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Solution Structure of Dynorphin A (1–17): a NMR Study in a Cryoprotective Solvent Mixture at 278 K

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Abstract: Dynorphin A, the endogenous agonist for the κ opioid receptor, has been studied by NMR spectroscopy in methanol, acetonitrile, DMSO and in mixtures of hexafluoroacetone/water and DMSO/water. NMR data in the DMSO/water cryomixture at 278 K are consistent with a conformer in which the *N*-terminal part, like the corresponding message domain of enkephalins, is poorly ordered, whereas the *C*-terminal part is folded in a loop centred around Pro[10]. The folded structure of the *C*-terminal part (address moiety) may shed light on the role of the essential residues Arg[7], Lys[11] and Lys[13].

Keywords: dynorphin A; opioid peptides; NMR structure; cryoprotective mixture; linear peptides

INTRODUCTION

Dynorphin A [1], YGGFLRRIRPKLKWDNQ, is the endogenous opioid 17-residue peptide that interacts with the κ opioid receptor (KOR). The interest in dynorphin A and other peptides related to the prepordynorphin gene [2] is based on the hope that κ opioid agonists can be powerful analgesics without the clinically limiting side effects that characterize morphine and all the other μ opioids agonists. Such an interest has been recently rekindled by the discovery of new peptides encoded by the prepronociceptin gene [3] and by the finding that, although κ-selective agonists produce analgesia, activation of the κ-receptor antagonizes μ-receptor-mediated analgesia [4].

Previous conformational analyses based on NMR include studies of dynorphin A in an aqueous solution of DPC micelles [5,6], a study in water of a 17-residue analogue [7] and investigations on dynorphin-(1–13), both in aqueous solution [8] and in methanol/water [9]. Most of the media previously employed in the study of dynorphin [5–9] were chosen in the hope of detecting the so-called bioactive conformation; in particular Lancaster *et al.* [9] and Kallick [5] looked for solution conditions that could favour helical conformers, in order to test Schwyzer's hypothesis of membrane catalysed receptor selection [10]. According to this hypothesis the key factor to explain κ-selectivity of dynorphin is the formation of an α-helix from Tyr[1] to Arg[9] that favours the insertion of the *N*-terminal message domain of the peptide into the hydrophobic phase of the cell membrane [10]. Although the first qualita-

Abbreviations: DMSO, dimethylsulphoxide; DQF-COSY, double-quantum filtered correlation spectroscopy; DPC, dodecylphosphocholine; HFA, hexafluoracetone; KOR, κ opioid receptor; NMR, nuclear magnetic resonance; NOE, nuclear Overhauser effect; NOESY, nuclear Overhauser effect spectroscopy; TAD, torsion angle dynamics; TFA, trifluoroacetic acid; TFE, 2,2,2-trifluoroethanol; TM, transmembrane; TOCSY, total correlation spectroscopy; TPPI, time proportional phase incrementation; standard IUPAC single- and triple-letter codes for amino acids are used throughout.

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tive results in the DPC micelles solution seemed to speak in favour of Schwyzer's hypothesis, a recent more quantitative structural determination by Tessmer and Kallick [6] finds a slightly different helical segment (Gly^3-Pro^{10}). 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.

Recent studies on opiate and nociceptin receptors have shown that the second extracellular loop (e2) of these 7TM receptors plays a crucial role for selectivity [11–13]. Figure 1 shows the relationships among the e2 sequences of μ, κ and nociceptin [14] receptors. Replacing the sequence of the e2 human μ receptor with the corresponding sequence of the human κ opiate receptor generates a chimeric receptor with dramatically increased affinity for dynorphin-like ligands. Considering the high concentration of acidic residues in the e2 loop, it seems likely that it interacts with the basic address domain of dynorphin. This interpretation of κ selectivity, based on the specific properties of the address domain, is consistent with the fact that the (Tyr^1-Phe^5) message domain is the same for μ, δ and κ natural peptides [10] and has weak conformational preferences. These results speak against a major role of a direct interaction of an α-helix extending from Tyr^1 to Arg^9 with the membrane and call for a detailed investigation of the conformational preferences of dynorphin in different environments. Thus, it seems important also to look for conformational preferences of the address domain of dynorphin A (Arg^6 through Gln^{17}) in a hydrophilic environment, closer to the extracellular medium.

We have studied the conformational properties of dynorphin A in a wide range of solution conditions: 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).

MATERIALS AND METHODS

Materials

Dynorphin A was purchased from Bachem Inc. (Torrance, CA, USA). HFA trihydrate, perdeuterated DMSO, methanol and acetonitrile were obtained from Fluka AG. The purity was checked by HPLC and by NMR spectroscopy. Analytical HPLC analysis was performed on a Vydac C_{18} column (150×4.6 mm, 5 μm particle size) using a linear gradient of acetonitrile in 0.1% TFA.

NMR Measurements

NMR samples were prepared by dissolving appropriate amounts of peptide in 0.5 ml of solvent to make 2 mM solutions. NMR spectra were run on Bruker DRX-400, Bruker DRX-500 and Bruker DRX-800 spectrometers. TOCSY [15] and NOESY [16] experiments were run in the phase-sensitive mode using quadrature detection in ω_1 by TPPI [17].

Structure Calculation

The input data for the structure calculation with the program DYANA [18] were generated from the peak volumes obtained from NMRView (Johnson BA, Merck Research Laboratories, Rahway, USA). Based on the peak volumes observed on the NOESY spectra, the upper distance limits were generated with the program CALIBA [18]. Computations were performed on SGI O2 computers. In the final refinement step, these restraints were converted to the input data format used in the AMBER program [19,20] and used to perform restrained energy minimization. The AMBER calculation was also performed on SGI O2 computers.

The conformers corresponding to the best target functions of DYANA were subjected to restrained energy minimization using the AMBER 5.0 package. The 1991 version of the all-atom force field was

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human μ      .  TKYRQGSIDCTCTFSHPTWYWENLV...
human κ      .. TKVREDVDVIECSLQFPDDDYSWWDLFM..
human ORL1   ..GSAQVEDEEIECLVEIPTPQDYWGPVFA..
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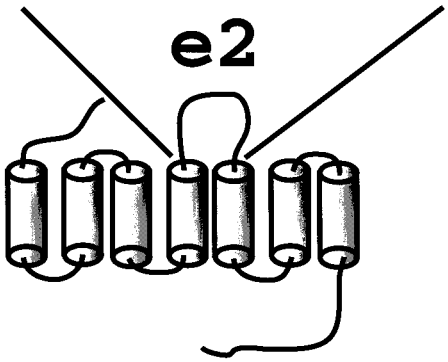

Figure 1 Comparison of the sequences of the second extracellular loop (e2) of μ, κ and nociceptin receptors.

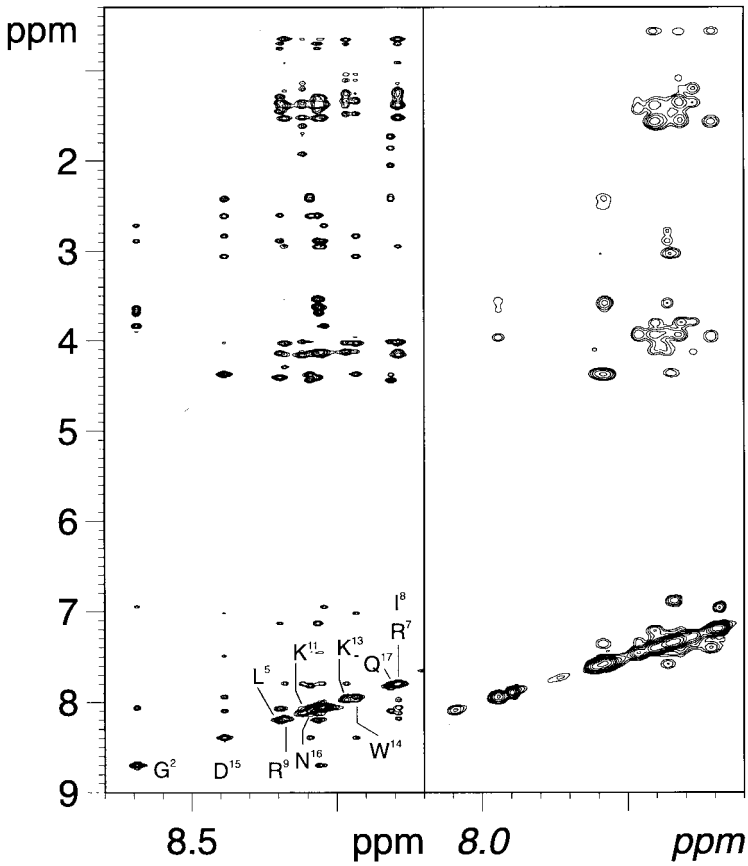

Figure 2 Comparison of the partial 400 MHz NOESY spectrum of dynorphin A in 50:50 HFA/water (v/v) at 298 K (right) and the corresponding one, at 800 MHz, in the 80:20 DMSO/water (v/v) cryomixture at 278 K (left).

used [19], with a distance-dependent dielectric constant $\varepsilon = r_{ij}$. In order to reduce the artefacts which can arise during *in vacuo* simulation, the charge of the ionizable groups was reduced to 20% of its full value. A distance cut-off of 12 Å was used in the evaluation of non-bonded interactions. Distance restraints were applied as a flat well with parabolic penalty within 0.5 Å outside the upper bound, and a linear function beyond 0.5 Å, using a force constant of 3.2 kcal mol^{-1} $Å^{-2}$.

RESULTS AND DISCUSSION

We have examined dynorphin A in a variety of solutions in order to test the intrinsic tendency of the dynorphin sequence to assume helical conformations and the possibility of structuring dynorphin in media that reproduce physicochemical conditions comparable to those of the interface between transport medium (extracellular aqueous solution) and membrane.

Alcohols, either neat or mixed with water are the most popular media used to induce helicity in peptides [21–25]. In addition to the quoted mixture of methanol and water employed by Lancaster *et al.* [9], we ran spectra of dynorphin in neat methanol and in an aqueous mixture of HFA. The last mixture has been recently shown to behave like TFE/water mixtures but with a much higher helix-inducing propensity at the same water/fluoroalcohol composition [26]. None of these mixtures was able to induce helical structures in dynorphin, albeit short.

In order to explore media compatible with the interface between extracellular aqueous solution and membrane we employed a variety of organic solvents, looking for values of the dielectric constant intermediate between that of water ($\varepsilon = 80$) and that of the lipid phase of the membrane (ε close to 1) and for other physicochemical features, e.g. a fairly high viscosity, that characterize cytoplasm. The neat solvents employed included, for instance, methanol, acetonitrile and DMSO characterized by values of the dielectric constant of 32.6, 37.5 and

45, respectively. Qualitative evaluation of the NMR data shows that dynorphin in these solvents retains a conformational flexibility comparable to that observed in water [8].

A decisive improvement was obtained by the use of a mixture of DMSO and water, a solvent medium that among several positive features, has a viscosity close to that of the intersynaptic fluid. Typical viscosities of cytoplasm range from 5 to 30 cp [27] and it has been postulated [28] that they play an important role in cell communication processes. Viscosities of the order of those of cytoplasm can be easily reproduced by cryoprotective mixtures [29–31]. In addition, the use of a viscous solvent medium can affect the equilibrium among isoenergetic conformers, selecting the more ordered conformers [32].

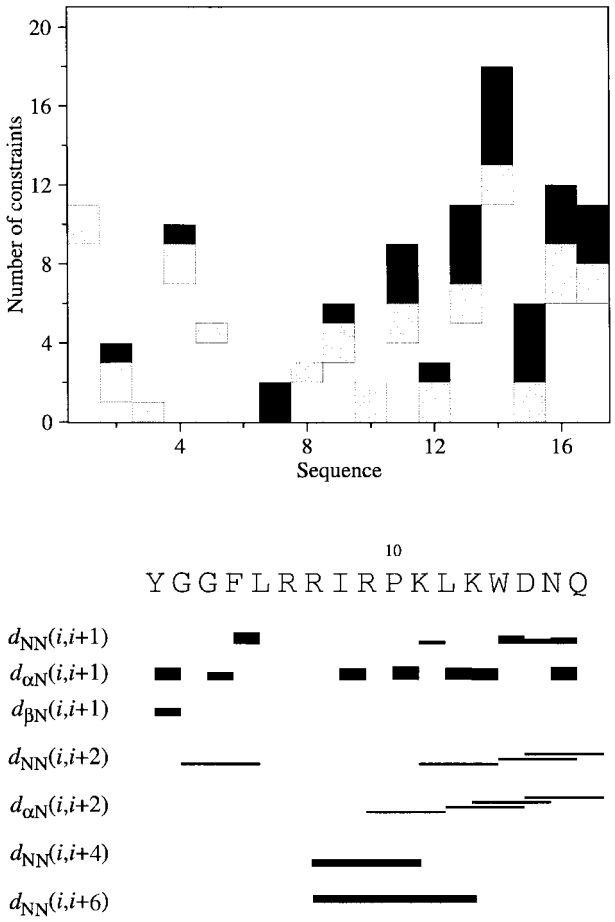

Figure 3 (a) Number of NOEs versus residue number for dynorphin A in the 80:20 DMSO/water (v/v) cryomixture at 278 K. Intraresidue effects are shown as white bars, sequential as gray bars and medium-range as black bars. (b) Bar diagram that summarizes diagnostic NOEs of dynorphin A in the 80:20 DMSO/water (v/v) cryomixture at 278 K.

2D NMR spectra of dynorphin A in the cryoprotective mixture DMSO/water (80:20; v/v) were run in the temperature range 305 to 268 K. At 278 K viscosity induces a sizeable increase in the number and intensity of NOEs with respect to room temperature, without excessive line broadening. Figure 2 shows a comparison of the partial NOESY spectra of dynorphin A in the DMSO/water cryomixture at 278 K and the corresponding one in 50:50 HFA/water (v/v) at 298 K. It is interesting to note that the number of cross peaks, even those typical of helical structures, is much smaller in the strongly helicogenic HFA/water mixture. In fact, even sequential NH–NH effects are virtually absent in the latter medium. The number of cross peaks in the cryomixture at 278 K is essentially the same at 400, 500 and 800 MHz, but it proved necessary to resort to the high field experiments to resolve a few difficult assignments that would otherwise require a very costly labelling procedure. Variable temperature NOESY experiments at 500 MHz proved also useful to untangle the mentioned difficult assignments. Sequential assignment was performed at 500 and 800 MHz by standard methods [33].

Qualitative analysis of the NOEs shows that those of the *N*-terminal part are similar to the ones previously observed for Leu-enkephalin in the same solvent system [34,35]. That is, the *N*-terminal part of the peptide lacks medium range NOEs but is characterized by a series of sequential NH–NH effects consistent with a mixture of poorly structured conformers. On the other hand, from Arg^{7} to Gln^{17} it was possible to observe several diagnostic NOEs suggesting the presence of structured conformers (Figure 3). Particularly, the two long range connectivities between the NH of Arg^{7} and those of Lys^{11} and Lys^{13} suggest the presence of a loop centred around Pro^{10} and involving most of the *C*-terminal sequence. It is interesting to note that the absence of any splitting of resonances of residues adjacent to Pro^{10} hints that the possible *cis*/*trans* isomerism around Arg^{9}-Pro^{10} is totally shifted towards the trans form. The relevant assignment was based on the observation of Arg^{9}-$C_{\alpha}H$/Pro^{10}-$C_{\delta,\delta'}H$ cross peaks and the concomitant absence of the (*cis*) Arg^{9}-$C_{\alpha}H$/Pro^{10}-$C_{\alpha}H$ cross peak. Figure 3(b) shows the bar diagram that summarizes diagnostic NOEs. Judging from the presence of substantial $C_{\alpha}H_{i}$-NH_{i+1} effects, typical of extended structures, it is not possible to exclude the coexistence of extended and folded conformers. Nonetheless, considering the very high number of observed NOEs we did try to derive a single structure from a combination of

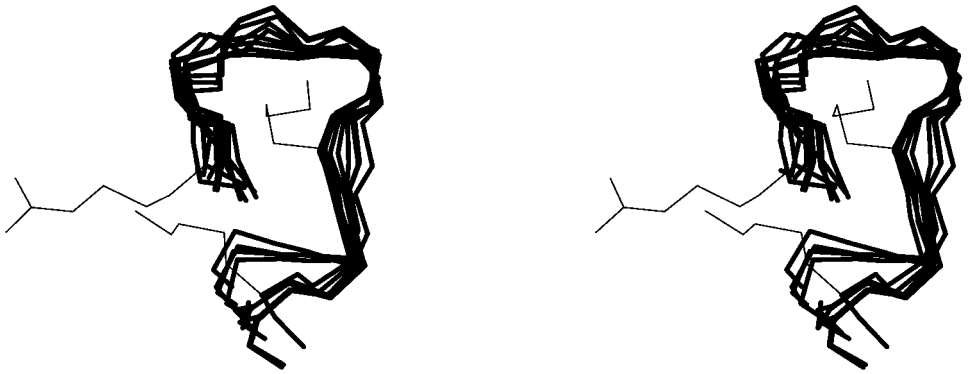

Figure 4 NMR-derived solution structures of dynorphin A. Superposition of the backbone N, C_a and C′ atoms of the segment from Arg^7 to Lys^{13} of the ten final structures calculated by DYANA. The side chains of three crucial basic residues, Arg^7, Lys^{11} and Lys^{13} in the first structure are shown as thinner lines.

torsion angle dynamics (TAD) and restrained energy minimization.

Introduction of restraints derived from intra-residue, sequential and medium range NOEs in DYANA [18] generated ten structures of dynorphin A with good values of the usual target function [18] out of 50 random generated initial conformers. All ten structures have similar values of the backbone torsion angles for the *C*-terminal part but diverge in the *N*-terminal region (message domain) even if there is some tendency to cluster around the conformers found in the crystal state for enkephalin [36]. Accordingly, it proved difficult to identify an average structure for the whole sequence.

The central part is fairly ordered as anticipated in a comparison with nociceptin [37], although it does not correspond to a recognizable, canonical structure, i.e. it is neither a helix nor a β-sheet. It can be described as a large loop that has Pro^{10} at its apex. The RMSD value for the backbone atoms of the loop, extending from Arg^7 to Lys^{13}, is 0.81 Å for the ten structures with the best values of the target function. In order to improve the quality of the structure determination we resorted to restrained energy minimization of the ten best structures generated by DYANA. This procedure led to improvements in the quality of molecular parameters, as judged from deviations from idealized covalent geometry and from canonical ϕ, ψ pairs of the torsion angles. Figure 4 shows the segment from Arg^7 to Lys^{13} of the 10 best structures. The side chains of three crucial basic residues, Arg^7, Lys^{11} and Lys^{13} (*vide infra*) in the structure with the lowest target function are shown as thin lines. There is not a sufficient number of NOEs to define a precise average orientation of the side chains, not even for residues of the common, ordered segment (from Arg^7 to Lys^{13}), but this representation emphasizes the fact that the central loop allows a similar orientation of the side chains of these residues.

The final structure is consistent with all observed NOEs and also with two prominent absent NOEs between the NHs of Lys^{11} and Leu^{12} and of Leu^{12} and Lys^{13}. It is interesting to note that the same solution conditions employed to observe this structure of dynorphin did not yield a single folded conformer in nociceptin, a circumstance that emphasizes the importance of a precise address message in these peptides. Indeed, the *C*-terminal part of nociceptin, although rich in basic residues like the address message of dynorphin, lacks Pro and has a different distribution of the basic residues [37].

The fact that no helical structure was observed in isotropic media, even those that are known to induce helicity very strongly [21–25], and the concomitant detection of a new structure in an isotropic hydrophilic medium of high dielectric constant and high viscosity hints that this structure may be the basis for a new model of interaction of dynorphin A with the e2 extracellular loop of KOR [11,12]. In particular, it is interesting to note the position of three crucial basic residues, Arg^7, Lys^{11} and Lys^{13} in the final structure. These residues have been identified long ago [38] as essential residues for the selectivity of dynorphin. It can be seen (Figure 4) that the side chains of these residues are exposed on the same side with respect to the average plane of the central part of the structure and may interact easily with acidic residues of the e2 loop of KOR. On the basis of the structure of Figure 4, such an interaction does not require a

specific (e.g. helical) predetermined conformation of the first half of the sequence of the e2 loop, as hypothesized by Paterlini *et al.* [39]. On the contrary, it is reasonable to suggest that the structure of dynorphin might induce a specific structural organization in an otherwise unstructured e2 loop, also including the second half of e2 that contains another 'patch' of acidic residues.

CONCLUSION

A recent model for the mechanism of action of dynorphin A on the κ opioid receptor depicts the crucial interaction as the packing of two helices, that found for a small segment of dynorphin in DPC micelles [6] and that hypothesized for the e2 loop of the receptor [39]. Our conformational study, based on a 'solvent scan', indicates that 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 a (80:20) DMSO/water (v/v) cryomixture at 278 K. The prevailing structure in this medium can be described as a large loop, extending from Arg^{7} to Lys^{13}, that has Pro^{10} at its apex. A structure like ours may favour an optimal interaction of three essential basic residues of dynorphin, Arg^{7}, Lys^{11} and Lys^{13}, with acidic residues of the e2 loop of the κ receptor and induce a mutual conformational rearrangement. It is interesting to note that the essential role of the three residues we find on the exposed surface of our structure, Arg^{7}, Lys^{11} and Lys^{13}, is consistent with the fact that dynorphin-(1–13) retains most of the κ activity of the parent peptide [1].

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