to be an unreasonably high-energy one.

In order to overcome some of these weaknesses, we have devised an extensive search strategy for linear peptides, that has not yet been reported. We are testing its validity using Met-enkephalin as an example, since this peptide has been studied using many other strategies. The procedure combines high and low-temperature molecular dynamics simulations and energy minimizations in an iterative fashion. Different dielectric constants were used to simulate different environmental conditions.

One of the principal aims of the work presented here is the evaluation of the ability of the new search strategy to scan the conformational space of short chain peptides. Reports in the literature show the advantages of search strategies based on the use of molecular dynamics for unrelated compounds [28]. This study of Met-enkephalin will provide information on its usefulness for a future systematic characterization of the conformational space of other short chain peptides, that could be applied to the deduction of a bioactive structure for the opiate receptors.

Another goal of this work is to characterize the influence of the environment on the structure of Met-enkephalin, as measured by the use of a distance-dependent dielectric constant $\varepsilon = r$ and two constant dielectrics of 10 and 80. The distance-dependent dielectric corresponds to the tendency of the field lines connecting interior atoms in an apolar environment to spread into the high dielectric solvent region. This condition corresponds to the physical environment of a ligand in a buried cavity of a protein. The higher dielectric constants simulate a membrane environment ($\varepsilon = 10$) and an aqueous phase ($\varepsilon = 80$). Embedding the peptides in a continuum dielectric results in a partial screening of the electrostatic interactions that make an important contribution to the internal energy for any force field. Therefore, two different effects can be expected based on the results. The first is a reduction in the strength of the hydrogen bonds that stabilize folded conformations, and the second is an energy reordering of the structures. However, the magnitude of these effects has not been explored for opiate peptides. If the effects are significant, then the deduction of the bioactive structure cannot be done assuming a particular environment, since the characteristics of the recognition site for the opiate receptors are unknown. Rather, such identification of the bioactive form would require parallel studies simulating various environmental conditions.

While the purpose of this study is not the characterization of a bioactive form, the information gained by the evaluation of the search strategy and of the influence of the environment on the low-energy conformational domains is a necessary first step for the selection of the most adequate strategy for its characterization.

METHODS

The search strategy adopted in this study combines two procedures. The first step involved a systematic nested grid search of the backbone torsion angles of Met-enkephalin, using standard bond angle distances and torsion increments of 60°. Only for this step were the side chains kept in an extended conformation. Each possible structure was generated and checked for close contacts. If the distance between any two nonbonded atoms was less than 1.5 Å, the conformer was discarded. Otherwise, a single point energy calculation was performed using the ECEPP potential [2]. In this potential, only electrostatic interactions, dispersion, hydrogen bonding, van der Waals and torsional energies were considered. The conformers obtained were rank ordered by energy and those within 50 kcal/mol of the lowest energy conformation were stored. This procedure yielded ninety-two unique conformers that constituted the initial set used as starting points to scan the potential surface of the peptide.

Each of the 92 conformers was minimized using the CHARMm program distributed by Polygen Inc. The potential in this program has been discussed in detail elsewhere [29]. All minimizations in this study were carried out allowing relaxation of all geometric parameters, including side chains, using the adopted-basis Newton Raphson algorithm. All hydrogen atoms were explicitly considered in this study. The criterium for termination of the minimizations was that the norm of the gradient was smaller than 10^{-6}.

All calculations were carried out in parallel using three environmental conditions: (1) a distance-dependent dielectric constant, $\varepsilon = r$; (2) $\varepsilon = 10$; and (3) $\varepsilon = 80$.

The second step of this search strategy, as shown schematically in Fig. 1, consisted of iterative high and low-temperature molecular dynamics simulations of sets of candidate conformers in the three environments chosen. All simulations were preceded by 10 ps of heating and 10 ps of equilibration and the time steps used were 1 fs. The cycle was continued until no new conformers within 5 kcal/mol of the current lowest energy conformer were found. The first working set of 33 conformers chosen (set 1, Fig. 1) was obtained by minimization of the structures derived from the systematic nested rotations. Each structure was subjected to 30 ps of molecular dynamics (MD) simulations at 1500 K. During this simulation, 50 structures were stored at regular intervals of 0.6 ps and subsequently minimized. From the 1650 minimized structures obtained by this procedure, all those within 5 kcal/mol above the lowest energy structure were chosen as a second working set for the low-temperature part of the cycle and subjected to 75 ps of low-temperature MD at 310 K. During each of these trajectories, 50 conformations were stored at regular intervals of 1.5 ps and subsequently minimized. The results led to the selection of a third set of structures (set 3) for the next cycle of MD simulations. In this set only, since there were so few conformers within the 5 kcal/mol cutoff, it was increased to 7.5 kcal/mol which resulted in 27 candidate structures, a number comparable to that in all other working sets, where a criterion of 5 kcal/mol was used.

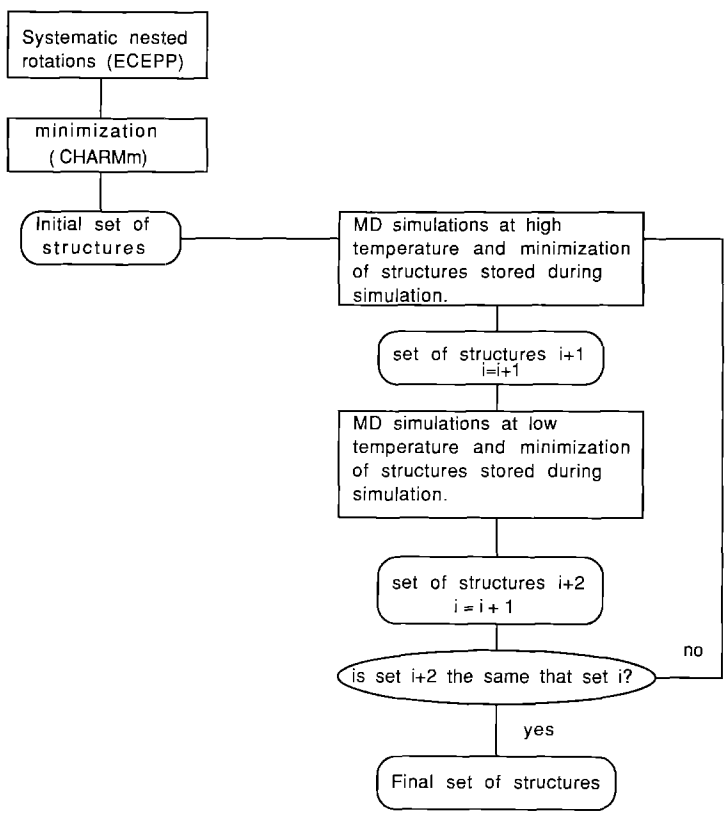

Fig. 1. Schematic representation of the strategy adopted for the scanning of the conformational space of Met-enkephalin.

As shown in Fig. 1, two additional cycles of high and low-temperature MD simulations, at 900 K for 30 ps and 310 K for 75 ps respectively were performed. In each case, 50 structures were saved and subsequently minimized. All structures with energies within 5 kcal/mol of the then lowest energy structure were chosen as the set to be subjected to the next MD simulation. The procedure was terminated when the set of working structures did not change after a cycle of high and low-temperature molecular dynamics simulations.

The high-temperature MD simulation allowed a large portion of the conformational hypersurface to be scanned, while the low-temperature simulations allowed a more detailed exploration of the area surrounding each minimum, and permitted the relaxation of structures that could have been trapped in high-energy domains after the high-temperature simulation.

The neutral form of Met-enkephalin was used throughout this study, in order to facilitate the comparison of our searching procedure with those previously reported. Moreover, the use of a nonionic form should also provide a more appropriate comparison to study the influence of the environment on the conformation, partly filtering out the electrostatic component that could be dominant in ionic structures.

Finally, each of the lowest energy structures in each environment simulated was subjected to 200 ps of molecular dynamics simulations at 310 K, in order to analyze their conformational flexibility.

RESULTS AND DISCUSSION

Evaluation of the search strategy in the $\varepsilon = r$ media

Over 6000 conformers were obtained in the process outlined above within 20 kcal/mol of the lowest energy conformer. Of all the structures generated, only 741 differed by at least 30° in one backbone angle, which is the criterion used for uniqueness.

The analysis of the unique conformers found by each step of this procedure revealed that after each iteration, a new global minimum energy structure with lower energy was found. The improvement can be visualized using Fig. 2, which shows a stacked histogram of the energy distribution of unique conformers found at each step in this search strategy. Table 1 complements Fig. 2. It gives (a) the energy of the lowest energy conformer found after each step relative to the lowest energy conformer found at the end of the study, (b) the number of structures on which MD was performed at each step, and (c) the number of unique structures characterized after minimization. For example, the lowest energy conformer after the first MD simulation at 1500 K (set 2) was only 3 kcal/mol lower in energy than the lowest energy structure generated by minimization of the nested grid rotation (set 1). In contrast, the first MD at 310 K (set 3) resulted in a dramatic drop of 7 kcal/mol in the energy of the lowest energy structure compared to the previous minimum.

No simple relationship was found between the relative energy of a conformation at a given step and that of the conformations derived from it by MD simulations in the next step of the procedure. For instance, the lowest energy conformer found in this study was derived from a conformer that was 4.1 kcal/mol above the lowest energy one in the first set of minimized structures. Conversely, the lowest energy structure in the first minimized set did not contribute any new low-energy structures after the first two MD iterations. Consequently, the prediction of the future relevance of a structure in the working set at any step in this search strategy is a difficult task. However, the

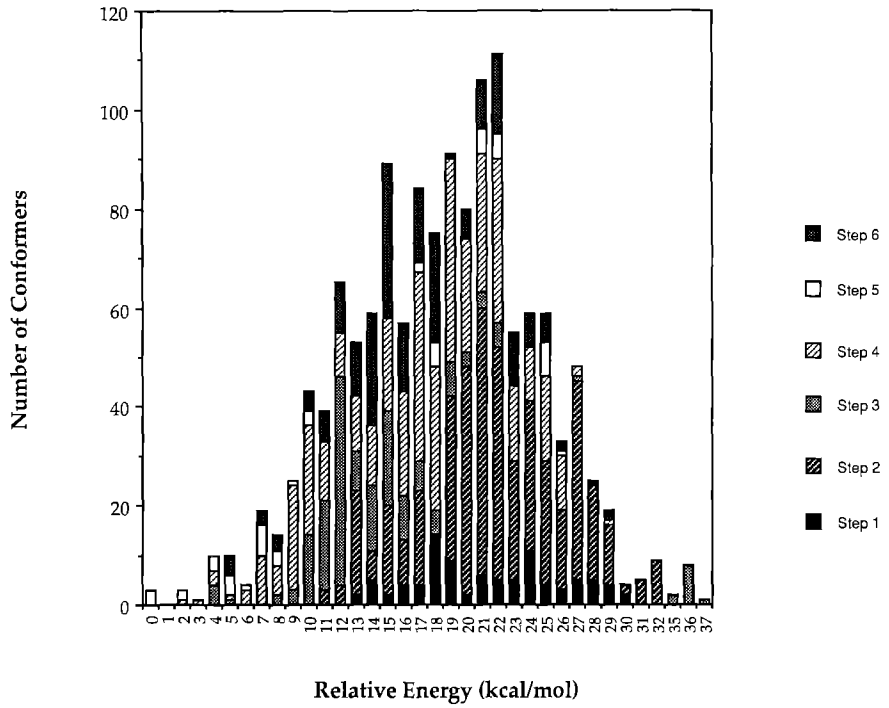

Fig. 2. A histogram of the energy distribution of the diverse unique minima found throughout this study: (1) after minimization of the structures generated by nested grid rotations; (2) after MD simulations at 1500 K and minimization; (3) after MD simulations at 310 K and minimization; (4) after a first set of MD at 900 K and minimization; (5) after a second set of MD at 310 K followed by minimization; (6) after a second set of MD at 900 K and minimization. A last step involving MD at 310 K was carried out but no new unique structures were derived within the working set.

energy analysis of the evolution of the conformations suggested that, in most cases, working with structures within a threshold of 5 kcal/mol was sufficient to scan the conformational surface for this peptide, provided that a significant number of structures is used at each iteration. For exam-

TABLE I
NUMBER OF STRUCTURES FROM WHICH THE SET WAS GENERATED (NMD), RELATIVE ENERGY IN KCAL/MOL OF THE LOWEST ENERGY CONFORMER FOUND (E) AND TOTAL NUMBER OF UNIQUE MINIMA FOUND AT EACH STEP (N)

Set	NMD	E	N
1	–	13.5	92
2	33	11.0	319
3	45	3.7	402
4	27	1.7	618
5	18	0.0	625
6	11	0.0	741
7	14	0.0	741

ple, after the first MD simulation at 310 K only six structures were found with energy lower than 5 kcal/mol. The relatively small number of structures found within this range was caused by the sudden drop in the lowest energy found in this step. Hence, we extended our threshold to 7.5 kcal/mol for this set, which included 27 unique structures, a number comparable to the working sets used in previous and subsequent iterations.

Description of the unique low-energy conformational domains of Met-enkephalin

Apolar distance-dependent dielectric environment

The thirty structures found within 10 kcal/mol of the lowest energy one all correspond to some type of bend. These 30 conformers could be grouped into 9 unique types of structural domains. Figure 3 shows the lowest energy conformer of each domain, labeled A-I, while their torsion angle values are reported in Table 2.

Four domains (A, B, C and E), are all G-P β-II′ turns with varying hydrogen bonding patterns, and are shown in Table 3. The lowest energy domain (A) has 3 internal hydrogen bonds. Domains C and E, represented by conformers 3 and 8, are also stabilized by three hydrogen bonds but in different arrangements. Domain B represented by the second lowest energy conformer, is stabilized by four hydrogen bonds. This conformer presents a bifurcated hydrogen bond from the OH group of the Tyr^1 and the NH of the Met^5 residues to the carbonyl group of Gly^2. In addition to these G-P β-II′ turns, domain D, represented by conformer 7 in Fig. 3, is a more globular structure that can be classified as a G-P β-V turn. Domains F and G, represented by conformers 9 and 20 respectively, are less structured bends. Domain H, represented by conformer 20 in Fig. 3, is a G-P β-II turn. Finally, conformer 29, representing domain I, corresponds to a G-G β-II′ turn. In this domain, the peptide is bent as a result of a hydrogen bond between residues Tyr^1-Phe^4, a clear difference from the other structures. The conformers belonging to each conformational domain span a similar energy range, in general corresponding to the 10 kcal/mol cutoff used.

Since there is no known method to verify the completeness and effectiveness of a search strategy in scanning the conformational space, the only possible means of validation is by comparison with the structures characterized by other procedures, even though different force fields were used. The lowest energy conformer obtained here, conformer 1, representing domain A, is a G-P β-II′ type similar, but not identical, to those previously reported. The main difference between this structure and the global minimum structure (a) reported by Li and Scheraga [6] is that the one found here has a hydrogen bond between the H of the terminal carboxyl group and the Gly^3 carbonyl group, which corresponds to a *cis* configuration of the terminal ω_5 angle involving the carboxyl group. This conformer would never be found by any search strategy that uses a potential that includes a penalty function to maintain all ω angles in the *trans* position, as is the case in the ECEPP potential used by Scheraga and co-workers. Thus, it is understandable why this conformation, and any others involving ω_5 *cis*, were not found. Since this last ω value simply fixes the position of the terminal carboxyl group, it is not a reasonable assumption to constrain it to a *trans* position as if it were an internal peptide bond. The search strategy used here does indeed find a conformer equivalent to the global minimum previously reported. It is conformer 8, representing domain E and is 5.0 kcal/mol above our global minimum. Alternatively, if we fix the torsion angles of structure (a) to be exactly as reported by Scheraga et al. and perform a geometry relaxation to a local minimum using the CHARMm potential, we obtain a conformer that is 8.3 kcal/mol higher than our global minimum.

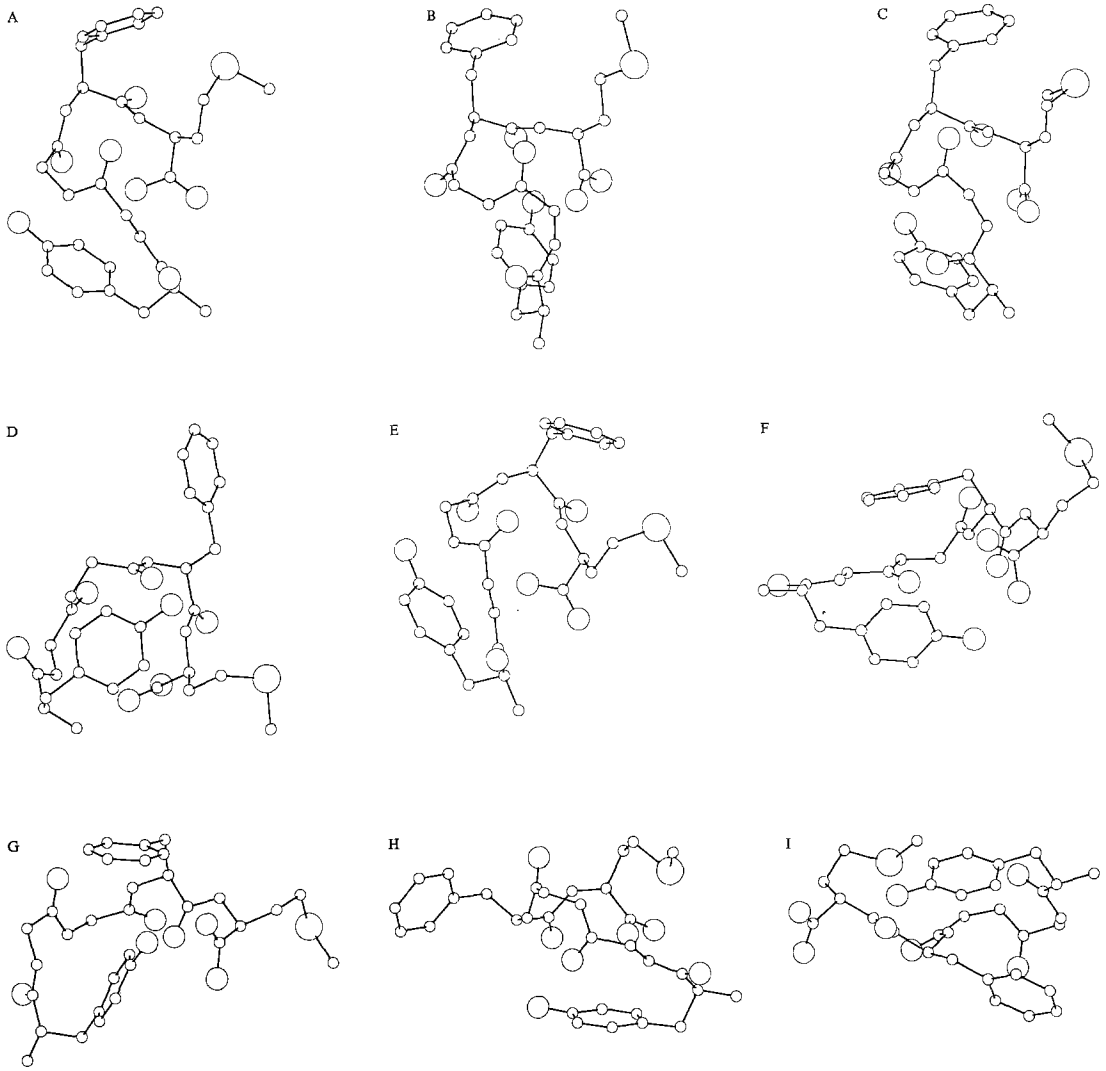

Fig. 3. Lowest energy conformers from each of the different conformational domains within the 30 lowest energy conformers. (A) domain A and lowest energy conformer; (B) conformer 2, domain B; (C) conformer 3, domain C; (D) conformer 7, domain D; (E) conformer 8, domain E; (F) conformer 9, domain F; (G) conformer 19, domain G; (H) conformer 20, domain H; (I) conformer 29, domain I. All hydrogen atoms are omitted for clarity.

The structure reported by Moskowitz et al. [9,30] was obtained using a simulated annealing procedure and the AMBER force field, which does not have any constraint on the angle ω. It is also a G-P β-II′-type turn and does have the ω_5 angle in a *cis* configuration. In fact, this reported structure corresponds to our structure 3, which is in domain B and lies 3.4 kcal/mol above the lowest energy configuration found here.

Although some caution must be exercised in comparing our results with those previously reported, a few useful inferences can be made. It is obvious that the results obtained depend not

TABLE 2
DIFFERENT STRUCTURAL DOMAINS CHARACTERIZED FOR MET-ENKEPHALIN USING A DISTANCE-DEPENDENT DIELECTRIC CONSTANT $\varepsilon = r$ [a]

Conformer		1	2	3	7	8	9	19	20	29
Domain		A	B	C	D	E	F	G	H	I
Tyr[1]	Φ	−62	−62	−179	177	−62	59	−61	−61	−60
	Ψ	160	152	95	19	163	173	168	169	59
	ω	180	−172	−175	175	180	−177	−176	179	−164
	χ1	177	−174	−178	60	180	−179	−170	−167	−165
	χ2	72	80	80	90	78	61	−93	83	79
	χ3	−35	−155	−129	−43	−42	−33	−27	−33	−127
Gly[2]	Φ	−169	−85	−82	81	180	−175	−75	−173	−65
	Ψ	65	46	61	−43	67	−169	35	−130	78
	ω	177	−176	176	177	−179	180	178	179	178
Gly[3]	Φ	66	65	65	−68	67	−72	77	−69	77
	Ψ	−93	−81	−78	86	−90	77	−49	84	18
	ω	177	173	172	−176	176	176	179	177	−175
Phe[4]	Φ	−75	−94	−82	78	−83	−159	−73	77	−82
	Ψ	−30	−43	−41	−55	−31	180	177	−47	45
	ω	174	−174	−177	174	174	−178	180	172	180
	χ1	58	179	179	−55	58	59	66	−54	−52
	χ2	−87	86	86	−80	−86	−92	−86	99	157
Met[5]	Φ	−74	−74	−78	−74	−73	−54	−58	−68	−63
	Ψ	86	128	80	84	−73	119	125	151	125
	ω[b]	−8	−174	−7	−7	−12	−178	−178	0	−179
	χ1	−60	−60	−59	−59	172	−59	−59	61	62
	χ2	−54	−178	−175	−52	−54	62	−53	69	62
	χ3	−64	180	−63	−64	−62	63	−62	180	−106
	χ4	−51	180	−46	−52	−170	171	−51	56	177
Energy		0.0	1.8	3.4	4.5	5.0	5.4	7.8	7.8	9.2

[a] The conformer number indicates the rank order of energy from the lowest energy structure, while its relative energy is given at the bottom in kcal/mol.
[b] ω5 refers to the carboxy terminus.

only on the completeness of the search strategy but also on the limitations of the potential used. A conclusion can be drawn in comparison of our results with other previous attempts, using the ECEPP force field and many different search strategies, to find a global minimum and to obtain a set of low-energy conformers [1,4–7]. Differences in the results obtained must be due to the limitations imposed by the geometry constraints of the ECEPP force field rather than differences in search strategies. This conclusion is reinforced by the fact that our search strategy does indeed find many of the low-energy conformers found previously that were accessible to the ECEPP potential. For example, domains B (structure 2), D (structure 7) and E (structure 8) all have counterparts in the low-energy conformers reported by Li and Scheraga [6], specifically corresponding to conformers (d), (f) and (a) respectively, in that reference. The structure characterized using the build-up procedure is significantly higher in energy, approximately 7 kcal/mol. The higher energy

TABLE 3
LIST OF THE HYDROGEN BONDS OBSERVED FOR THE LOWEST ENERGY CONFORMATIONAL DOMAINS CHARACTERIZED USING THE DISTANCE-DEPENDENT DIELECTRIC CONSTANT $\varepsilon = r$ [a].

Domain	H-Donor	H-Acceptor	Domain	H-Donor	H-Acceptor
A	OH-Tyr^1	Gly^3	F	OH-Tyr^1	Phe^4
	Gly^2	Met^5		Phe^4	Gly^2
	Met^5	Gly^2			
			G	OH-Tyr^1	Met^5
B	OH-Tyr^1	Phe^4		Gly^3	Tyr^1
	Gly^2	Met^5		Phe^4	Gly^2
	Gly^3	Tyr^1			
	Met^5	Gly^2	H	OH-Tyr^1	Phe^4
				Gly^2	Met^5
C	Gly^2	Met^5		Phe^4	Gly^2
	Gly^3	Tyr^1		Met^5	Gly^3
	Met^5	Gly^2			
			I	OH-Tyr^1	Phe^4
D	OH-Tyr^1	Gly^2		Phe^4	Tyr^1
	Gly^2	Met^5		Met^5	Gly^3
	Gly^3	Tyr^1			
	Met^5	Gly^2			
E	OH-Tyr^1	Gly^3			
	Gly^2	Met^5			
	Met^5	Gly^2			

[a] Unless otherwise stated the proton donor is always the NH in the backbone while the acceptor is the carbonyl group, from the residues indicated.

of this conformer is in accord with the results obtained when it is compared to the global minimum reported for the ECEPP potential. The AMBER and CHARMm force fields are more closely related to each other than to the ECEPP potential since they contain the same terms in the force fields and both allow full geometry optimizations. Therefore, it is not surprising that we find domain B, characterized as the lowest energy form using the AMBER potential as described above, closer in energy than the minima characterized with the ECEPP potential. However, the purpose of the simulated annealing study [9,30] was to demonstrate the use of the technique without any indication that the form reported was the lowest energy form for that potential. Thus, it might be possible that both methods, CHARMm and AMBER, find the same lowest energy structure, as could be expected from their conceptual similarities.

Moderately polar environment $\varepsilon = 10$

The most obvious difference obtained when the dielectric constant is increased is that the density of conformational states becomes larger. In this case, we found 50 unique conformers within a 5 kcal/mol range. Moreover, in all cases the structures show significantly fewer hydrogen bonds than those characterized in vacuo. Nevertheless, all structures are still bent but cannot be clustered into the well-defined conformational domains as those found using $\varepsilon = r$.

Table 4 shows the torsion angles for selected conformers in this environment. The lowest energy conformation is a G-P β-II′ turn, according to the values found for the torsion angles. However, the distance between the atoms in residues 1 and 4, that typically form a hydrogen bond that characterizes this type of conformer, is 3.5 Å. The second lowest energy structure is a G-P β-I turn conformation, with its characteristic hydrogen bond between residues Gly^2 and Met^5. As the energy increases, the structures continue to be bent, even when they cannot be classified according to the values of the dihedral angles as any of the classical bends. Most of those structures can be loosely described as β-IV turns. The first G-G β-II turn is 4.5 kcal/mol above the lowest energy minimum, compared to 9 kcal/mol found with $\varepsilon = r$. Therefore, this type of structure is stabilized in more polar environments. A conformer where the Gly^2 and Gly^3 residues are in an extended conformation is also found at 7 kcal/mol from the lowest energy structure. While this is not a totally extended conformation, it illustrates a tendency towards less compact structures in more polar environments.

In summary, the use of a moderately polar environment, whilst somewhat altering the relative energies among the different conformers and leading to less compact conformers, does not appear to change the qualitative nature of the structures found.

Polar environment $\varepsilon = 80$

A further increment in the density of conformational states is found in this increasingly polar environment. In this case, 50 structures are found in an energy range of 4 kcal/mol from the lowest energy structure. Following the same trend observed for $\varepsilon = 10$, the conformations found are less structured.

In Table 4 we report two of the conformers in this media. As in the other two simulated environments, the lowest energy structure can also be classified as a G-P β-II turn. The lowest energy G-G β-II turn is only 1.6 kcal/mol higher in energy from the lowest energy structure, a much lower energy than in the other environments simulated. Therefore, this type of structure appears to be stabilized in more polar environments and could play a role in the observable properties of Met-enkephalin.

Finally, the overlap of the three lowest energy conformers, shown in Fig. 4, found independently in each of the three environmental conditions reveals the striking similarity among them.

Flexibility of the peptide

For the global minimum, we have carried out a 200 ps dynamics trajectory calculation at 310 K. The results shown in Fig. 5 and Table 5 indicate that for $\varepsilon = r$ the structure is very stable with small fluctuations. Figure 5 shows the actual superposition of 10 snapshots at regular intervals recorded along the trajectory followed during the MD simulation in the three environmental conditions simulated. As can be seen in Fig. 5A there is not much deviation from the average structure along this trajectory, pointing towards a remarkable stability of the bend form for $\varepsilon = r$. This observation is also supported by the values reported in Table 5 of the root-mean-square deviations of the atoms of every residue along the dynamics trajectory, as a measure of the flexibility of the different amino acids reported.

The analysis of the elongations of the hydrogen bonds along the dynamics trajectory show a concerted movement that does not allow breaking of more than one hydrogen bond at any given

TABLE 4
TORSION ANGLES FOR THREE DIFFERENT STRUCTURAL DOMAINS CHARACTERIZED FOR MET-ENKEPHALIN IN $\varepsilon = 10$ AND TWO DOMAINS CHARACTERIZED IN $\varepsilon = 80$ [a]

Dielectric		10			80	
Conformer		1	2	39	1	9
Domains		G-P β-II′	β-I	G-G β-II	G-P β-II′	G-G β-II
.Tyr[1]	Φ	60	60	−179	61	−61
	Ψ	163	166	50	−51	157
	ω	−178	175	−175	−178	177
	χ1	−178	−179	−57	−172	60
	χ2	−104	−101	−86	−89	−90
	χ3	171	168	180	177	1
Gly[2]	Φ	−177	−157	−69	78	66
	Ψ	49	159	142	−84	16
	ω	179	−177	175	180	−176
Gly[3]	Φ	79	−64	82	175	83
	Ψ	−86	−28	8	−27	27
	ω	−179	178	178	179	178
Phe[4]	Φ	−66	−71	−161	−66	−159
	Ψ	−21	−24	−32	−28	−166
	ω	−177	180	177	175	180
	χ1	62	59	59	61	58
	χ2	−89	−89	−87	94	−88
Met[5]	Φ	−65	−73	62	−71	−62
	Ψ	122	119	−111	−13	123
	ω	180	180	180	−2	0
	χ1	−60	−55	−54	−61	−64
	χ2	−177	−174	−58	−172	61
	χ3	−178	−171	173	−166	66
	χ4	179	−179	−61	−55	53
Energy		0.0	0.1	4.5	0.0	1.6

[a] The conformer number indicates the rank order of energy from the lowest energy structure in that environment, its relative energy is given at the bottom in kcal/mol.

time, in the distance-dependent dielectric. The hydrogen bond distances fluctuate between 1.8 and 2.3 Å. The Gly[2](CO)...(NH)Met[5] hydrogen bond exhibits the largest fluctuations (1.8–2.7 Å) while the Tyr[1](OH)...(CO)Gly[3] is the most stable showing the smallest oscillations (1.8–2.0 Å) during the trajectory. Paradoxically, during the simulations made, the peptide can reach many different minima on the hypersurface, while retaining its bend structure.

The same analysis for the molecular dynamics simulations when the peptides are embedded in the other dielectrics reveals an increased motility of the molecule in the more polar environments. The structures become less compact along the MD trajectories. The rms values per residue in Table 5 and Figs. 5B and C illustrate the larger flexibility that can be attributed to the weaker nature of the electrostatic term in the more polar environment.

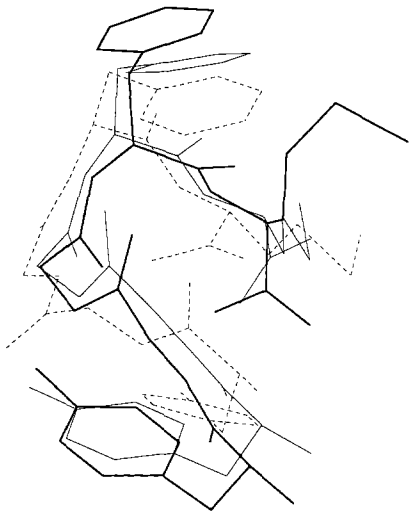

Fig. 4. Overlay of the lowest energy conformers found in each of the three environmental conditions simulated. The thick line corresponds to $\varepsilon = r$, the thin line to $\varepsilon = 10$, and the dotted line to $\varepsilon = 80$.

CONCLUSIONS

In this study we have combined an extensive systematic search strategy with the use of an empirical potential that allows total geometry optimization with no backbone angle constraints to characterize the lowest energy and accessible conformational domains of Met-enkephalin. A search procedure was used that started with systematic nested rotations of the backbone torsion angles and was followed by iterative MD simulations, that alternate high and low temperature using sets of optimized structures obtained from each simulation for the next. One advantage of this procedure is that it could be repeated until no new low-energy structures were obtained, ensuring closure, at least within the limitations of the strategies and approximations made.

Comparisons of the results with previous theoretical studies and with experimental results indicate that the strategy used is a promising one for locating a very wide range of low-energy minima of peptides. Not only were all low-energy conformers found that had been previously reported by different investigators, using other potentials and other strategies, but also additional ones, including a new, lower energy 'global minimum' than heretofore reported. While there may be more cost- and time-effective methods for finding a structure close to the global minimum, as was demonstrated by Moskowitz et al. [9,30], this is not enough information to identify a bioactive conformer. The results obtained showing low-energy conformers with a G-P bend and hydrogen bond between Gly^2 and Met^5 are also consistent with the NMR studies in DMSO as the most populated conformer [14–17]. The extended structures obtained in the X-ray studies appear to be the result of interstrand interactions leading to the dimerizations found in the crystal. No extended structure was found within 10 kcal/mol, with the assumption of a distance-dependent dielectric constant. While the lowest energy structure showed very little flexibility during a 75 ps dynamics simulation at 310 K, it nevertheless can undergo fluctuations that transform it to different conformational domains. This accessibility allows it to change from globular structures to more open β-II-type turns but does not include any extended structures. In the more polar environments simu-

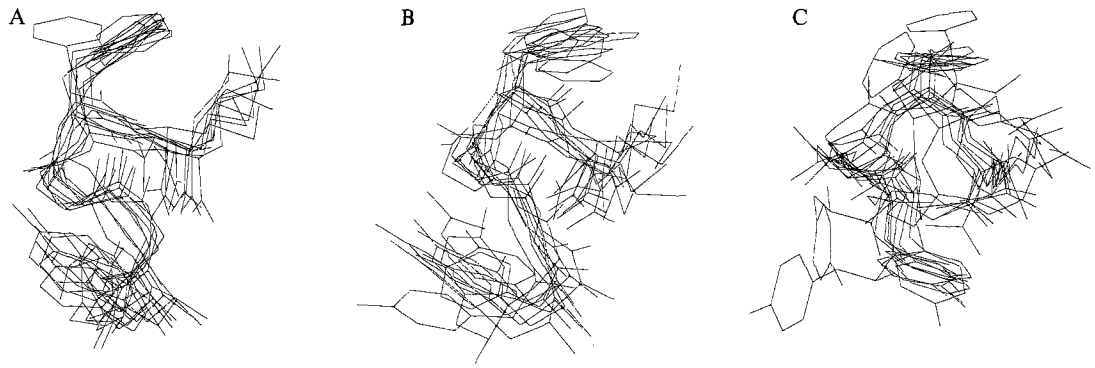

Fig. 5. Overlay of 10 structures observed at regular intervals during the MD simulations in the distance-dependent dielectric approximation for the lowest energy conformer at 310 K during 200 ps, in the three environments simulated (A) $\varepsilon = r$, (B) $\varepsilon = 10$, and (C) $\varepsilon = 80$.

lated, the flexibility of the peptide increases compared to $\varepsilon = r$, while some high-energy partly extended conformations can be found.

Among the low-energy conformers found from this extensive search was a G-G β-II′-type turn long postulated by several authors to be a plausible bioactive form for recognition of peptides by the μ-opioid receptor. Interestingly, an increase in the dielectric constant increases the stability of this type of conformer.

Procedures such as the modified build-up [13] appear highly convenient for short chain tetra- and pentapeptides, if combined with an additional procedure, since, as shown, it may miss part of the conformational surface. However, slightly longer peptides that may be required to deduce the bioactive structure for the opiate receptor cannot be handled as effectively, since the number of initial conformers required varies exponentially with the number of residues. Alternative procedures, where fewer initial conformations suffice to scan the conformational space, are necessary, particularly for longer peptides. The procedure outlined here is based on the use of a limited number of conformations at each step.

The influence of the dielectric constant is significant, not so much in determining the low-energy domains found as in the dynamic properties that they display. The number of conformers found per unit of energy is significantly larger for the higher dielectric constants. Moreover, fewer hydrogen bonds are formed in the more polar environments, which results in less-structured minima.

TABLE 5
MEAN RMS VALUES (IN Å) PER RESIDUE FOR THE LOWEST ENERGY G-P β-TURN IN THE THREE ENVIRONMENTAL CONDITIONS SIMULATED AFTER 200 ps MD SIMULATION AT 310 K

Residue	$\varepsilon = r$	$\varepsilon = 10$	$\varepsilon = 80$
Tyr^1	1.3	2.3	2.0
Gly^2	1.0	1.6	2.3
Gly^3	1.1	1.5	1.9
Phe^4	0.8	1.4	1.0
Met^5	0.9	1.5	1.3

At present we are using the same search strategy to continue our systematic studies of several other linear and cyclic opiate peptides with varied pharmacological profiles in the search for their bioactive form at the μ- and δ-opiate receptors.

ACKNOWLEDGEMENTS

One of us (J.J.P.) wishes to express his gratitude for financial support from the 'Laboratorios del Dr. Esteve' through the 'Fundacion Condes de Barcelona'. This work was supported by NIDA Grant No DA-02622.

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