Journal of Peptide Science
J. Peptide Sci. **8**: 497–509 (2002)
Published online in Wiley InterScience (www.interscience.wiley.com). DOI: 10.1002/psc.412

Review

Solution Structure of Nociceptin Peptides

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Received 28 March 2002
Accepted 14 May 2002

Abstract: Peptides embedded in the sequence of pre-pro-nociceptin, i.e. nociceptin, nocistatin and orphanin FQ2, have shed light on the complexity of the mechanisms involving the peptide hormones related to pain and have opened up new perspectives for the clinical treatment of pain. The design of new ligands with high selectivity and bioavailability, in particular for ORL1, is important both for the elucidation and control of the physiological role of the receptor and for their therapeutic importance. The failure to obtain agonists and antagonists when using, for nociceptin, the same substitutions that are successful for opioids, and the conformational flexibility of them all, justify systematic efforts to study the solution conformation under conditions as close as possible to their natural environment. Structural studies of linear peptides in solution are hampered by their high flexibility. A direct structural study of the complex between a peptide and its receptor would overcome this difficulty, but such a study is not easy since opioid receptors are membrane proteins. Thus, conformational studies of lead peptides in solution are still important for drug design. This review deals with conformational studies of natural pre-nociceptin peptides in several solvents that mimic in part the different environments in which the peptides exert their action. None of the structural investigations yielded a completely reliable bioactive conformation, but the global conformation of the peptides in biomimetic environments can shed light on their interaction with receptors.

Keywords: allodynia; analgesics; NMR; nociceptin, opioids

INTRODUCTION

The discovery of nociceptin (NC) [1,2] has opened up new perspectives for the clinical treatment of pain and has shed light on the complexity of the mechanisms involving the peptide hormones related to pain. NC, also known as orphanin FQ, is a heptadecapeptide whose sequence (FGGFTGARKSARKLANQ) is remarkably similar to that of dynorphin A (DA), (YGGFLRRIRPKLKWDNQ). NC was discovered [1,2] in a direct search for a possible agonist of ORL1, a G protein-coupled receptor whose amino acid sequence is closely related to those of the opioid receptors. The nociceptin receptor contains an acidic

Abbreviations: DA, dynorphin A; DMF, N,N-dimethylformamide; DMSO, dimethylsulphoxide; DPC, dodecylphosphocholine; EM, energy minimization; GPCR, G protein-coupled receptor; HFA, 1,1,1,3,3,3-hexafluoroacetone; MD, molecular dynamics; MM, molecular mechanics; MVD, mouse vas deferens; NC, nociceptin; NOESY, nuclear Overhauser spectroscopy; NS, nocistatin; OFQ2, orphanin FQ2; ORL1, opioid receptor like 1; PPNOC, pre-pro-nociceptin; SA, simulated annealing; SAR, structure–activity relationship; SDS, sodium dodecylsulphate; TAD, torsion angle dynamics; TFE, 2,2,2-trifluoroethanol; 7TM, 7-transmembrane; Standard IUPAC single- and triple-letter codes for amino acids are used throughout.

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extracellular loop similar to that required for high affinity binding of DA to the κ-opioid receptor [3]. Systematic structure–activity studies on NC [4] suggested that, as in the case of DA, the message domain is probably coincident with the sequence of the four *N*-terminal residues, thus leaving a highly basic *C*-terminal address domain [5] that differs from that of DA mainly in the detailed distribution of the basic residues. In spite of these similarities NC displays no opioid activity. In binding, as well as in biological assays it was shown that nociceptin does not interact with the opiate receptors and acts through the activation of a selective and specific functional site (receptor) which has been shown to inhibit electrically evoked contractions in several isolated organs suspended *in vitro* [6,7]. When applied *in vivo*, nociceptin is algogenic [1,2] and tends to reverse or reduce opioid-mediated analgesia in the mouse [8]. It therefore appears that, despite the structural similarities of nociceptin with dynorphin and the identity of some biological effects, e.g. inhibition of sympathetic, parasympathetic and peptidergic nervous activities, as well as inhibition of the forskolin induced accumulation of cAMP, the functional sites of opioids and that of nociceptin are pharmacologically different. This surprising behaviour may be ascribed to constitutional and/or conformational differences. In either case a clear identification of the origin of this discrepancy could lead to a better understanding of opioid selectivity.

It was soon discovered that pre-pro-nociceptin (PPNOC) [9,10] contains two other very interesting 'pain heptadecapeptides', namely orphanin FQ2 (OFQ2), (FSEFMRQYLVLSMQSSQ) and nocistatin (NS), (TEPGLEEVGEIEQKQLQ) [1,2,11]. OFQ2 is a potent analgesic given either supraspinally or spinally. While supraspinal OFQ2 analgesia is readily reversed by naloxone, implying activation of opioid receptor systems, spinal OFQ2 analgesia is insensitive to opioid antagonists [12]. NS plays a role opposite to that of NC in pain transmission but it is not related in any obvious way to known opioid peptides.

These peptides differ profoundly both in their SAR and in conformational properties. DA [13] and NC [14] show little tendency to form well-defined structures in solution, not only in water or DMSO, but also in media that normally favour the formation of helices. NC, on the other hand, forms a well-defined structure extending from Gly^4 to Leu^{16}, with an unusually well-defined helix (for a short peptide) in the *C*-terminal part [15]. In opioids and NC the message domains coincide with the *N*-terminal part (YGGF and FGGF, respectively) [5,13], whereas the shortest fragment of NS that retains allodynia-blocking activity is the *C*-terminal hexapeptide (EQKQLQ), which is conserved in bovine, human and murine species [11].

The design of new ORL1 ligands with high selectivity and bioavailability is important both for the elucidation and control of the physiological role of the ORL1 receptor and for their therapeutic importance. Several potential therapeutic applications have been recently reported for the ORL1 receptor system [7]. ORL1 agonists have been proposed as potential anxiolytics, stimulants of food intake, suppressants of drug abuse, anti-epileptics and for the management of water-retaining syndromes [7]. ORL1 receptor antagonists, on the other hand, have been evaluated as anorectics, analgesics as well as nootropic agents [7]. It would be desirable to be able to complement these ligands with counterparts from OFQ2 and NS.

The failure to obtain agonists and antagonists when using, for NC, the same substitutions that are successful for opioids, and their conformational flexibility, justify the systematic efforts to study the solution conformation under conditions as close as possible to their natural environment.

CHOICE OF THE ENVIRONMENT

The primary structure, biological environment and the interplay of these two factors are most important for the conformation of bioactive peptides. In particular, the relative abundance and distribution of hydrophobic residues along the sequence are key features in determining solubility, conformational preferences and the overall response to different environments. Since the stability of canonical secondary structures, or even simple ordered structures, depends on both polar and apolar interactions in a complex way, reliable predictions are possible only for a few well-characterized situations, e.g. transmembrane and amphipathic peptides. In general it is very difficult to predict the influence of a solvent on conformation. Solvent effects relevant to the conformation of bioactive peptides can be roughly subdivided into viscosity effects, the influence of the macroscopic dielectric constant, the global anisotropic organization, e.g. in micelles, or microenvironmental anisotropic partitions that can stabilize different regions of the

peptide in a different way. The latter case, exemplified by the microenvironment created by mixtures of water and alcohols, is particularly relevant for peptides interacting with receptors characterized by irregular distributions of polar and apolar patches. Within this framework the well-known induction of helicity promoted by fluorinated alcohols represents only an extreme case highlighting conformational preferences already embedded in the sequence [13 and references therein]. Thus, the study of peptides in a variety of solvent media, akin to a 'solvent scan', can furnish unexpected hints on the conformational stability as a function of environment, a precious clue for the identification of putative biological environments.

Peptides derived from PPNOC, like most linear peptides, are too flexible to assume a single structure in solution, particularly in aqueous solutions [16]. This conformational flexibility generates values for most NMR parameters that are close to average or absent, as is the case for diagnostic NOEs. This difficulty, well documented for opioid peptides [17], originated in part from their intrinsic flexibility and in part from the unfavourable correlation times of small molecules in high magnetic fields. Although the ROESY experiment [18] circumvented the problem of unfavourable correlation times, the number of diagnostic nuclear Overhauser effects in small peptides remains very small in ROESY spectra. The observation of NOEs of opioid peptides was made possible by the introduction of solvents of elevated viscosity, the so-called cryoprotective mixtures [19,20]. The influence of the environment on the conformational state of the peptides reminds us that it is crucial to study bioactive peptides in biologically relevant media. PPNOC peptides, like opioid peptides, can be found in several biological environments that can be grouped in extracellular fluids, membranes and receptor cavities.

Extracellular Fluids

As already mentioned, peptides are generally unordered in water, the paradigmatic environment for transport fluids. A valid alternative to aqueous environments is represented by cryoprotective mixtures, i.e. simple mixtures of water and organic solvents. The most commonly employed solvents are alcohols, DMSO and DMF, but also osmolytes — protective molecules found in many organisms living under extreme conditions [21,22]. Cryomixtures can have a dielectric constant identical to that of water and are fully biocompatible as shown by classical biochemical and structural studies on proteins [23]. The main effect of cryomixtures is to increase the viscosity of the solution with respect to water, thus shifting the correlation time of the dissolved peptides to a range typical of macromolecules, hence allowing the measurement of NOEs even at high fields. In addition, high viscosity seems to favour compact conformers over disordered ones [24]. Cryomixtures represent, in part, a mimic of cellular environments since they have a viscosity close to that of cytoplasm [25].

Membranes

The receptors of OFQ2 and NS are not known but it is certain that the receptor of NC is a 7TM-helices GPCR receptor, very similar to the opioid receptors. Opioid peptides have often been studied in media similar to the membrane environment, usually aqueous micellar solutions. The micelles generally used are based on SDS, a detergent easy to find in perdeuterated form [26–29], but there are also published studies of micelles of phospholipids [30–36].

The membrane environment plays a central role in the model of membrane-assisted-selection [37] that tries to explain the selectivity of related peptides mainly on the basis of the role played by the membrane. According to this model, the key factor to explain κ-selectivity of dynorphin, the opioid peptide closest to NC, is the formation of a helix comprising the first nine residues that favours the insertion of the message domain of the peptide into the hydrophobic phase of the cell membrane [5]. An exploratory study of dynorphin in DPC micelles [34] was consistent with Schwyzer's hypothesis [5], but later on a more quantitative structural determination [32] found a different helical segment (Gly^3-Pro^{10}) induced by the negatively charged surface of the micelles and not by insertion into the lipid phase. The receptors of DA and NC, i.e. 7TM GPCRs, have the shape of irregular funnels with the active site at the bottom. Thus it seems unlikely that the agonists may find their way to the active site through the lipid phase.

However, even taking into account the difficulty of using micelles to reproduce the specific environment inside 7TM GPCR receptors, it is important to note that studies in micellar solutions are interesting from a general point of view. Indeed, the discontinuity between the aqueous phase and the apolar phase offered by micelles is similar to the discontinuity between extracellular fluids and the apolar environment typical of the receptors' active sites.

Receptor's Cavities

The active site of GPCR receptors is essentially a hydrophobic cavity [38]. In studies of several bioactive peptides [39–44] aqueous alcoholic mixtures were employed to reproduce a local apolar environment. Alcohols are *not* apolar molecules of course, but their alkyl radicals, particularly those of fluorinated alcohols, mimic the apolar walls of the active site by surrounding the peptides with an apolar surface [44].

Alcoholic mixtures proved particularly useful in the study of longer peptides, either opioids like DA [13] and endorphin [45] or derived from PPNOC [14,15,46]. In the case of DA the use of mixtures of water and alcohols was useful for checking the hypothesis that the peptide must assume a helical conformation to interact with its receptor [5]. By studying the conformational properties of DA in a wide range of solution conditions, including methanol and mixtures of organic solvents with water such as 50 : 50 HFA/water (v/v), we found that DA has no tendency to assume a helical conformation in any media that are known to favour this ordered secondary structure [13]. The behaviour of β-endorphin is the opposite; in fact, it represents an interesting example of the tendency of specific amino acid sequences to form helical structures in alcoholic mixtures. The whole peptide has no tendency to assume ordered structures in water, but in mixtures of water and alcohols it has a helical address domain (from P^{13} to Y^{27}) and a still unordered message domain. In particular, only the *C*-terminal half of the molecule assumes a very regular helical conformation, whereas the *N*-terminal segment, containing the enkephalin message (YGGFL), remains unordered. Thus, it can be concluded that the *N*-terminal segment behaves as an internal probe, hinting that the role of the sequence prevails in this case and that its conformation is not dominated by the properties of the solvent.

As mentioned above, the ORL1 receptor, a 7TM GPCR transmembrane protein, was discovered before the natural agonist. In principle, the interaction of NC with its receptor is well characterized and it would be possible to choose the proper conditions discussed above to simulate the best possible environment. In practice, the extreme flexibility of NC required a wider exploration of structuring conditions. In the case of the other two peptides derived from PPNOC very little is known about possible receptors. Accordingly, a conformational study in many different (from very polar to very apolar) environments can yield useful information on the nature of the receptor interaction. The search for suitable structuring conditions led in all cases to a kind of 'solvent-scan'. To quote but a few examples of the 'solvent scan', we used the solvent series methanol, acetonitrile, DMSO and mixtures of organic solvents with water such as 50 : 50 HFA/water (v/v) and 80 : 20 DMSO/water (v/v) for the conformational study of DA [13]; methanol, acetonitrile, DMSO, 90 : 10 DMSO/water (v/v) and aqueous solution of SDS micelles in the case of NC [14], and water, DMSO and aqueous mixtures of methanol, ethylene glycol, TFE, HFA and DMSO for the solution structure study of β-endorphin [45].

NOCICEPTIN

Owing to the close relationship with DA, the conformational properties of NC have been initially studied in media similar to those employed for DA [13,47]. Previous NMR studies of DA were performed in an aqueous solution of DPC micelles [32,34], in water [48] and, for the dynorphin-(1–13) fragment, both in aqueous solution [49] and in methanol–water [47]. Most of these media were chosen in the hope of detecting a plausible bioactive conformation; in particular Lancaster *et al.* [47] and Kallick [34] looked for conditions favouring helical conformers, to test Schwyzer's hypothesis of membrane catalysed receptor selection [5].

In order to widen our 'solvent scan' approach we employed also acetonitrile, DMSO and a DMSO/water cryomixture [21]. A detailed conformational study showed that this solvent furnishes the best structuring conditions [13]. The NOESY spectra of NC in all solvent systems exhibit a limited spread of the NH resonances. It was still possible, however, to compare the conformational preferences of NC to those of DA, at least qualitatively.

Figure 1 shows a comparison of the partial NOESY spectrum of DA in the 80 : 20 (v/v) $DMSO_{d6}/H_2O$ cryoprotective mixture at 278 K, corresponding to the best structuring conditions for this peptide [13], with the spectra of NC in an aqueous solution of SDS micelles, in DMSO and in the 90 : 10 (v/v) $DMSO_{d6}/H_2O$ cryoprotective mixture at 278 K. It can be seen that in all experimental conditions the conformational state of NC is less ordered than that of DA. The number of inter-residue cross peaks for the resonances is small, insufficient for a detailed structural description. However, the NOEs of the

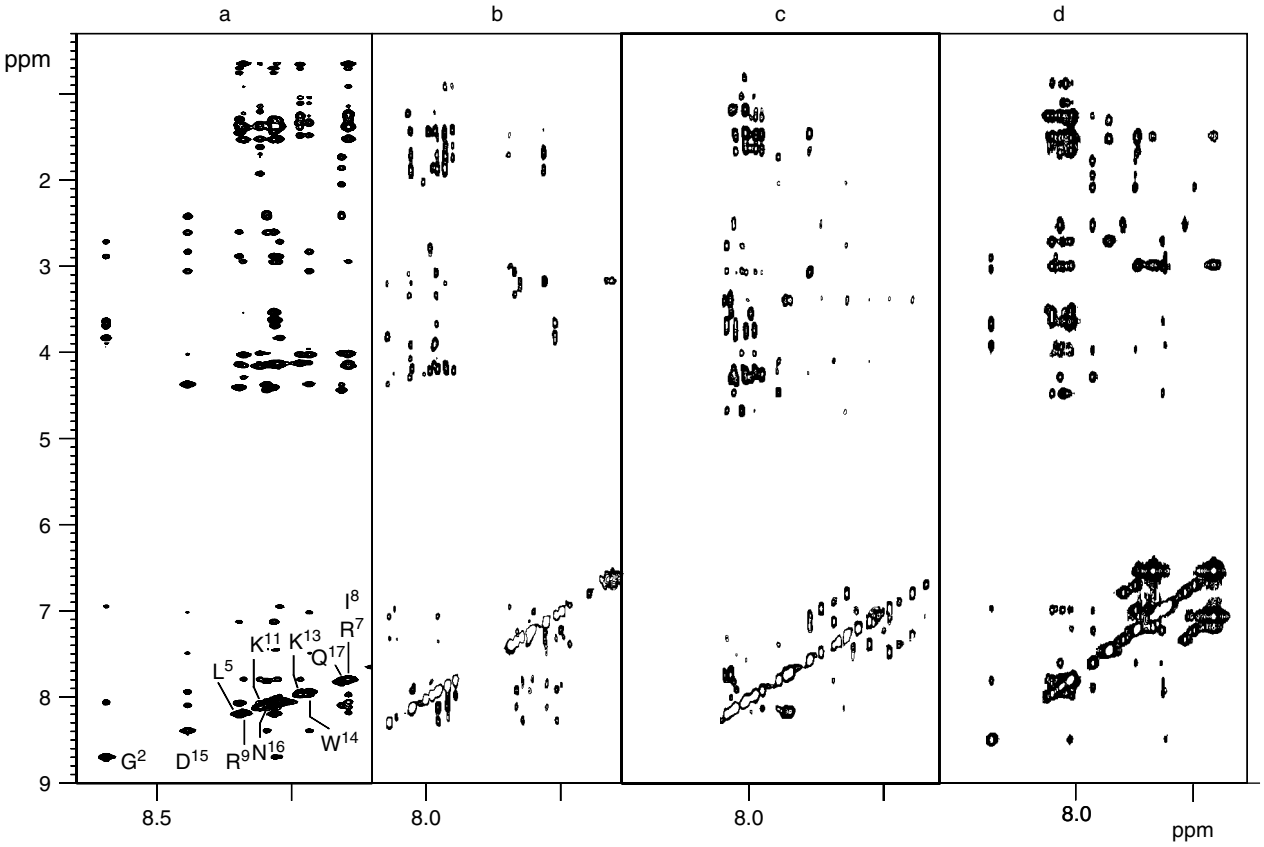

Figure 1 Comparison of the partial NOESY spectrum of (a) dynorphin A in the 80 : 20 (v/v) $DMSO_{d6}/H_2O$ cryoprotective mixture at 278 K with the spectra of nociceptin in (b) an aqueous solution of SDS micelles, (c) in neat DMSO and (d) in the 90 : 10 (v/v) $DMSO_{d6}/H_2O$ cryoprotective mixture at 278 K.

N-terminal part in the DMSO/water cryoprotective mixture are similar not only to those of DA, but also to those observed for Leu-enkephalin [28,50]. Thus, it was assumed that the relevant conformers consistent with the $d_{NN(i,i+1)}$ effects observed for the first four residues are similar to the single-bend and double-bend structures that characterize several enkephalin analogues in the crystal state [51].

The lack of opioid activity of NC could be linked either to the different constitution of the message domain, i.e. the substitution of Tyr^1 with Phe^1 [6], or to an increased flexibility with respect to DA. [Tyr^1]-NC regains an opioid activity comparable to that of enkephalin and shows good μ selectivity, but contrary to DA it lacks κ selectivity [14]. Both DA and [Tyr^1]-NC owe their opioid activity to the Tyr-Gly-Gly-Phe- message, but selectivity with respect to opioid receptor subtypes seems completely due to the *C*-terminal segment.

The main conformational differences between NC and DA seem confined to the address moiety. The conformation of the address domain of DA is dominated by the presence of Pro^{10} that separates two groups of basic residues [13] and reduces the flexibility of the whole *C*-terminal part, whereas the corresponding domain of NC does not contain relevant constitutional constraints. The pharmacological activity of [Pro^{11}]-NC in the MVD is characterized by a pEC_{50} of 5.67, nearly two orders of magnitude lower than that of NC [52]. That is, insertion of a Pro residue in a position corresponding to that of Pro^{10} in DA (FGGF-TGARKSPRKLANQ) that increases the rigidity of the address domain is not beneficial for ORL1 activity. Since [Tyr^1]-NC retains an ORL1 activity nearly identical to that of NC [6], we can infer that the ORL1 address does not have stringent conformational requirements or that its conformation is completely induced by the interaction with the receptor.

All attempts to change the activity of NC by systematic variation of the message, in a fashion similar to that employed successfully with opioids, have met

only a limited success [53]. Conventional conformational studies were likewise disappointing, mainly owing to the extreme flexibility of NC (see above). To circumvent these difficulties, we have tried a radically different approach, based on structurally relevant residues of NC(1–13)NH_2, a template that retains all activity of NC (R. Iscaro, thesis in Chemistry, University of Naples 'Federico II', 2001). The four essential residues of the message domain (Phe-Gly-Gly-Phe) are separated from the main residues of the address domain of NC(1–13)NH_2 (Arg-Lys-Ser-Ala-Arg-Lys) by three rather flexible residues, namely Thr-Gly-Ala. Substitution of these with Pro can tell us whether there are specific topological restrictions in the relative positions of the two domains. At the same time, the very presence of Pro residues in the flexible hinge should make structural studies in solution more amenable than those on NC or on NC(1–13)NH_2.

[Pro^6]-NC(1–13)NH_2 was prepared as described by Salvadori *et al.* [52], [Pro^5]-NC(1–13)NH_2 and [Pro^7]-NC(1–13)NH_2 were prepared and purified similarly to the analogues described by Guerrini *et al.* [54] for other NC analogues. Only [Pro^6]-NC(1–13)NH_2 has an appreciable activity, whereas both [Pro^5]-NC(1–13)NH_2 and [Pro^7]-NC(1–13)NH_2 are devoid of any activity. The pharmacological activity in the MVD of [Pro^6]-NC(1–13)NH_2 is characterized by a pEC_{50} of 5.9, nearly two orders of magnitude lower than that of NC(1–13)NH_2, but comparable to those of many NC(1–13)NH_2 analogues [52]. It is also in order to mention that, according to Lapalu *et al.* [55], internal to the sequences of both NC and DA, amino acids in positions 5 and 6 contribute significantly to receptor selectivity. The chimeric peptide DA(1–6)/NC(7–17) containing the Leu^5-Arg^6 sequence prefers the κ-opioid receptor, while chimera DA(1–4)/NC(5–17) containing the NC sequence Thr^5-Gly^6 prefers the ORL1 receptor. A hybrid chimera DA(1–5)/NC(6–17), containing the Leu^5-Gly^6 sequence, is unselective.

The biological data can be analysed in terms of structural data in solution. The peptides were studied by NMR in 90 : 10 (v/v) $DMSO_{d6}/H_2O$, the cryoprotective mixture corresponding to the best structuring conditions for NC and NC(1–13)NH_2 [14].

Figure 2 summarizes relevant backbone NOEs for NC(1–13)NH_2 and its three Pro analogues. It can be seen that all three analogues are more structured than NC(1–13)NH_2 and probably characterized by a β-turn centred on the Pro-Xaa bond. Starting from the β-turn types I and II [56–58], consistent with experimental data, it proved possible to build reliable models by MM and MD calculations. Figure 3 shows a schematic representation of the best models obtained for [Pro^6]-NC(1–13)NH_2 with unrestrained energy minimization, starting from initial conformations based on type I and type II

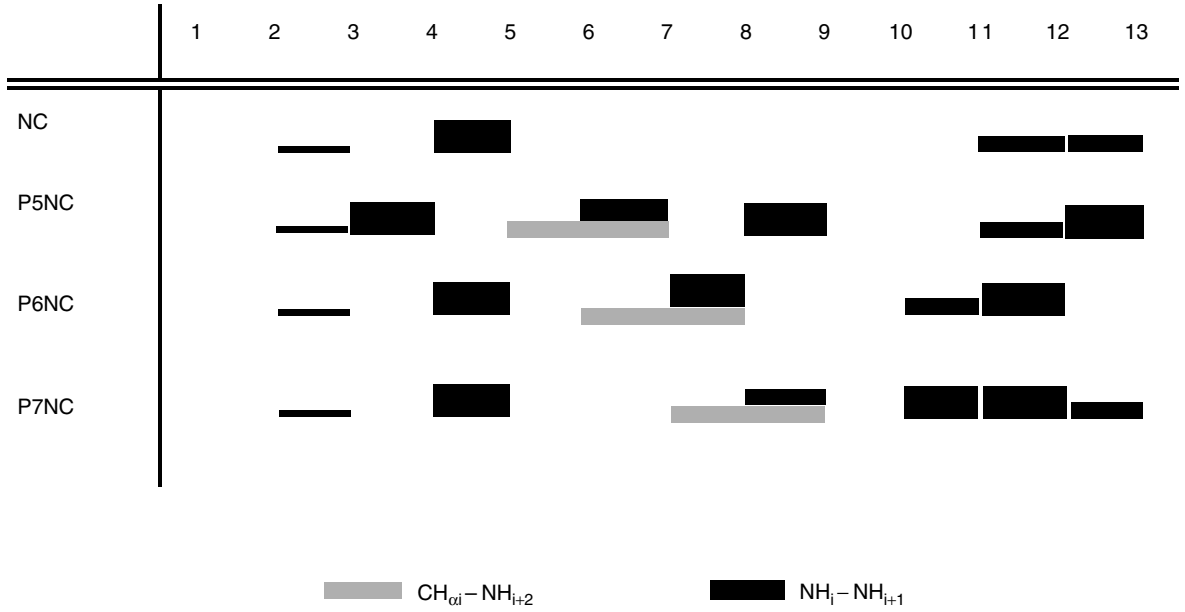

Figure 2 Comparison of relevant NH_i-NH_{i+1} and $CH\alpha_i$-NH_{i+2} NOE effects of NC(1–13)NH_2, [Pro^5]-NC(1–13)NH_2, [Pro^6]-NC(1–13)NH_2 and [Pro^7]-NC(1–13)NH_2 in the 90 : 10 (v/v) $DMSO_{d6}/H_2O$ cryoprotective mixture at 283 K.

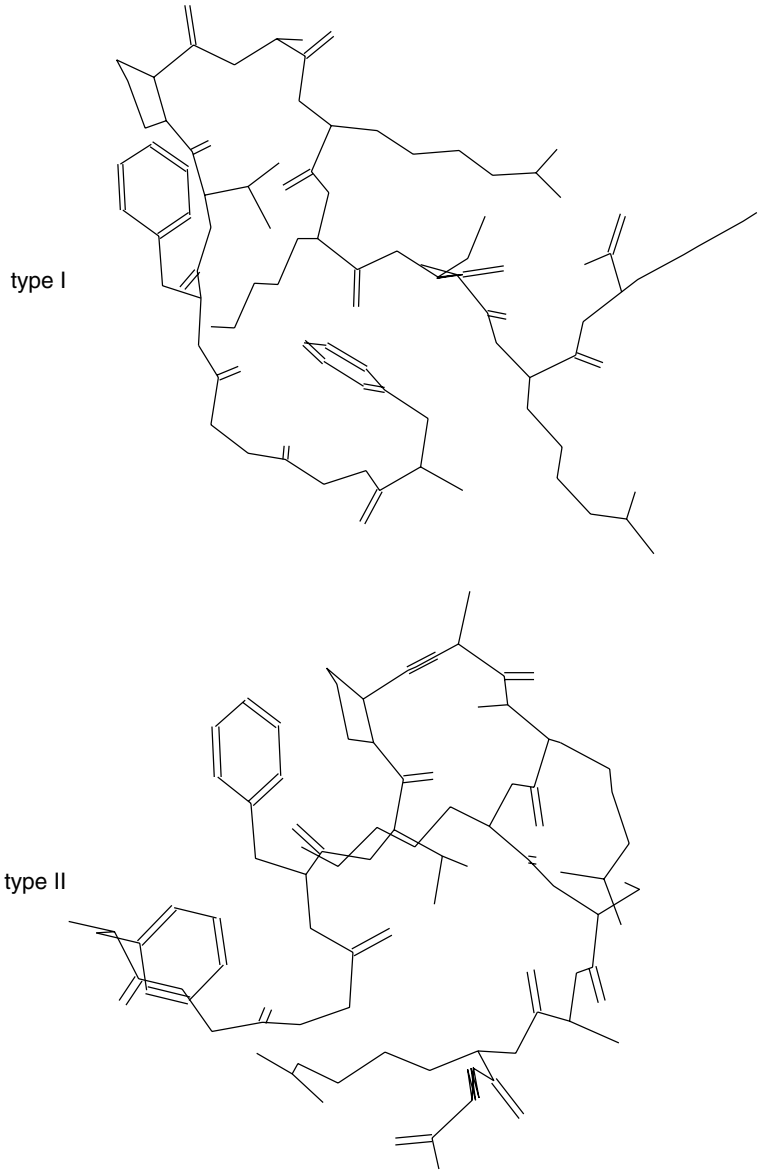

Figure 3 Schematic representation of the best 'type I' and 'type II' models obtained for [Pro^6]-NC(1–13)NH_2 with unrestrained energy minimization, starting from models in which only the Pro^6 and Ala^7 were initially restrained into canonical type I or type II β-turn conformations.

β-turns in which only the residues at the apex of the turn were initially restrained.

It is interesting to see that residues characterizing both β-turns retain a conformation close to the canonical one even after unrestrained minimization. Crucial residues are placed symmetrically with respect to the type I β-turn. All basic residues of the address domain are placed on the right-hand side of the turn, whereas the message domain is on the left-hand side. The side chains of the two aromatic residues that characterize the message, namely Phe^1 and Phe^4, are placed on opposite sides with respect to the average plane of the β-turn, respectively above and below the plane of the turn. In the type II model the side chains of both Phe^1 and Phe^4 are placed below the average plane of the β-turn and are arranged in an edge-to-face fashion. The experimental results seem to favour the latter type of β-turn, although the data are not sufficient for a detailed structural determination.

This result, albeit simple, tells us that only a β-turn centred on Gly^6-Ala^7 is consistent with

ORL1 activity, whereas β-turns centred on adjacent residues, i.e. Thr^5-Gly^6 or Ala^7-Arg^8, prevent any biological activity. It might be argued that the activity of [Pro^6]NC (1–13)NH_2 is substantially lower than that of the parent peptide, but the strong decrease in activity in going from NC (1–13)NH_2 to [Pro^6]NC (1–13)NH_2 can be explained by the conformational rigidity introduced by the incorporation of Pro^6 with respect to the flexibility of a *native* β-turn centred on Gly^6-Ala^7 residues.

ORPHANIN FQ2

A 'solvent scan' was only attempted in the case of OFQ2, owing to the poor solubility of this peptide in most of the media previously mentioned. OFQ2 is almost insoluble in water and DMSO and very little soluble in water/DMSO cryoprotective mixtures. At any rate, spectra of OFQ2 in cryomixtures are not of sufficient quality to yield structural information. On the contrary, OFQ2 is soluble in media that favour helical conformations. In these solvents it shows NOESY spectra consistent with an ordered structure. The best structuring conditions were found in mixtures of water with HFA, an alcohol with helix inducing properties even stronger than TFE [44].

Owing to the lack of tertiary structure, quantitative analysis of the sequential and medium-range NOEs was sufficient to yield a reliable solution structure of OFQ2. In this case a combination of NMR data with MM and MD calculation led to a detailed 3D-structure. Distance restraints calculated from intraresidue, sequential and medium-range NOEs, when introduced in SA calculations performed with both AMBER 5.0 [59,60] and DYANA [61] packages, show a helical structure ranging only from Met^5 to Ser^{16}. After EM refinement with the SANDER module of AMBER 5.0, a well-defined helical structure from Ser^2 to Ser^{16} could be observed. The finding of a shorter helix length and larger deviations from an ideal helix in the structures derived from DYANA SA calculations can be ascribed to the ability of the AMBER force field to reconstruct stretches of secondary structure even from partial NMR input.

Hydrophobic residues do not cluster according to any known motif (amphiphilic helix, Leu-zipper, coiled-coil) but are irregularly scattered along the helix surface, forming one larger and two smaller hydrophobic pockets. This distribution of hydrophobic residues in OFQ2, shown in Figure 4, could suggest close interaction of this peptide with a hydrophobic core or, at least, complementary hydrophobic regions in the intact precursor of the peptide.

The role of OFQ2 in the complex chain of events controlling pain is not established with certainty, but a comparison of its primary and secondary structure with those of other 'pain peptides' may shed some light on this issue. The likely message domain of OFQ2 (Phe-Ser-Glu-Phe) is reminiscent of that of NC (Phe-Gly-Gly-Phe) and, in part, also that of DA (Tyr-Gly-Gly-Phe). Indeed, aromatic residues in the *N*-terminal sequence of opioid and ORL1 peptides play a major role in receptor binding and receptor activation [4,38,54]. Otherwise, there is little sequence similarity, e.g. DA and NC owe their selectivity to the presence of several cationic residues in the address domain, whereas OFQ2 has but one cationic residue (Arg) in position 6. The potent analgesic activity of OFQ2 both spinally and supraspinally, not mediated by opioid receptors (μ, δ, κ and ORL1) hints that this new peptide could interact with different receptors or, according to Rossi *et al.* [12] it may activate downstream naloxone-sensitive opioid mechanisms.

Our structural data show a distribution of hydrophobic residues along the helix, consistent with the presence of an apolar environment *in vivo*, such as that of the lipid phase of membranes that hosts the transmembrane helices of ORL1 and opioid receptors. It is tempting to suggest that OFQ2 interacts with the transmembrane helices of a receptor of this kind in a fashion similar to that proposed for β-endorphin [45].

NOCISTATIN

NS, corresponding to the bPNP-3 sequence of pre-pro-nociceptin (TEPGLEEVGEIEQKQLQ), plays a role opposite to that of nociceptin in pain transmission but it does not interact with any known opioid receptor [11]. Its sequence hints to a structure–activity relationship different from that of nociceptin. Contrary to opioids and nociceptin, the message domain is not located in the *N*-terminal part: the shortest fragment, conserved in bovine, human and murine species that retains allodynia-blocking activity is the *C*-terminal hexapeptide (EQKQLQ) [11]. Knowledge of the conformational state in solution may shed light on the mechanism of action of nocistatin and on the characteristics of its receptor. The *C*-terminal octapeptide and the full heptadecapeptide were thus studied in a

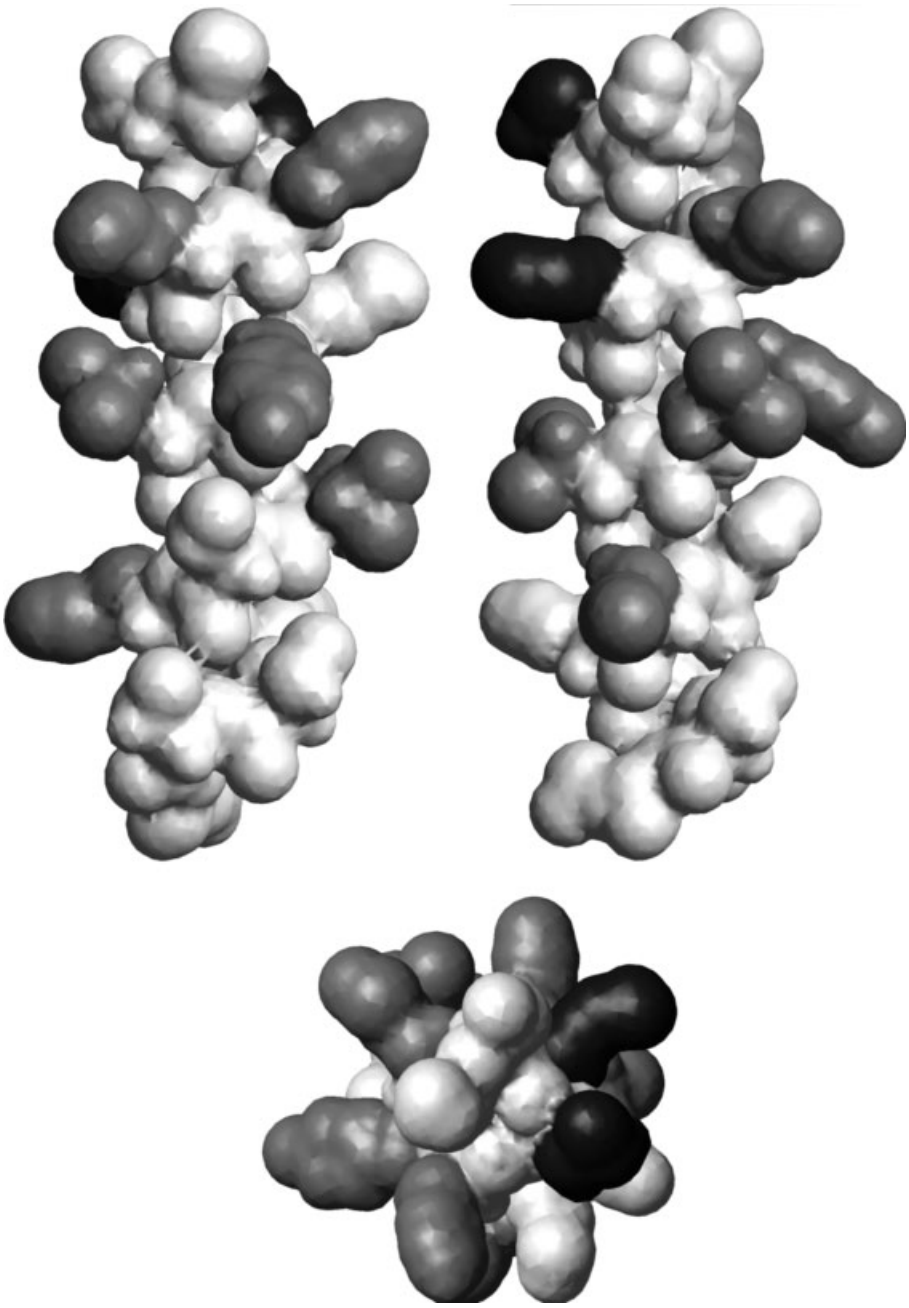

Figure 4 Different views of OFQ2. The van der Waals surfaces of atoms in side-chains of hydrophilic residues are represented in light grey, those of hydrophobic residues in dark grey and charged residues in black. The upper views are related by a 90° rotation around the helix axis, while the bottom view is along the helix axis. The models were generated by MOLMOL [62].

variety of solvent and temperature combinations to find the best structuring conditions: water in the temperature range 278–298 K, DMSO at 298 K, the cryomixture DMSO/water (80 : 20, v/v) in the temperature range 278–298 K, TFE/water (30 : 70, v/v) at 298 K and HFA/water (50 : 50, v/v) at 300 K.

NOESY spectra in water, neat DMSO and in the DMSO/water cryomixture show little ordering. The spectrum in the cryomixture does exhibit a tendency, albeit limited, of nocistatin to assume a helical conformation, but the number of diagnostic NOEs is insufficient to define a single structure.

On the other hand, all samples dissolved in media that favour helical conformations show rich NOESY spectra consistent with high helix content. As mentioned above, alcohols, either neat or mixed with water have been widely used to induce helicity in bioactive peptides [39–44]. We ran spectra of NS in mixtures of water with TFE and HFA. This last mixture behaves like TFE/water mixtures but with a much higher helix-inducing propensity [44]. The structuring effect of HFA/water does not overrule intrinsic residue tendencies, i.e. it does not induce helicity in sequences that have a natural propensity to be unordered. For instance, the *C*-terminal part of β-endorphin, another long-chain opioid, in HFA/water assumes a regular helical structure but the first 12 residues, comprising the five residues of enkephalin, remain completely unordered [45].

Figure 5 shows a comparison of partial 500 MHz NOESY spectra of NS and of its *C*-terminal octapeptide in HFA/water at 300 K.

The data in HFA/water show features typical of helical structures, e.g. the presence of a variety of NH-NH cross peaks and several 'medium range' NOEs for resonances of residues from Gly^4 to Leu^{16} that were used for a quantitative structure determination of the full peptide. The whole sequence from Gly^4 to Leu^{16} is a fairly regular α-helix. Small deviations from a canonical α-helical structure may originate from an insufficient number of constraints in the refinement procedure. It proved possible to

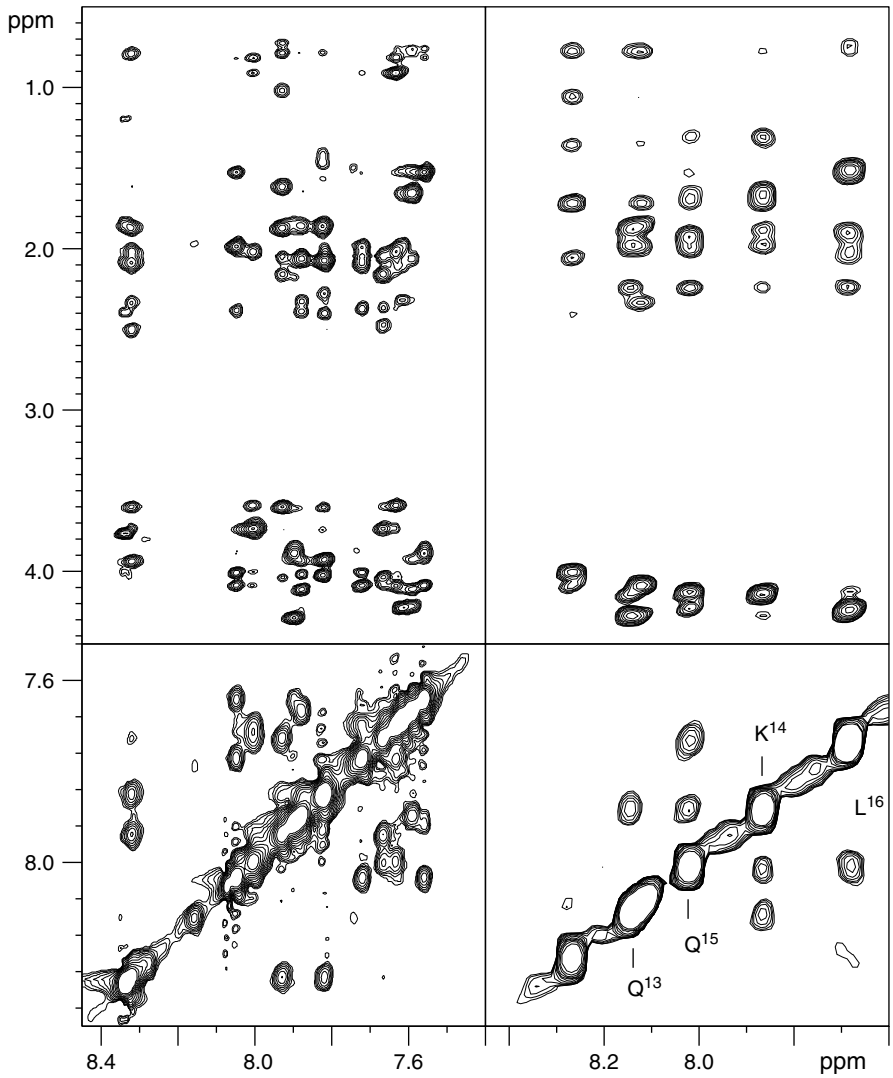

Figure 5 Comparison of partial 500 MHz NOESY spectra of nocistatin (left) and of its *C*-terminal octapeptide (right) recorded in 50 : 50 v/v HFA/water at 300 K. The labels in the spectrum of the octapeptide refer to the numbering of whole nocistatin.

add H-bonding constraints based on the observation of slowly exchanging amide hydrogens. In particular, the resonances of Val^8 and Ile^{11} showed half-lives of the order of up to 1 hour. Such a behaviour indicates a remarkable stability of the helix even in the absence of a tertiary structure. The introduction of restraints corresponding to only two intramolecular H-bonds (those involving residues Gly^4-Val^8 and Glu^7-Ile^{11}) in the DYANA TAD procedure improved the regularity of the helical parameters. Restrained MM and restrained MD procedures led to further improvements in the quality of molecular parameters. In the final refinement a standard simulated annealing protocol in Cartesian space was applied to obtain an ensemble of structures having no violation greater than 0.4 Å in distance from the input restraints.

The NMR data of the octapeptide (bPNP-3-8P) in the same solvent suggest a fairly high tendency, for such a short sequence, to assume a regular conformation. In particular, it was possible to detect strong sequential NH-NH NOEs in the central region from Gln^{13} to Leu^{16}. As one might expect, the other NMR parameters are not sufficient to perform a detailed structure computation, but it is possible to fit the NMR data in a model using the structure of the full peptide as template. According to Okuda-Ashitaka *et al.* [11], bovine NS binds to a membrane receptor of mouse brain and/or of spinal cord with high affinity but it is not related to known opioid peptides nor to other peptide hormones.

At any rate, although NS does not bind to an opioid receptor or to ORL_1, it does antagonize the action of NC by binding to a membrane receptor of the nervous system [11]. Accordingly, it is tempting to use the structural results of our study to hypothesize possible specific interactions with other receptors connected, albeit indirectly, with pain transmission but distinct from opioid and ORL_1 receptors. The *C*-terminal part of nocistatin is an unusually well-defined helix (for a short peptide) and, even more interesting from the point of view of drug design, the structure of the last eight residues in bPNP-3 is consistent with the conformation of bPNP-3-8P, indicating that even this segment has an intrinsic tendency to assume a similar conformation, at least when interacting with its receptor.

CONCLUSION

NC, NS and OFQ2 are peptide hormones related to pain and endowed of many additional biological properties. The solution studies described in this review article confirm that, like most linear peptides, they are too flexible to yield detailed structural information on the bioactive conformation. A direct structural study of the complexes of the peptides with their receptor would overcome this difficulty, but such a study is not easy since opioid receptors are membrane proteins. Thus, it was natural to resort to conformational studies in a series of solution media. A 'solvent scan' in several solvents, that mimic the different environments in which the peptides exert their action, showed that their behaviour can be clearly differentiated. NC shows little tendency to form well-defined structures in all solvents, whereas NS and OFQ2 form well-defined helices in mixtures of alcohols and water. Although none of the structural investigations yielded a bioactive conformation, the behaviour of its Pro^6 analogue indicates that the active conformation of NC is consistent with a type II β-turn centred on Gly^6-Ala^7. The global conformation of these peptides in biomimetic environments hints to possible modes of interaction with their receptors.

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