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Review

Natural peptide analgesics: the role of solution conformation

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Abstract. Endogenous opioids have been studied extensively since their discovery, in the hope of finding a perfect analgesic, devoid of the secondary effects of alkaloid opioids. However, the design of selective opioid agonists has proved very difficult. First, structural studies of peptides in general are hampered by their intrinsic flexibility. Second, the relationship between constitution and the so-called 'bioactive conformation' is far from obvious. Ideally, a direct structural study of the complex between a peptide and its receptor should answer both questions, but such a study is not possible, because opioid receptors are large membrane proteins, difficult to study by standard structural techniques. Thus, conformational studies of opioid peptides are still important for drug design and also for indirect receptor mapping. This review deals with conformational studies of natural opioid peptides in several solvents that mimic in part the different environments in which the peptides exert their action. None of the structural investigations yields a convincing bioactive conformation, but the global conformation of longer peptides in biomimetic environments can shed light on the interaction with receptors.

Key words. Opioid; peptide; NMR; biomimetic environment; osmolyte; conformation.

Introduction

Opiates are the most ancient analgesics [see e.g., ref. 1]. Although not in pure form, alkaloids of opium have been used during the last 40 centuries to alleviate pain and for their euphoric effects. Alkaloid analgesics are very powerful and difficult to substitute with other drugs, especially for acute pain, but are plagued by several undesirable side effects: addiction, nausea, and respiratory depression. All attempts to find the 'perfect analgesic,' that is, an alkaloid retaining analgesic power comparable to that of morphine but devoid of its secondary effects failed. Discovery of enkephalin, the first of several endogenous opioids [2] rekindled the hope of finding a perfect analgesic since it seemed unlikely that endogenous opioids would carry secondary effects comparable to those of alkaloids. This hope was reinforced by the concomitant discovery that all opioids act in the framework of a complex neurotransmitter system that comprises at least three opioid receptors that, in principle, might correspond to different actions [3]. Selective opioids, it was argued, would display only part of the many effects exerted by unselective opioids and would eventually be devoid of the most noxious secondary effects.

Endogenous opioids are not very selective with respect to μ, δ, and κ receptors. Enkephalins (YGGFL and YGGFM) are weak δ agonists, with selectivity ratios (δ/μ) slightly greater than unity, dynorphin A (YGGFLRRIRPKLKWNNQ) is κ selective and β-endorphin (YGGFMTSEKSQTPLVTLFKNAIIKNAYKKGE) binds to μ and δ receptors with similar affinity. According to Schwyzer [4], the sequences can be subdivided in two functional domains, the recognition or message domain and the address domain. The message domain, common to most natural and synthetic opioid peptides, resides in the N-terminal part of the sequence (YGGF) and is necessary

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for recognition, whereas the address domain is responsible for selectivity and varies greatly among different opioids. It seemed a simple task, at least in principle, to vary the selectivity by appropriate changes in the address domain. In practice, design of peptide analgesics proved very difficult, mainly for two reasons. First, because peptides in general are very flexible and enkephalins particularly so, since their message domain contains two Gly residues. This intrinsic flexibility makes all structural studies extremely difficult. Second, the relationship between primary structure (constitution) and the so-called 'bioactive conformation' is far from obvious.

Thousands of linear analogues were produced as a result of classical structure-activity relationship synthetic studies based mainly on trial-and-error substitution, e.g., Ala scan, but little improvement in activity and/or selectivity over enkephalins or other 'endorphins' was obtained, at least for linear analogues [5]. The best 'analogues' came later from natural sources: the belief that the message domain of enkephalins (Tyr-Gly-Gly-Phe) is essential for opioid recognition was shaken by the discovery of dermorphin [6] and deltorphins [7–9], powerful μ- and δ-selective agonists, respectively. These peptides are found in the skin of South American frogs and have the peculiarity that their message domain (Tyr-D-Xaa-Phe) contains one D-amino acid residue, generally D-Ala. Nuclear magnetic resonance (NMR) conformational studies [10–15] on dermorphin and deltorphins indicate that the backbone conformation of the message sequence in solution is similar for both μ and δ peptides, whereas a different orientation of the side chains is possible but not easily detected in solution. The structural contribution to selectivity can thus be attributed to a different address domain, different backbone conformation in the message domain, or different side chain conformation in the message domain. These causes are not mutually exclusive, since a different address domain can induce different backbone conformation in the message domain and/or different side chain conformations in the message domain.

However, even after extensive studies on many opioids with different message domains, the main question – which is the bioactive conformation? – remains unanswered. This question has both theoretical and practical relevance. The ideal answer could only come from a direct structural study of the complex of an opioid peptide and its receptor, but such a study is not possible (yet), since opioid receptors are large membrane proteins, difficult to study by standard structural techniques. By the same token, we do not know the precise structure of the uncomplexed receptor, although modelling studies yield acceptable pictures of the receptors at low resolution [16]. Alternatively, we may use rigid molecules (as molds) to infer the shape of the bioactive conformation of peptides [17] or to validate, as possible bioactive conformations, experimentally determined conformations [18]. The use of moulds is allowed only if they interact with the same receptors and in the same active site as the peptides. This assumption cannot be taken for granted [16] but it is true that receptor modelling has greatly benefited from experimental work based on the systematic use of rigid opioids. The key role of aromatic transmembrane residues of the delta-opioid receptor in ligand recognition was probed by interacting mutated receptors with several rigid opioids [19]. The use of rigid molds poses another problem: why do we have to study the conformation of peptides at all? There are at least two reasons: (i) they are 'the real thing,' i.e., even if we assume that natural or synthetic alkaloids interact with the same active site of the opioid receptors, the true endogenous ligands are the peptides, and (ii) peptides are generally larger and consequently may have further 'interaction points' with the receptors.

Thus, conformational studies of opioid peptides are still important for receptor studies and drug design. This review deals mainly with the solution conformation of linear opioid peptides, their conformation-activity relationship, and its relevance for indirect mapping of opioid receptors and drug design. Such a choice, dictated by space, excludes the enormous literature on synthetic analogues, notably cyclic analogues. A brief mention of the main results of solid-state studies and a priori calculations on linear, natural opioids will be made for comparison purposes.

Conformations in the solid state

Solid-state structures of flexible molecules may not reflect conformational tendencies of isolated molecules since lattice forces are not negligible. In fact, they are sometimes the prevailing forces. The first two structures of enkephalin in the solid state illustrate this situation well. The first is a compact, folded conformer [20] whereas the second is completely extended [21]. Accordingly, the biological significance of solid-state structures is difficult to assess, not only because of the above-mentioned influence of crystal packing on conformation but also because the lattice is certainly different from any of the biological environments in which the peptide can be found (see below). However, despite these limitations, solid-state structures are very valuable: they are actual low-energy conformations that represent reference structures to which conformers predicted by computational methods (in silico conformers) and found in solution can be compared.

Linear peptides are not easy to crystallize, mainly because of their tendency to exist in several quasi-isoenergetic conformations [22]. Opioid peptides are particularly difficult, owing to the presence of the -G-G- motif in most of their sequences. Nonetheless, thanks to the ef-

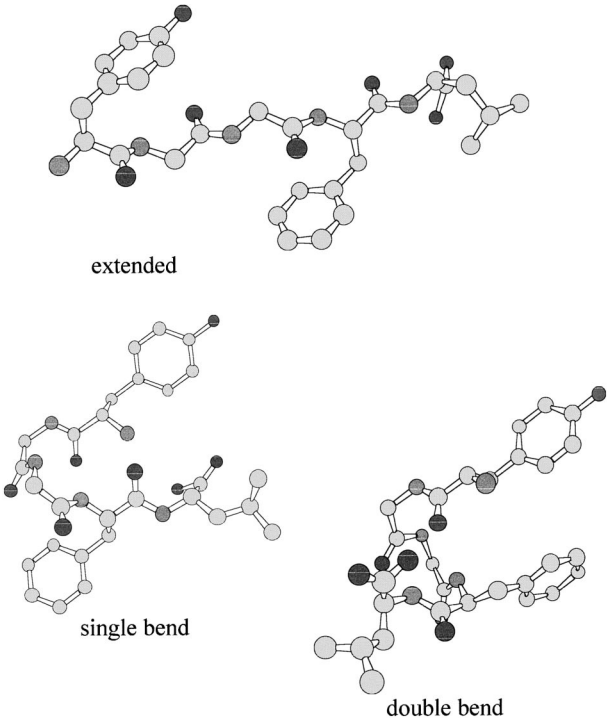

Figure 1. Schematic models of the most populated conformations of enkephalins in solid-state structures: fully extended, single bend, and double bend. Carbon atoms are shown with a uniform light-gray filling, nitrogens with gray filling, and oxygens with dark-gray filling.

forts of clever crystallographers, several structures of linear opioid peptides are now available [20, 21, 23–33]. These structures are classified and dissected most efficaciously in a review by Deschamps et al. [33]. Although no definite conclusion could be reached on their possible biological relevance, the solid-state structures of enkephalins, as determined by X-ray diffraction analysis, have revealed a limited number of backbone conformations. The most populated conformations are the fully extended ($\phi_i = \psi_i = \approx 180$, i = 2–4), the single bend ($\phi_2 = \approx 60$, $\psi_2 = \approx 30$, $\phi_3 = \approx 90$, $\psi_3 = \approx 0$, $\phi_4 = \approx -120$, $\psi_4 = \approx 150$), and the double bend ($\phi_2 = \approx -60$, $\psi_2 = \approx -30$, $\phi_3 = \approx -60$, $\psi_3 = \approx -30$, $\phi_4 = \approx -90$, $\psi_4 = \approx 0$). Models corresponding to representative conformers of linear opioids [33] are shown in figure 1.

Calculated conformations

Linear peptides, when considered as isolated molecules (i.e., in solution or in vacuo), exist in several quasi-isoenergetic conformations, or microstates [22]. This feature complicates the search for the bioactive conformation even if we can restrict the search to a limited number of low energy conformations. In other words, unlike proteins, small linear peptides do not have a 'native conformation' and thus the emphasis is shifted toward the search of the dominant microstates and the determination of their populations with the combination of experimental and theoretical calculations. Nevertheless, several attempts have been made to develop conformational search methods for finding the so-called global energy minimum (GEM) [34–37] and all the energy-minimized structures in certain energy ranges above the GEM. Between these, the Monte Carlo minimization method (MCM) [37] proved to be a useful tool for finding the lowest-energy states of small peptides. Over the past three decades, much effort has been invested in developing novel simulation techniques to enhance the sampling of low-energy conformations. Probably the most commonly used method for finding low-energy conformations is simulated annealing [38]. A molecular dynamics or a Monte Carlo simulation starts at high temperature, which is decreased gradually, and the system is expected to reach the low-energy region on the potential energy surface of the molecule. However, in many cases this process is not efficient. For linear peptides [39], the MCM method [37] is significantly more efficient than simulated annealing as generator of low-energy minimized structures.

Another approach to enhance sampling in protein folding and peptides simulations is to perform simulations in the so-called generalized ensembles. The most prominent example of this newer and more elaborate technique is probably the multicanonical algorithm (MUCA) [40]. The greatest advantage of generalized ensemble algorithms lies in the fact that, from a single simulation run, one can obtain not only the lowest energy conformation, but also any thermodynamic quantity at any temperature. The first application of generalized ensemble techniques to the protein-folding problem can be found in Hansmann and Okamoto [41], in which the MUCA technique was used. Testing this methodology for Leu-enkephalin [42], the authors minimized the energy of the structures selected from the MUCA trajectory and found very satisfactory results, particularly for coverage of the conformational space in the low-energy regions in comparison with results obtained previously with a conformational search technique, the MCM of Li and Scheraga [37]. In a recent study, Hansmann and Okamoto [43] report the free-energy landscape of Met-enkephalin. Their data were obtained from a generalized-ensemble Monte Carlo simulation taking the interactions among all atoms into account. They showed that the free-energy landscape, as in the case of a protein, resembles that of a funnel, indicating that this peptide is a good folder. This result reflects the elegance of these new algorithms but can hardly be taken as evidence of the existence of a 'native conformation' since all experimental data in solution for enkephalins contradict this view. It seems difficult, in general, to think of small linear peptides as molecules characterized by a

„native conformation“: proper folding can occur only when the peptide is complexed with the receptor.

Rather than trying to use increasingly complex calculation schemes in the case of flexible molecules, it is wiser to try to combine calculations and experimental data. Elucidating their dynamic three-dimensional structure by NMR is complex, since the experimentally measured nuclear Overhauser enhancement (NOE) intensities represent averages over individual contributions. Peptide conformations have been studied quite extensively with restrained molecular dynamics, using pseudopotentials based on NMR data, and time-averaged restraints [44]. A procedure called MEDUSA has been set forth by Bruschweiler et al. [45]. The underlying hypothesis is that individual conformations might violate some of the NOE distance restraints, which were fulfilled only by the entire dynamic set of substates. Only pairs of exchanging conformations were considered and the best combinations in terms of structural similarity were delineated [45]. Several researchers have proposed methods of different levels of sophistication for analyzing intermediate flexibility [46, 47]. These methods share a common basis, that is, a set of reasonable conformations are generated using any of the currently available force fields, conformations are retained if they differ significantly, their energies do not exceed some threshold, and they are consistent with a subset of the NMR restraints. The main disadvantage of this approach is the arbitrariness inherent in the selection of the conformations, which turn the populations into fitting parameters, rather than thermodynamic variables. Meirovitch and collaborators [22, 48–50] developed a statistical mechanics methodology for treating flexibility that has been used for analyzing NMR data. The first stage of this methodology is also based on an extensive conformational search using the local torsional deformation (LTD) method for identifying a large number of the lowest-energy-minimized structures within 2/3 kcal above the GEM. These structures are compared to each other and a subset of significantly different structures is selected. Each of these structures then becomes a seed for a canonical Monte Carlo calculation that spans its vicinity. The free energy of the corresponding sample is calculated with the local-states method from which the relative populations of these regions (microstates) are obtained. This methodology was applied initially to the linear peptide Leu-enkephalin (H Tyr-Gly-Gly-Phe-Leu OH) described by the potential-energy function ECEPP [51]. We can take their results as representative of *in silico* conformations. Figure 2 shows the five significant energy-minimized backbone structures found within the 2 kcal/mol range above (and including) –50.25 kcal/mol, which was considered to be the GEM of Leu-enkephalin. The five best conformers have the following backbone dihedral angles: a ($\phi_2 = 170$, $\psi_2 = 42$, $\phi_3 = 74$, $\psi_3 = 35$, $\phi_4 = -94$ $\psi_4 = -19$), b ($\phi_2 = 164$, $\psi_2 = -70$, $\phi_3 = -178$, $\psi_3 = 60$, $\phi_4 = -158$, $\psi_4 = -52$), c ($\phi_2 = -83$, $\psi_2 = -39$, $\phi_3 = -176$, $\psi_3 = 51$, $\phi_4 = -100$, $\psi_4 = -43$), d ($\phi_2 = -177$, $\psi_2 = 50$, $\phi_3 = 94$, $\psi_3 = 25$, $\phi_4 = -158$, $\psi_4 = 156$), e ($\phi_2 = -146$, $\psi_2 = 87$, $\phi_3 = 152$, $\psi_3 = -72$, $\phi_4 = -158$, $\psi_4 = 156$). None of them corresponds completely to the three representative solid state conformers of figure 1, but the majority of the backbone angles of conformers a and d are close enough to the single-bend structure, and conformer e, although characterized by different angles, has a global shape reminiscent of a double-bend structure.

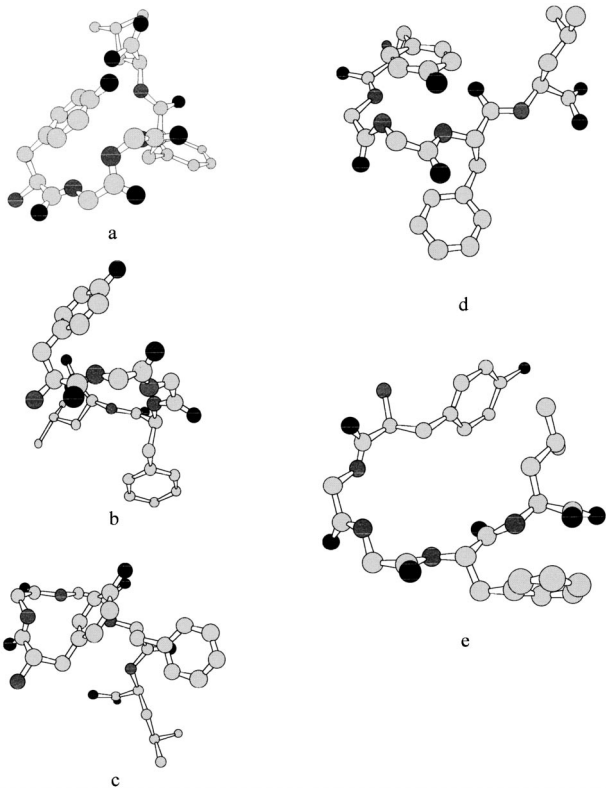

Figure 2. Schematic models of the most populated conformations (*a*–*e*) of Leu-enkephalin (H Tyr-Gly-Gly-Phe-Leu OH) calculated in vacuo with the ECEPP force field. Conformers *a* and *d* are close enough to the single-bend structure of solid-state models and conformer *e* has a global shape reminiscent of a double-bend structure of solid-state models. Carbon atoms are shown with a uniform light-gray filling, nitrogens with gray filling and oxygens with dark-gray filling.

Conformations in solution

Choice of the solvent

The first two papers dealing with the solution structure of opioid peptides appeared soon after the discovery of enkephalins [52, 53]. These studies, like the majority of early structural studies on bioactive peptides, were performed in water and were based on the assumption that all

observable NMR parameters originated from a single conformer. This assumption proved wrong, since all linear peptides of short sequence are too flexible to assume a single structure, or even a limited number of conformations, in aqueous solution [54]. The flexibility of linear peptides is reflected by NMR parameters, e.g., by the fact that $J_{NHC\alpha H}$ scalar couplings are distributed around the average value of 6.5 Hz and, most of all, by the failure to observe diagnostic NOEs. In fact, for several years nobody could observe NOEs, except for a few intraresidue ones, in the spectra of enkephalins [55] in any solvent. This difficulty originated in part from the intrinsic flexibility of these peptides and in part from shortcomings of the instruments. The introduction of the ROESY experiment [56], which circumvents specific difficulties associated with unfavorable correlation times, allowed the measurement of a larger number of NOEs in small peptides, but still of low diagnostic value.

The first observation of NOEs in the spectra of enkephalins was possible only at low temperatures, in solvents of elevated viscosity [57, 58]. This experimental observation emphasizes the great influence of the environment on the conformational state of the peptide and raises the question whether it is meaningful, from a biological point of view, to search for *any* environment that favors the existence of regular conformers. Finding a suitable structuring environment is not too different to finding the right crystallization conditions: in both cases we end up with useful static pictures of a low-energy conformation. However, the main goal is not simply to find the best structuring conditions per se but to study opioid peptides in biologically relevant environments. Opioid peptides can be found in several biological environments: (i) transport fluids, (ii) membranes, and (iii) receptor active sites. Can we simulate them?

Transport fluids

The paradigmatic environment for transport fluids of course is water, or rather aqueous solutions containing a wide range of solvents in diverse biological fluids. As already mentioned, opioid peptides are essentially disordered in water. The only apparent exception was described by Gupta et al. [59], who reported large NOEs among aromatic protons of Tyr1 and Phe4 of enkephalin in water. This claim was only a false positive, based on a trivial technical error, but, while checking it, we showed that in a mixture of dimethylsulfoxide (DMSO) and water, i.e., a cryoprotective mixture, it was indeed possible to observe both intra- and interresidue NOEs [57, 58]. Cryoprotective mixtures, or cryomixtures for short, are simple mixtures of water and organic solvents such as alcohols, DMSO or dimethylformamide (DMF), which are fully biocompatible according to numerous biochemical and crystallographic studies on proteins [60]. They can be regarded as an 'in vitro version' of osmolytes, protective solutions found in many organisms living in extreme conditions [61, 62]. Cryomixtures can be used in a wide range of temperatures and, at low temperature, they can have a dielectric constant identical to that of water at room temperature. From this point of view, they can be considered representative of transport fluids. The main effect of cryomixtures is to change the correlation time of the dissolved peptides, allowing the measurement of NOEs. However, their action is not simply limited to the technical influence of viscosity on NOEs but can be likened to a 'conformational sieve': high viscosities favor folded (more compact) conformers over disordered ones [63]. Accordingly, we postulated that the viscosity of these fluids contributes, in addition to the membrane catalysis proposed by Schwyzer [4], to overcoming the so-called entropic barrier to the transition state of peptide-receptor interaction, by selecting ordered conformations prior to receptor interaction. In addition, they represent a mimic of the intracellular environment since they have a viscosity close to that of cytoplasm. Cytoplasm viscosities range from 5 to 30 cP [64] and may play an important role in cell communication processes [65]. Opioids exert their action at synapses; they are excreted by specialized vesicles of the presynapse and reach membrane receptors on the postsynaptic membrane by crossing a cleft of 100–500 Å occupied by the intersynaptic fluid, an aqueous solution whose viscosity is higher than that of cytoplasm because of the ordering effect of membrane heads and unstirred-layer phenomena [66].

The use of cryomixtures in the solution study of enkephalin yielded the measurement of the first NOEs that, in turn, allowed sophisticated calculations on the composition of conformers in equilibrium [48] but could not possibly point to a single, or even a few, prevailing conformation. However, we showed that it is possible to adopt a reverse strategy. Rigid molds are used initially as filters to find a range of likely bioactive conformations [18] and only at a later stage are the NMR data in cryomixture checked for consistency with the selected conformations. Some of them, even after extensive free minimization, retain a shape consistent with that of rigid molds. Figure 3 shows the superposition of one of these structures, dubbed F in the original work [18], with 7-spiroindanyloxymorphone (SIOM), a rigid delta-selective opioid. Both Tyr1 and Phe4 of enkephalin are very close in space to the corresponding aromatic rings of SIOM, and have a very similar orientation. However, conformer F, as well as the other structures found by this strategy, bears no resemblance either to solid-state conformations or to calculated conformers. This finding is consistent with the difficulty of threading a peptide sequence into the alkaloid skeleton, but it also shows that the very flexibility of enkephalins is probably needed to adopt an 'unnatural' bioactive conformation. In the case of less flexible opi-

Figure 3. Best fit of the most significant structure of Leu-enkephalin [F in ref. 18] with the shape of 7-spiroindanyloxymorphone (SIOM), a rigid delta-selective opioid. Leu-enkephalin is shown with dark grey balls. The rigid mould is represented with light grey balls.

oids, the conformational state could be described in more detail. The most interesting examples are deltorphins [13, 15, 54, 67]. Once again the use of biomimetic environments characterized by a viscosity higher than water but closer to typical values of cytoplasm proved very useful. Another commonly used solvent representative of transport fluids is neat DMSO that has a smaller dielectric constant than water, but is a more effective hydrogen bond acceptor, and has a higher viscosity than water (3.5 cP vs 1.0 cP at room temperature). Dermorphin, a very potent and specific μ agonist, was studied mainly in DMSO [68, 69]. The prevailing conformations of this peptide are linear.

Membranes

Long before opioid receptors were cloned and classified as seven-transmembrane (7TM) helices G-protein-coupled receptors (GPCRs), they were known to be membrane proteins. This fact alone could explain the interest in studying the conformational preferences of opioid peptides in media similar to the membrane environment. As Deber and Behnam [70] put it 'In the course of their biological function, peptide hormones must be transferred from an aqueous phase to the lipid-rich environment of their membrane-bound receptor proteins.' The media commonly employed to mimic the membrane environment are aqueous micellar solutions. Many of these studies, particularly the older ones, are in micelles of sodium dodecyl sulfate (SDS), a detergent easy to find in perdeuterated form [71–74], but there are also several studies in micelles of phospholipids.[70, 75–81]. A series of structuring properties were found in micellar solutions. For example, according to Fiori et al. [71], who studied endomorphin-1 (Tyr-Pro-Trp-Phe-NH2) and endomorphin-2 (Tyr-Pro-Phe-Phe-NH2), two recently discovered opioid peptides with high affinity and selectivity for the μ receptor, amphipathic SDS micelles and even more efficiently, the AOT reverse micelles of bis(2-ethylexyl) sulfosuccinate sodium salt constitute with their electrostatic and hydrophobic potential an excellent mimetic of amphipathic surfaces as present on lipid bilayers and on ligand recognition and ligand-binding sites of proteins. Yan et al. [72] studied the structural and dynamic properties of opioid peptide E in SDS micelles and found that the intermediate-order parameters observed for peptide E suggest a degree of dynamic mobility that may enable facile interconversion between helical and β-turn geometries in the N-terminal agonist domain.

The drastic change of environment from an aqueous phase to the lipid-rich environment of their membrane-bound receptor proteins plays a central role in a model that tries to explain not only the role of the membrane but also the selective interaction of similar peptides with three receptor subtypes [4]. In his membrane-assisted selection, Schwyzer [4] pointed out that the state of linear peptide hormones in water is probably random but that, even before a peptide binds to a protein receptor, the change from the bulk of the aqueous environment to the water-membrane interface, and finally to the apolar environment of the membrane lipids can induce a transition from more or less disordered 'random coils' to fairly ordered folded conformations. The model implies that the peptide enters the receptor coming from the lipid phase, a view that was plausible when the receptor was unknown but that is more difficult to reconcile with the fact that all opioid receptors are 7TM helices that form a sort of tunnel crossing the membrane. This difficulty has not escaped the attention of some researchers studying opioid peptides in micellar solutions. According to Schwyzer's hypothesis of membrane-catalyzed receptor selection [82], the key factor to explain κ-selectivity of dynorphin is the formation of an α helix from Tyr1 to Arg9 that favors the insertion of the N-terminal message domain of the peptide into the hydrophobic phase of the cell membrane. Although the first qualitative results in the dodecyl phosphocholine (DPC) micelles solution [83] seemed to speak in favor of Schwyzer's hypothesis, a more quantitative structural determination by Tessmer and Kallick [77] hypothesizes that the central helical segment they detect in the revised determination (Gly3–Pro10) is induced by the negatively charged surface of the micelles and not by insertion into the lipid phase.

Even taking these difficulties into account, it is important to note that studies in micellar solutions are interesting from a general point of view, even if strictly speaking they do not correspond to a biological environment visited by opioid peptides in their approach to the active site of re-

ceptors. In fact, the discontinuity between aqueous phase and apolar phase offered by micelles is similar to the discontinuity between extracellular fluids and the (apolar) receptor active site.

Receptor cavities

When opioid peptides were discovered, all one knew about the receptor active sites was that they were probably apolar cavities, with one or a few charged groups to anchor the agonists, specifically, in the case of opioids, an anionic site that can interact with the basic site common to all opioids, both peptidic or alkaloidic. This view is confirmed by contemporary views of the receptors, coming from modelling work. The active site is a largely hydrophobic cavity, lined by several aromatic side chains [ref. 16 and reference therein]. The many studies of different bioactive peptides in alcohols or in aqueous alcoholic mixtures [84–89] reproduce at least in part the apolar environment. Alcohols are *not* apolar molecules of course, but their radicals can mimic the apolar walls of the active site by presenting an apolar face to the peptides. For example, according to Rajan et al. [89], a mixture of water and a fluorinated diol, hexafluoroacetone hydrate (HFA), can surround the helix with a sort of 'teflon coating.'

Enkephalins are so flexible that even in these environments they are completely random. Alcohol/water mixtures, however, proved very useful in the case of longer opioids, particularly dynorphin [90], β-endorphin [91], and enkelytin [92]. Most of the media previously used in the study of dynorphin [77, 83, 93–95] were employed in solution conditions that could favor helical conformers, in order to test Schwyzer's hypothesis of membrane-catalyzed receptor selection [82]. Although the first qualitative results in the DPC micelles solution seemed to support Schwyzer's hypothesis, a recent more quantitative structural determination by Tessmer and Kallick [77] found a slightly different helical segment (Gly3–Pro10). These authors hypothesize that the central helical segment they detect in the DPC micelles solution is induced by the negatively charged surface of the micelles and not by insertion into the lipid phase. This hypothesis emphasizes the fact that aqueous solutions of micelles are intrinsically heterogeneous media and calls for further investigations that can determine conformational tendencies in homogeneous media. We have studied the conformational properties of dynorphin A in a wide range of solution conditions, including methanol and mixtures of organic solvents with water such as 50:50 (v:v) HFA:water, i.e., media that are known to favour helical conformations. However, dynorphin has no tendency to assume even partially helical conformations in any solvent. The only solvent medium in which dynorphin is at least partially ordered is an 80:20 (v:v) DMSO:water cryomixture at 278 K. The prevailing structure in this medium can be described as a large loop, extending from Arg7 to Lys13, that has Pro10 at its apex. Such a structure may favor an optimal interaction of three essential basic residues of dynorphin, Arg7, Lys11 and Lys13 with acidic residues of the e2 loop of the κ receptor and induce a mutual conformational rearrangement. Figure 4 shows a model of this structure. The essential role of the three residues we find on the exposed surface of our structure, Arg7, Lys11 and Lys13, is consistent with the fact that dynorphin(1–13) retains most of the κ activity of the parent peptide [96].

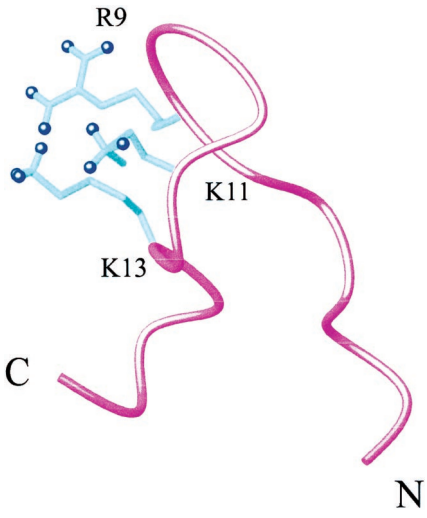

Figure 4. Predominant structure of dynorphin A in a 80:20 (v:v) DMSO:water cryomixture at 278 K. The backbone is shown in a magenta, the side chains of the three essential basic residues, Arg7, Lys11, and Lys13, are shown in blue.

Human β-endorphin, composed of 31 residues, contains the enkephalin sequence in its N-terminal part (i.e., the message domain). This peptide has very little tendency to assume an ordered structure in water but a strong tendency to assume a helical structure in its address domain (from P13 to Y27) in mixtures of water and alcohols, particularly in TFE:water (30:70 v:v) and HFA:water (50:50 v:v). The structure of β-endorphin yields an interesting example of an internal probe for the tendency of given amino acid sequences to form helical structures in helicogenic solvents [91]. The finding that only half of the molecule assumes a very regular helical conformation, while the N-terminal dodecapeptide moiety, containing the enkephalin message, remains disordered, hints that the role of the sequence is prevailing in this case. Once found the nature and location of the secondary-structure elements may reveal something about the function of each segment. In other words, the fact that the initial 12 amino acids do not show any tendency to go helical even in a strong helix-inducing solvent such as HFA:water (50:50 v:v) hints that the N-terminal mes-

sage domain must remain very flexible to favor an induced fit with the receptor active site, whereas the C-terminal address domain can assume a regular helical structure that favors an interaction with stable elements of secondary structure of the apolar cavity of the 7TM-helices receptor. A 'two-point' attachment involving an interaction of the helical part of β-endorphin (the address domain) with either an extracellular loop or with one or more of the transmembrane helices and the (triggering) interaction of the message domain (YGGF) with the receptor subsite common to all opioid receptors has been proposed [91]. Figure 5 shows a schematic model of the μ receptor hosting the helical segment of β-endorphin: the helix of endorphin (P13–Y27) interacts with all five transmembrane helices that contribute most in forming the active site (III–VII). The helix of the peptide is antiparallel to both helices III and V. It is certainly possible to put the relevant pharmacophores of endorphin close to the hypothesized residues [16] of the receptor active site if the message adopts a folded conformation.

The lack of any structure in the N-terminal part of endorphin (YGGFL) is at variance with the behavior of the corresponding sequence (YGGFM) in the C-terminal part of enkelytin, a peptide corresponding to the bisphosphorylated form of proenkephalin-A 209–237 [97]. Although coming from proenkephalin A, which can yield several copies of enkephalin, this peptide has not opioid but antibiotic activity. An NMR study showed that synthetic PEAP-209–237, which is unstructured in water, folds into an α-helical structure in trifluoroethanol:water (1:1) [92]. As observed by these authors, although many studies have focused on the three-dimensional structure of enkephalins, finding a wide range of conformations in the crystal state and in solution, this is the first observation of a helical structure for the sequence of enkephalin. The ability to assume this conformation parallels the absence of any binding (of enkelytin) to opioid receptors. The N-terminal charge is possibly essential both for binding and to induce the helix.

Ascertaining whether the environment afforded by alcohol:water mixtures is truly apolar is difficult. On the other hand, peptides cannot be studied in truly apolar solvents because they are not sufficiently soluble. Solubility is precluded by the presence, on peptides, of charged

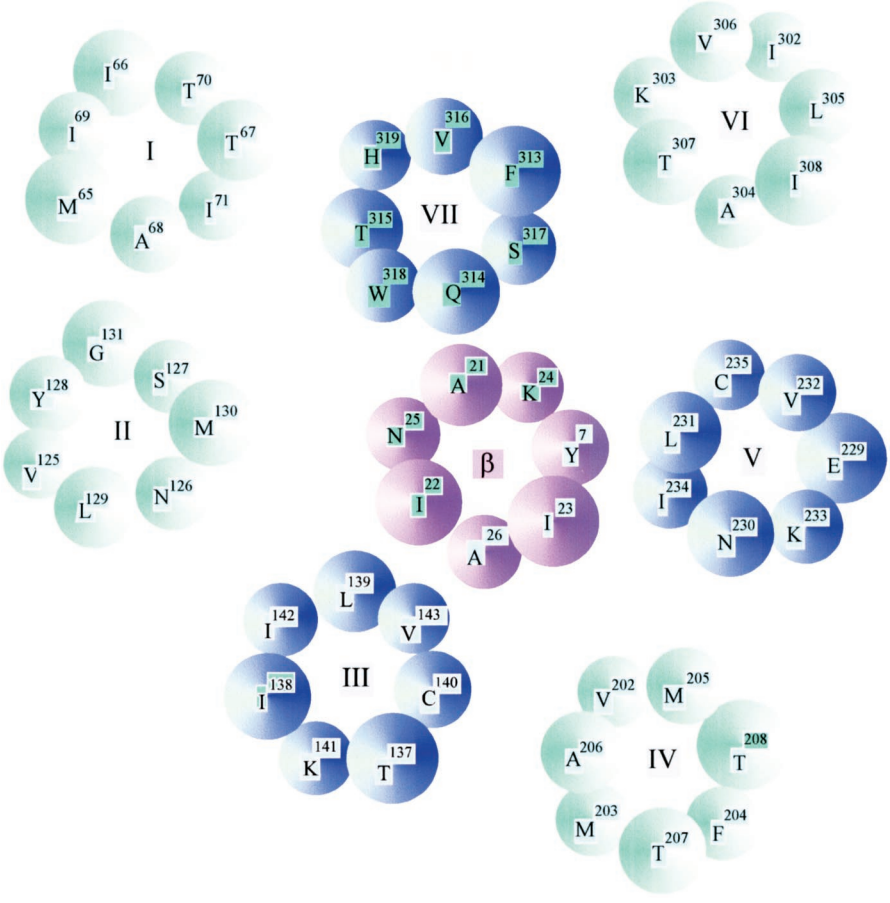

Figure 5. Schematic model of the interaction of the helical segment of β-endorphin (P13–Y27) with the transmembrane helices forming the active site of the μ receptor (III–VII). The μ model is represented by the extracellular view of the uppermost seven residues of the seven transmembrane helices. Helices I, II, IV, and VI are represented in cyan whereas the helical segment of β-endorphin, labeled with a β, is represented in magenta. The helices that are thought to interact more strongly (III, V, and VII) are represented in blue.

groups, in particular the N-terminal ammonium group that is essential for an interaction with the receptor, at least for agonists [98]. A possible way to circumvent this difficulty was proposed by Temussi et al. [99]. Complexation of the $-NH_3^+$ group of enkephalin amides with a crown ether can be likened to the binding of the same group to the anionic subsite of the receptor, whereas a relatively apolar solvent, like $CDCl_3$, can play the role of the hydrophobic cavity. These experimental conditions favor folded conformations of the family of β turns [10, 99, 100].

Conclusion

Peptide opioids have been studied in a wide variety of solution conditions. The goal of inferring the 'bioactive conformation' from experimental data in solution has indeed proved elusive [101]. Even if one chooses reasonably biomimetic conditions, all linear opioid peptides are too flexible to yield spectroscopic data consistent with a single conformation, particularly in the crucial message domain that coincides with the five residues of enkephalin for human opioids and with the first four residues of deltorphin for opioids derived from frog skin. However, the numerous low-energy conformations emerging from solution studies constitute a useful data base for modelling studies, particularly those attempting to depict the complex of a receptor with its agonist. Last but not least, solution studies of long-chain opioids suggest possible mechanisms of interaction with parts of the receptors other than the active site, probably related to selectivity.

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