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Proposals for the μ-Active Conformation of the Enkephalin Analog Tyr-cyclol(-N$^{\gamma}$-D-A$_2$-bu-Gly-Phe-Leu-)

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SUMMARY

The conformational behavior of the sterically restricted cyclic peptide Tyr-cyclo(-N$^{\gamma}$-D-A$_2$-bu-Gly-Phe-Leu-), proposed recently as an enkephalin analog with high opiate activity, is examined by theoretical investigations. The method used allows the search of conformational energy minima associated with cyclic structures fitting a hypothetical opiate pharmacophore. The results obtained show that, despite the fact that many cyclic structures of low conformational energy can be found for this compound, only one of them can be retained as a conformer presenting the characteristic features of the imposed pharmacophore. This conformation is stabilized by an intramolecular H-bond between the D-A$_2$bu-carbonyl and the Leu NH group so that a β-turn is formed. This structure also presents a high mobility of the Tyr^1 side-chain which can fit the tyramine moiety of rigid opiates with minor loss of conformational energy. A two-step binding mechanism is proposed for the interactions of this cyclic peptide with its receptor which could be an intermediate between the "zipper" model proposed for flexible linear peptides and the "lock-and-key" model adapted to rigid molecules. The selectivity of enkephalin analogs for μ and δ opioid receptors is discussed in light of the present theoretical investigations.

Since the discovery of the opioid activity of the enkephalins, correspondence between critical functions in the peptides and in opiate alkaloids has been tentatively established from structure-activity studies performed on enkephalin analogs (1–5).

In fact, the enkephalins interact with at least two opioid receptor-subtypes, designated μ and δ (6), but the supraspinal analgesic properties of these endogenous peptides very likely result from their stimulation of the μ-receptor subtype (7), which corresponds to the binding site of morphine and surrogates.

Therefore, the knowledge of the spatial disposition of the important chemical groups in opioid peptides is of particular relevance, and several theoretical and experimental approaches led to the determination of several conformational models of enkephalin-like compounds in solution, in the crystalline state, or bound to specific receptors (reviewed in Ref. 5). Nevertheless, no consensus about the conformational behavior of these molecules has been reached, and the question remains whether enkephalin peptides bind to their different receptor subtypes with a conformation already existing in solution (7), or adapt to the specific geometry of the binding sites (8–10). In previous communications (9, 11) some information about these questions has been given, as it appears that none of the stable conformations proposed for enkephalin in solution present the geometrical characteristics of the critical chemical functions found in rigid opiates: it was shown that the conformations that well fit the opiate pharmacophore belong to a family of conformers that are not predominant in aqueous solutions. Therefore, the differences observed between the conformations of tetra-peptide opiates in solution (deduced from NMR) and at the receptor level (proposed from computed similarity with morphine) suggested a stepwise mechanism for the receptor recognition process of enkephalins (8, 11).

Another way to get information about this mechanism and also about the receptor-bound conformations is to study conformationally restricted enkephalin analogs. Such an approach is now possible, as some recently synthetized peptides present severe steric constraints which limit their conformational possibilities (12–15). One of the most interesting molecules of this kind is the cyclic peptide, H-Tyr-cyclo(-N$^{\gamma}$-D-A$_2$-bu-Gly-Phe-Leu-), recently proposed by Di Maio, Schiller, and colleagues (10, 12). This molecule presents high affinity and relatively good selectivity for the μ-receptor, so that its conformations can be related to those of other μ-specific agonists including peptides and rigid opiates. Theoretical analysis of the conformational possibilities of this compound can thus be undertaken, so that results can be compared to recently proposed features of peptides acting preferentially on the μ-receptor (7, 10, 14, 15–18).

Methods

The method followed in this paper used a constrained energy minimization procedure in order to find conformations of low energies fitting the μ-opiate pharmacophore. The CONMIN procedure of Haar-

hoff *et al.* has been used as in our previous work (11, 19) to tackle the constrained search of low energy conformers. This algorithm has been developed to solve the general nonlinear problem:

$$\begin{aligned} &\text{minimize} \quad f(X) \qquad X \in E^n \\ &\text{subject to} \quad g_i(X) = 0 \qquad i = 1, \cdots m \\ &\text{and} \quad h_j(X) > 0 \qquad j = m+1, \cdots p \end{aligned}$$

where $f(X)$ is the objective function (here, the molecular energy), $g_i(X)$ is an equality constraint, and $h_i(X)$ is an inequality constraint. X is the vector built from the variables of the problem (here, the ϕ, ψ, $\{\chi\}$ dihedral angles).

This approach has been found to be better than the more usual ones which use unconstrained minimizers in which the constraints are treated as penalty terms in the objective function itself.

The corresponding program has already been described and used for linear peptides (11). It is now adapted to cyclic molecules so that two constraints are presently used in the algorithm.

The first one drives the search of low energy conformations toward structures presenting the geometrical characteristics of the μ-pharmacophore by computing the maximum overlap between selected atom groups in the cyclic peptide and in the morphine. The atom groups presently used are (Fig. 1):

a: OH group and aromatic ring of Tyr ↔ A-ring,
b: N-terminal nitrogen in peptide ↔ N-atom of the D-ring,
c: CO carbonyl bond of Gly^3 ↔ C_6—O_2 bond of the C-ring.

So that the fitting constraint $g(1)$ looks like the distance:

$$g(1) = (d_a + d_b + d_c)^{1/2}$$

in a least squares sense, between selected atoms in the peptide and morphine as described above. This definition of the pharmacophore has been discussed in a previous paper (11).

Different fitting pathways thus can be explored, depending on the chemical functions of the peptide and morphine, which are first superposed and kept fixed in this position. For example, the aromatic ring of Tyr^1 in the peptide and the A-ring in morphine (parts a) can be fixed in a perfect, overlapped position at the beginning of the search (by choosing the coordinate origin at both the phenol rings, x and y axes in the ring plane, z axis perpendicular to this plane). In this case, the aim of the fitting procedure will be to find the best possible superposition between the remaining target groups b and c. One also should first superpose the C′═O carbonyl of Gly^3 in peptide with the C_6—O_2 bond in morphine, and then fit the remaining a and c groups of the molecules, etc. Besides this sequential fitting procedure, one also can fit simultaneously the three atom groups a, b, and c. In this last case, the results obtained will correspond to an average overlap between the different moities, no one being particularly favored as before.

The second constraint imposes the closure of the molecular ring

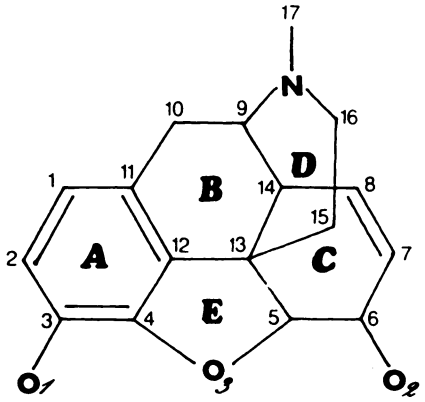
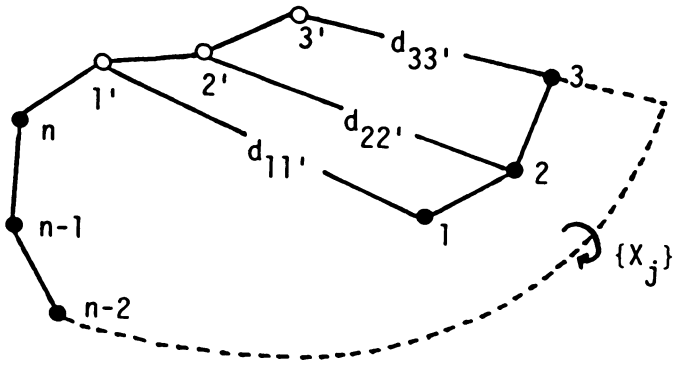


Fig. 1. Morphine structure and atom numbering.



SCHEME 1

according to a method used elsewhere (19). It involves the superposition of three dummy atoms added at the end of the molecular ring on the three first atoms at the beginning of this ring (Scheme 1). The criterion of cyclization is taken as:

$$d = \left(\sum_{i=1}^{3} d_{i,i'}^2 \right)^{1/2}$$

so that the ring closure constraint is $g(2) = d$, where n is the number of atoms in the ring, and $d_{i,i'}$ is the distance between atom i and the dummy atom i' associated with i. According to the possible variations in bond lengths and valence angles as compared to the fixed values used here, the ring is declared closed as soon as $d < 2$ Å (for a perfect closure $d = 0$).

The nomenclature and conventions adopted by the IUPAC-IUB Commission were used. The potential functions taken for the conformational energy calculations are those previously used in our Monte-Carlo calculations on enkephalin. A standard geometry is given to amino acids, and atomic coordinates associated with each (ϕ, ψ, χ) set of dihedral angles are calculated. Charges NH_3^+ groups are taken as end-groups, and solvent effects are not included in the calculations. The atomic coordinates of morphine are taken from Gylbert as before (11).

The strategy followed to search for the most stable cyclic conformations presenting the critical chemical functions of opioids in the right positions can be described in two steps, as follows.

1) Low energy conformations of the cyclic molecule are obtained without any condition but the ring closure. The variables used in the procedure are all the (ϕ, ψ, χ) dihedral angles of the molecule (the peptide-bonds are kept transplanar). The conformations that initiate the search are taken from physical molecular models, or from the dihedral angle values given in the literature for the most stable conformations of different enkephalin analogs.

2) The conformations obtained from step 1 are modified in order to get the proper geometrical requirements defining the pharmacophore. During these calculations, the two constraints $g(1)$ and $g(2)$ defined above are used, and the following procedures are used. First, one searches for conformers that fit the pharmacophore but are not far from the starting cyclic structures obtained after step 1. To prevent large modifications of the molecular conformations during this minimization, the variable dihedral angels are constrained to change as follows: for each residue in the peptide ring, the $(\phi_i,\psi_{i-1})_{i=3-5}$ are correlated so that the peptide-bond planes can rotate around the $C_{i-1}^{\alpha} - C_i^{\alpha}$ bonds which are thus kept almost fixed; such movements roughly imply that the variations allowed in the peptide ring for the angles ψ_{i-1} and ϕ_i satisfy the relation $\Delta\psi_{i-1} = -\Delta\phi_i$. The number of variable dihedral angles is then reduced to:

$$(\phi_1,\{\chi_1\};\ \phi_2,\{\chi_2\};\ \phi_3;\ \phi_4,\{\chi_4\};\ \phi_5,\{\chi_5\})$$

If the fit to the pharmacophore cannot be obtained satisfactorily from this procedure, calculations are made again, now using all of the

(ϕ, ψ, χ) dihedral angles without any restriction in their variations; hence, the starting conformations can now be deeply modified.

Results

Computed low energy conformations of the cyclic peptide. The conformations that present fairly good energies of the cyclic molecule without imposing any fit to the defined opiate pharmacophore are presented in Table 1. It appears from examination of the set of (ϕ, ψ, χ) dihedral angles in this table and from computer drawings of some of the associated conformations (Figs. 2–4) that a wide variety of acceptable cyclic structures can be found. As concerns the spatial organization of the cyclic-(A_2-bu-Gly-Phe-Leu) peptide backbone, several hydrogen-bonded conformations are found that present the usual β-bends requirements. γ-Turn structures can be obtained from molecular models, but such conformations are not stabilized enough to be retained. One also can find conformations that present the C_7 structures stabilized by intramolecular $CO_{i-1} \ldots HN_{i+1}$ hydrogen bonds. All of these conformations can be roughly classified according to the geometrical figures formed by the peptide bonds of the cyclic (C_2^α—C_3^α—C_4^α—C_5^α—C_2^α) moiety and by the positions of the Tyr[1], Phe[4], and Leu[5] side-chains in regard to the molecular ring. Two cases can thus roughly describe the peptide ring possibilities: the first one corresponds to an almost planar ring stabilized by intramolecular hydrogen bonds (β-turns or C_7 conformations), while the other presents a twisted ring without any H-bonds; the side-chains of the Tyr, Phe, and Leu residues can be found pointing outside of the peptide ring, or in alternating up/down positions with respect to the ring.

Biologically active conformation of the cyclic peptide at the μ-receptor subtype. Starting from all the acceptable cyclic conformations obtained above, attempts to fit the complete pharmacophore lead to severe steric hindrances or to energetic barriers which cannot be bypassed by the algorithm. Therefore, only one conformation presenting both a satisfactory fit and a good energy can be found.

This conformation is described as conformer 4′ in Table 1 and is presented in Fig. 3. It appears from the examination of the structural parameters which characterize this fitted stable conformation that only weak differences are observed between the fitted model and the starting conformer (conformation number 4 in Table 1): minor modifications are found in the cyclic (A_2-bu-Gly-Phe-Leu) moiety (dihedral angle variations less than 10°), except for the Leu side-chain ($|\Delta\chi_5^1| = 28°$, $|\Delta\chi_5^2| = 58°$) and for the aromatic Phe ring ($|\Delta\chi_4^2| = 28°$). The conformation of the Tyr[1] residue was not deeply modified during the fit as revealed by the dihedral angle variations observed between the initial and final conformers ($|\Delta\chi_1^1| = 12°$, $|\Delta\chi_1^2| = 16°$, $|\Delta\psi_1| = 16°$). Only the flexibility of a conformation which can already exist as a stable one in the physiological medium is required. As in the starting conformer, the fitted structure presents two intramolecular H-bonds, one of the C_7 type between the Tyr[1] carbonyl and the Gly[3] N-H groups, and the other of the β-turn type, between the A_2-bu carbonyl and the Leu[5] N-H groups. It can be noted that this type of folding satisfies the β-bend model already proposed for the bioactive conformation of enkephalins (8, 20, 21).

When looking at the distances usually defined as important in opiates, it appears that the structural requirements for acceptable receptor interactions are roughly satisfied in the proposed model, keeping in mind that rigid geometries are used: $N^+ \ldots$ phenol ring center distance of 5.1 Å (4.7 Å in morphine), phenol ring center... O=C_{Gly^3} distance of 5.6 Å (4.7 Å in morphine). The observed differences are in the range of the values observed for various opiate agonists, antagonists, and other similar drugs, showing that the stereospecificity of the receptor binding is conserved in the obtained conformation.

TABLE 1

Dihedral angles which define the more stable conformations for the peptide molecule (conformers 1–17) in solution, and for the postulated active conformer (4′) acting on the μ-receptor

Residue	Dihedral angles	Conformations numbers																	
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	4′
Tyr[1]	χ^1	−99	64	74	−147	60	54	−57	169	−100	163	159	−163	180	−155	−127	170	81	−159
	χ^2	−113	−80	−84	117	94	−76	−78	−89	−50	−108	−101	70	−126	78	111	79	−59	152
	ψ	−113	179	176	−54	135	42	168	154	118	−73	150	115	−61	144	143	157	−15	−70
D-A_2 bu[2]	ϕ	165	−71	94	103	136	53	−70	19	145	−174	−66	130	172	153	−62	86	178	89
	χ^1	−27	140	112	151	−14	−180	125	−13	−45	−27	176	23	−21	−58	−172	−105	−40	150
	χ^2	−68	90	58	35	−78	148	92	−149	−69	−124	100	−118	−133	−97	121	−81	107	32
	χ^3	−76	−96	50	155	−73	−132	−48	159	166	−171	137	45	−145	161	−169	5	147	150
	ψ	−166	69	−53	−36	173	35	71	69	−33	44	−125	150	63	−98	−117	27	41	−36
Gly[3]	ϕ	80	−57	80	76	62	−123	−67	−132	−85	−134	23	98	−129	−17	1	−61	−120	76
	ψ	−131	−101	−18	−78	109	22	−83	−60	−77	−78	83	53	−56	124	94	110	−105	−78
Phe[4]	ϕ	−71	−44	−163	−116	81	−127	−57	−119	−33	−57	133	121	−112	97	151	84	−31	−116
	χ^1	61	−67	−18	−163	−40	163	−66	−34	127	−54	172	−180	8	−51	152	−152	−98	−115
	χ^2	−99	109	−101	102	142	56	120	109	83	114	51	90	−71	−34	89	73	99	74
	ψ	1	−48	−58	−35	−57	−45	−39	−47	−39	−70	59	−34	−76	−58	−32	25	−61	−36
Leu[5]	ϕ	−140	−144	−152	−159	110	−138	−152	−150	173	−135	68	−166	−97	179	−160	125	−156	−161
	χ^1	−163	−151	−151	59	−73	−114	−95	−163	−135	−68	−160	−162	−161	−173	168	175	−121	31
	χ^2	165	−171	−172	105	88	46	166	−164	85	163	94	−139	155	−97	140	144	175	47
	ψ	−110	−79	145	39	−124	−134	−131	144	74	129	−79	143	103	111	−151	−61	137	48
Energy (kcal/mol)		−29	−28	−27	−26	−26	−24	−23	−23	−22	−21	−20	−20	−20	−20	−19	−19	−19	−20
Ring closure constraint for cyclization Å		1.03	0.06	1.9	0.03	0.4	1.0	0.5	0.6	0.5	0.9	1.7	1.8	0.8	0.1	1.2	1.1	0.8	0.001

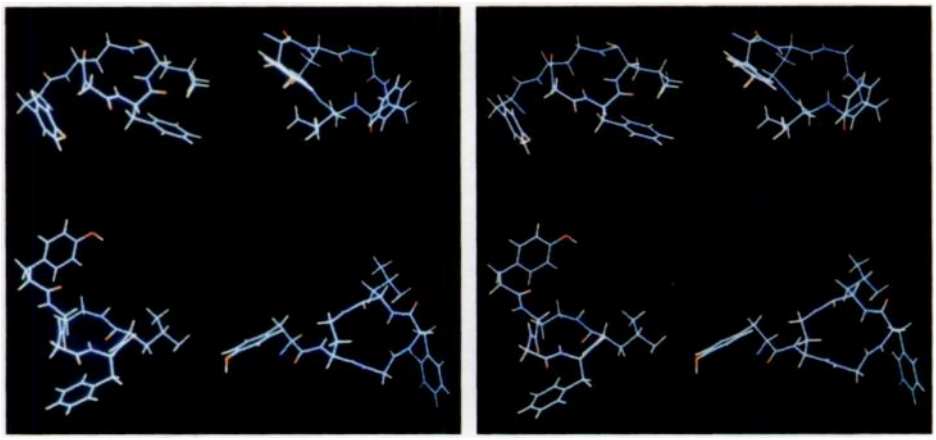


Fig. 2. Molecular drawings of some of the more stable conformations calculated for the cyclic peptide and defined in Table 1 (up-left conformer number 6, up-right conformer number 1, down-left conformer number 3, and down-right conformer number 5).

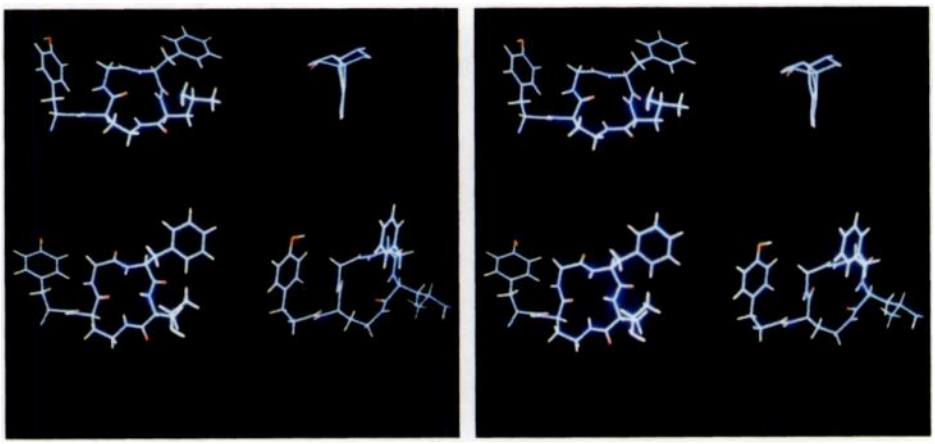


Fig. 3. Molecular drawings of the proposed active conformation (up-left conformer number 4′), of morphine (up-right), of conformer number 4 from which conformer number 4′ is obtained (down-left), and of conformer number 2 (down-right).

The distance obtained between the Tyr[1]...Phe[4] aromatic ring centers is 11.1 Å, in good agreement with Schiller's experimental results (22) (9–12 Å). In the proposed biologically active conformation, the phenylalanine side-chain is folded back on the Tyr[1] phenol ring. This distance and the relative positions of the aromatic rings confirm that the chemical substituents present in the first and fourth residues of enkephalin could be required for the activity, and that their relative orientation is important for the recognition of the molecules (8–10, 20, 22). Thus, the receptor-bound conformation of the μ-specific peptide could have the Phe[4] side-chain folded in the right position making very plausible the analogy between the Phe[4] side-chain in the peptide and the equivalent group in oripavine (4, 22).

One should note that the pharmacophore presently used drastically limits the number of acceptable fitted conformations, so that the choice could be questionable as it is not the only possible one. Indeed, other proposals have been made, the simplest one using the phenol ring and the N^+ atom, while another one adds two atoms of Phe[4] (2). Besides, when using the present pharmacophore, one can fit the C_6O_2 bond in morphine with the C=O bond of either Gly[3] or Leu[5] because of the cyclic structure of the molecule. Starting from the good cyclic conformations described in Table 1, if calculations are performed again with a modified definition of the pharmacophore according to the above indications, results show the following.

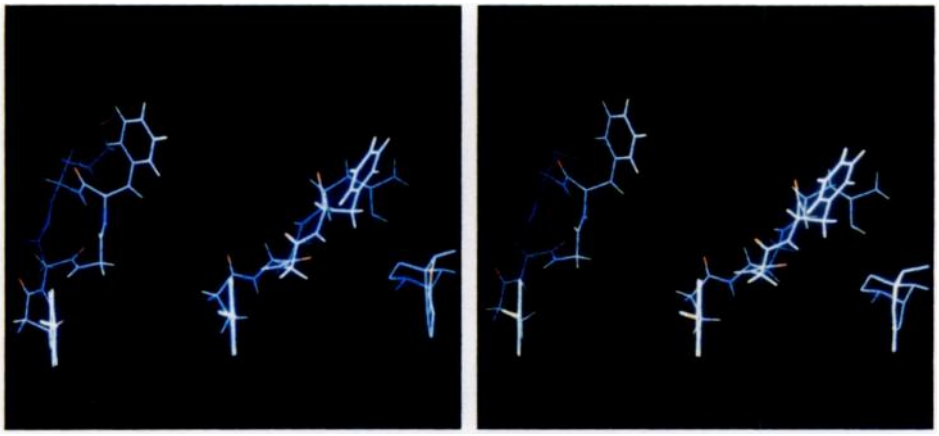


Fig. 4. Molecular drawing showing the structural analogy between the "solution conformation" (conformer number 4), the "active" conformation (conformer number 4′), and morphine. The usual "T" shape of rigid opioids can easily be recognized from this picture.

(a) One can easily obtain stable conformations fitting the simplest pharmacophore (phenolic ring + N^+). These conformations do keep the same peptide ring structures as the starting ones, but the dihedral angles of Tyr^1 are modified. The final conformations of Tyr^1 thus obtained are those one can get by fitting Tyr-NH_2 alone (see Table 2). This indicates that there is almost no interaction between the Tyr^1 residue and the peptide ring, so that the simple fit of the Tyr^1 part of the cyclic molecule can be realized with only a small increase of the conformational energy.

(b) It was impossible to obtain any conformation verifying the definition of the pharmacophore by Gorin *et al.* (2). This situation is mainly due to the constraint imposed by the peptide ring, which does not allow the movements necessary to put the Phe^4 side-chain in a good position.

(c) Finally, the C_6O_2 bond of morphine can hardly be fitted to the C═O of Leu^5 (instead of that of Gly^3 used before). In this last case, conformations can be obtained, but they are always less stable than the best one found previously (conformation 4′ in Table 1).

Hence, it seems that, besides the fit of Tyr^1, which is easy to obtain, the only other part of the molecule which can satisfy an extended definition of the pharmacophore is the C═O bond of Gly^3. In this last case, one should note that, when the fit is realized, it corresponds to an increase of the conformational energy. Nevertheless, examination of molecular models corresponding to the conformations 4 and 4′ indicates that, as discussed below, the loss of energy (6 kcal/mol) due to internal molecular movements between the starting cyclic conformations and the fitted conformation can be compensated by intermolecular interactions.

TABLE 2

Calculated conformations for the Tyr-NH_2 model peptide

The dihedral angles of the conformers giving the best fit between the tyramine group of the postulated pharmacophore and the corresponding atoms of this molecule are also given.

	Dihedral angles (deg)			Energy	Fit
	χ^1	χ^2	ψ		
				kcal/mol	Å
Fitted conformations	−90	−160	−67	−8.1	0.34
	90	121	−176	−11.4	0.27
Lowest energy conformations obtained without any fit	−176	−99	155	−12.6	3.12
	64	91	172	−12.4	1.70
	−62	−76	−57	−10.0	3.67

Discussion

Stepwise model for the binding of opioid peptides to the μ-receptor subtype. According to the present results, one can propose a hypothetical scheme of interaction between opioid peptides and the μ-receptor subtype which could involve two steps in the case of cyclic peptides.

The first, step, corresponding to the binding of the "message" to the transduction site would involve the interaction of the Tyr^1 side-chain and of the N^+ atom with the corresponding subsite of the opioid receptor (8). The second step would implicate the positioning of the carbonyl of Gly^3 so that it can very likely interact by hydrogen bonding with an appropriate group of the receptor. This latter process could be associated with the required rotations around the single carbon bonds of the Phe and Leu side-chains in order to fit the proposed model.

The first step destabilizes the molecular conformation by about 3.5 kcal/mol, and the second corresponds to the adaptation of the peptide to the specific topology of the μ-receptor subtype by about 2.9 kcal/mol. Thus, the conformational energy requirements to achieve the whole binding "message and address" (23) of the molecule are quite low and could be easily counterbalanced by appropriate interactions within the receptor-site. Indeed the unfavorable loss of energy (3.5 kcal/mol) occurring in this initial nucleation step could be easily overcome by the simultaneous formation of: 1) an electrostatic interaction between the positively charged ammonium group of Tyr and a putative carboxylate group of the receptor [this assumption is strongly supported by the complete loss of affinity following acetylation of the amino group of Tyr (1)] and 2) hydrogen bond(s) and Van der Waals interactions involving the phenol group and the aromatic ring of Tyr, respectively [accordingly,

the replacement of the tyrosine by a Leu residue led to completely inactive compounds (1)].

All of the results of these structure-activity studies strongly suggest that all of the putative interactions between the tyrosine residue and the receptor are satisfied in the nucleation step. Binding studies of substrates or inhibitors with corresponding enzymes have shown that hydrogen bond formation, or ionic or Van der Waals interactions leads to a free-energy gain of between 3 and 7 kcal/mol for each (24, 25). Therefore, combination of these interactions leads to a favorable enthalpy change on binding which probably largely overcomes the penalty related to conformational adaptation.

Along this line, it is very interesting to observe that the apparent affinity of the dipeptide Tyr-D.Ala-NH_2 for the opioid μ-receptor (R_{Dapp} = 4.75 μM) remains very high even when it is compared to the affinity of the whole enkephalin analogue, Tyr-D.Ala-Gly-Phe-Leu-NH_2 (R_{Dapp} = 0.7 μM) (26). Starting from the binding constant of the dipeptide amide and assuming an association rate $k_{on} = 10^7\ M^{-1}\ s^{-1}$, the life time of the nucleation step ($\xi = 10^{-4}$) is considerably longer than the time required for rotation around single bonds (27). In the case of cyclic or linear enkephalins this very likely allows the subsequent adaptation of the "address" part of the peptide in the corresponding subsites of the receptor. Such a zipper mechanism (27) is in agreement with the slightly faster association rate of the linear μ-specific ligand [^{3}H]DAGO ($k_{on} = 1.4 \times 10^6\ M^{-1}\ s^{-1}$) as compared to [^{3}H]dihydromorphine ($k_{on} = 8.5 \times 10^5\ M^{-1}\ s^{-1}$) (28). It is highly probable that the rigid alkaloid binds to the opioid receptor through the "lock and key" model, and its relatively fast association rate shows that, as expected, the proportion of the right conformer in solution is very high (27).

Obviously, it would be of great interest to compare the binding kinetics of a cyclic enkephalin with that of its open-chain precursor. Unfortunately, at this time such compounds are not available as tritiated forms.

Relationships between the proposed model and relevant opioid peptides. The relevance of the proposed model of biologically active conformation of enkephalins at the μ-receptor subtype can be discussed taking into account the structure of the reported most selective μ- and δ-agonists. First, it must be observed that the affinity of the endogenous enkephalins for the μ-opioid receptor is only 10 times lower than for the δ-subtype (6). This suggests the occurrence of large similarities in the active site of both binding sites which could belong to slightly different subunits of a single opioid regulator. NMR studies (reviewed in Ref. 5) and crystallographic analysis (Ref. 21 and references therein) have shown that Leu-enkephalin and Met-enkephalin exist in equilibrium between folded and extended conformations of similar energy. According to these features, it has been proposed (9) that the compact conformation of the peptide could correspond to the active structure at the μ-receptor, whereas the extended form could interact more adequately with the δ-subtype.

Starting from these assumptions, a very large number of enkephalin analogs endowed with conformational constraints were synthesized. Almost all of the linear peptides that present conformational restrictions, related, for instance, to incorporation of proline (16) or Aib residues (17), are characterized by both folded structures in solution and higher affinity for the μ-receptor subtype. In the series of cyclic enkephalins retaining the required Tyr flexibility (8), introduction of more drastic restrictions of the overall conformations led to opposite results regarding the μ/δ selectivity. Indeed, when the covalent linkage was ensured by an unsubstituted alkyl chain containing an amide (12) or a thiomethylene bond (18), the cyclic compounds showed a large preference for the μ-receptor over the δ-receptor. Interestingly, comparative binding studies performed with the cyclic peptide studied in this paper, Tyr-cyclo (-N^{γ}-D-A_2-bu-Gly-Phe-Leu-) and its open-chain analog, [D-A_2-bu, Leu^5]enkephalinamide, have shown that the μ-selectivity of the cyclic peptide resulted from its loss of affinity at the level of the δ-site and not from an enhanced affinity for the μ-site (10). This finding suggests that the μ-selectivity of this first series of cyclic peptides is due to energetically unfavorable steps in the binding to the δ-receptor subtype through the zipper mechanism. As discussed further, such an unfavorable process could arise from a steric hindrance in the fit of a critical component of the peptide, i.e., the Phe^4 residue in the corresponding subsite of the δ-receptor.

In the cyclic peptide studied in this paper, the structural modifications of the peptide ring never destroy the β-turn, which stabilizes the internal ring. The resulting molecular shape thus defined is required to put the C=O carbonyl of the third residue and the aromatic ring of the fourth in the right positions. Conformational studies (5, 29) have shown that, in solution, the kinds of β-turns occurring in various μ-selective cyclic peptides were dependent upon both the length and the nature of the covalent linkage. However, the subsequent slight modifications in the structure of these peptides are probably unable to strongly modify the molecular shape required to reach the proposed fitted model.

More recently, a second class of cyclic enkephalins with covalent linkages characterized by a disulfide bond arising from oxidation of cysteine residues in positions 2 and 5 of enkephalins was reported to be nonselective regarding μ- and δ-receptors (5). By contrast, replacement of the Cys residues by β,β-dimethylcysteine (penicillamine) moieties led to highly δ-specific peptides (13). As for the μ-selective cyclic enkephalins, the δ-specificity of (D-Pen^2-L-Pen^5) enkephalinamide or (D-Pen^2-D-Pen^5) enkephalinamide results from a drastic loss of affinity for the μ-subtype (13). These results show that, for synthetic peptides, extended conformations are not a definite requirement for δ-receptor recognition, contrary to our first suggestion (9). Nevertheless, the δ-receptor selectivity of these compounds probably results from strongly increased rigidity of the ring structures (5). This feature could inhibit the proposed small changes in the dihedral angles of Phe and Leu side-chains required for an optimal interaction of the address part of the peptide within the appropriate μ-receptor subsite. Likewise, the considerable increase in the size of the linking chain, which contains both sulfur atoms and gem-dimethyl groups, might hinder its binding to the subsite of the μ-receptor which is hypothetically able to accept the side-chain of Leu or Met in natural enkephalins (9, 20, 21).

By contrast, the enhanced rigidity of the penicillamine-containing cyclic peptide could favor the fit of the Phe ring in a specific subsite of the δ-receptor. The crucial role of this aromatic moiety for δ-receptor recognition has been already underlined (9) and is reinforced by the large loss of affinity for δ-receptors following the replacement of the natural amino acid Phe^4 by its gem-diamino analog in both cyclic and linear modified enkephalins (14). This very interesting result could

be related to differences in the conformational degree of freedom for the Phe[4] ring in peptides containing natural or retro amide bonds. All of these assumptions have to be tested by synthesis of appropriate models containing, for instance, a linear and lipophilic chain in place of the Phe residue and by calculations of the energy changes of δ-selective peptides to fit the proposed model of μ-active conformation. These experiments are now in progress in our laboratories.

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