NMR studies of flexible peptides in cavities mimicking the synaptic cleft

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Abstract The interaction of neuropeptides with post-synaptic receptors is characterised by a high entropic barrier originating from the combination of nanomolar concentration with low conformer population. The influence of high viscosity environments on conformer distribution can help overcome this difficulty. In an attempt to simulate the physicochemical conditions of the synaptic cleft, ^{15}N-labelled enkephalin has been studied in polyacrylamide gels swollen by different aqueous solutions in the temperature range 273–293 K. Nuclear Overhauser enhancement spectra in the gel pores are consistent with a conformational selection or a slowing down of internal motions that can favour the interaction of the peptide with the receptor.

Key words: Acrylamide gel; Opioid peptide; Viscosity; Synapsis

1. Introduction

Signal transmission in the nervous system is modulated, at synapses, by small molecular weight compounds, including a large variety of neuropeptides that, binding to receptors, regulate ion channels indirectly. Neuropeptides interact with their post-synaptic receptors with binding constants in the order of nanomoles. Owing to the high flexibility of small linear peptides, the probability of finding a peptide agonist in solution with the proper conformation for interaction with the receptor, i.e. the so-called 'bioactive conformation', is very low. Even taking into account the possibility of induced fit or equivalent mechanisms, the combination of absolute concentration with low conformer population leads to a high entropic barrier in the peptide–receptor interaction [1]. Overcoming this difficulty amounts to hypothesising mechanisms of conformation pre-selection or conformation interconversion.

We have previously proposed that media of high viscosity, such as some mixtures of water with organic solvents called cryoprotective mixtures, can indeed act as a conformational sieve, favouring compact, folded conformations over extended, disordered ones [2]. The main difficulties with a mechanism of conformation pre-selection based on viscosity are whether the neuropeptides are constantly in a high viscosity environment prior to receptor interaction and whether viscosity can induce a conformation pre-selection. A specific difficulty of simulations, on the other hand, is the possible influence of solvation effects from cosolvents used to increase viscosity [2,3]. Neuropeptides, after being generated in vesicles of the pre-synapsis (a cytoplasmatic environment of high viscosity), must cross the synaptic cleft: the fluid of the synaptic cleft is that of an extracellular environment, nominally of lower viscosity with respect to the cytoplasm. The synaptic cleft, however, is not an open space; it is a very narrow environment that separates the pre-synapsis from the post-synapsis (the cell hosting the receptor). Fig. 1 shows a schematic representation of the synaptic cleft. The viscosity of the fluid trapped in the synaptic cleft can be much higher than the nominal one corresponding to a given composition in bulk, owing to unstirred layer phenomena [4] favoured by the dimension of the cleft, ranging from 10 to 30 nm, but also to the presence, on the surface of the two membranes, of complex carbohydrates and proteins that reduce even further the dimension of the cleft and modify the properties of the fluid.

The second question, i.e. whether viscosity can induce a conformation pre-selection, is answered by the observation that, in aqueous solutions, owing to the numerous hydrogen bonds between the solute and water molecules, the volume displaced by compact conformers is much smaller than the corresponding volume displaced by extended conformers. The smaller volume displaced corresponds to an energy gain [5], whereas the loss of energy due to the breaking of hydrogen bonds with the solvent is compensated, in folded peptide conformers, by formation of an equivalent number of intramolecular hydrogen bonds. Besides, it has already been shown that viscosity can influence both the stability of protein conformations and the folding mechanism of proteins [6].

A reasonable mimic of the cleft, at least from a geometrical point of view, is furnished by the pores of polyacrylamide gels that have recently been proposed, as an alternative to bicelles, to measure residual dipolar couplings in proteins [7,8]. The average dimension of the pores of the polyacrylamide gel, when using acrylamide concentrations in the range 10–15% w/v, should correspond to pore diameters of 12–8 nm [9], the right order of magnitude with respect to the dimension of the cleft. A moderate increase of viscosity, induced solely by the dimensions of gel pores, has been observed in nuclear magnetic resonance (NMR) spectra of ubiquitin and human immunodeficiency virus-1 Nef [7]. We expect that the apparent increase of viscosity for a small flexible peptide, owing to the interplay of extended conformers with the solvent, can be even higher and may affect the composition of conformational

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Abbreviations: MNEI, single chain monellin; DMSO, dimethylsulfoxide; NOESY, nuclear Overhauser spectroscopy

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mixtures and/or the kinetics of interconversion among conformers. The neuropeptide chosen for our study is [Leu]enkephalin[1] (YGGFL), both for its symbolic significance, i.e. it is the prototype opioid, the first endogenous opioid to be identified, and for its extreme flexibility.

2. Materials and methods

^{15}N-Labeled [Leu]enkephalin was prepared by standard solid phase methods as described in [10]. ^{15}N-Labeled single chain monellin (MNEI) was expressed in *Escherichia coli* as described in [11].

The gels were prepared by polymerising an acrylamide solution of the appropriate concentration in (previously sililated) glass tubes of diameter approximately 0.5 mm smaller than the inner diameter of 5 mm NMR tubes, as described in [7]. After preliminary trials we used two slightly different gels for the experiments on a small protein (10% w/v) and for those on the neuropeptide (15% w/v).

NMR measurements on ^{15}N-labelled MNEI samples were performed at pH 2.9 and 308 K, using a 2 mM solution of the protein in 90% H_2O/10% ^2H_2O with 18.5 mM potassium phosphate buffer. Samples of enkephalin for NMR measurements were prepared by dissolving ^{15}N-labelled [Leu]enkephalin in an aqueous solution containing 20 mM Na phosphate, pH 5.4, and eventually adding the appropriate amount of dimethylsulfoxide (DMSO) or glycerol to reach a final concentration of ^{15}N-labelled enkephalin of 1.1 mM in the stock solution.

^{1}H-NMR spectra were run at 500 MHz on a Bruker DRX-500 spectrometer equipped with XYZ pulsed field gradients ^{1}H/^{15}N probehead. Two-dimensional heteronuclear single quantum coherence experiments were recorded without ^{1}H decoupling and both in bulk and in the presence of the gel, using the IPAP sequence [12]. ^{15}N-Edited nuclear Overhauser spectroscopy (NOESY) were run as described in [13].

3. Results

A straightforward way to check whether the cavities are of the right dimension is to measure residual dipolar couplings on a protein of small molecular weight [7,8]. Checking experiments were performed on uniformly ^{15}N-labelled samples of MNEI (96 residues) whose solution structure has been recently solved [11], using the same solution conditions described in that paper. Typical residual couplings [12] ranging from −18 to +16 Hz were measured along the MNEI sequence, confirming that the pores are of the right dimensions.

The neuropeptide chosen for our study, [Leu]enkephalin, is so flexible that, from solution studies, it proved impossible to find not only the 'bioactive conformation' but even reliable indications on plausible representative conformations [14]. Accordingly, it is a clear candidate for a mechanism that helps to overcome the entropic barrier. Besides, thanks to its very flexibility, it is a sensitive indicator of changes in conformational equilibria. In order to further enhance the sensitivity of the detection of conformational effects, exploratory experiments were performed in cryoprotective mixtures whose viscosity, in the temperature range 273–293 K, can be easily adjusted just below threshold values that favour conformational selection. Cryoprotective mixtures are simple mixtures of water and organic solvents such as alcohols, DMSO, dimethylformamide, etc., that are fully biocompatible according to biochemical and crystallographic studies on proteins [15]. They can be regarded as an 'in vitro version' of osmolytes,

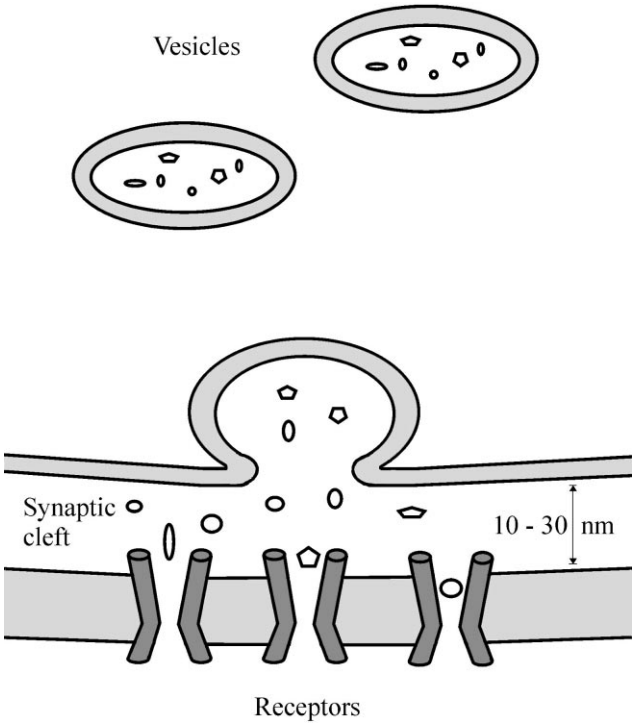

Fig. 1. Schematic representation of the synapsis showing the presynaptic vesicles, the synaptic cleft and the post-synaptic receptors.

protective solutions found in many organisms living in extreme conditions [16,17]. Typical threshold values found for deltorphin I, a potent δ selective opioid heptapeptide, are in the range 2–3 cp [2,3]. A mixture of DMSO and water (20:80, v:v, corresponding to a DMSO mole fraction of 0.050) has viscosities of 1.5–2.8 cp in the temperature range 293–273 K. The same viscosity, 1.5 cp at 293 K, is obtained by mixing glycerol, a normal component of the membrane heads and well-known osmolyte [18], with water to get a glycerol mole fraction of 0.036.

^{15}N-Edited NOESY [13] spectra of ^{15}N-labelled [Leu]enkephalin in the mixture of water and DMSO, show an outstanding difference between bulk solvent and gel phase, in the temperature range 273 to 293 K. Cross peaks are absent in the spectrum in bulk solvent at a temperature of 293 K and very few appear at 273 K whereas the spectrum in gel at 293 K has the same number of visible cross peaks of the spectrum in bulk at 273 K, i.e. the actual viscosity of the gel phase should be close to 2.8 cp. This observation allows an estimation of the increase in viscosity due to the confinement in a small cavity: it is of the order of 100%. The trend in the mixture of water and glycerol is similar.

When using cryomixtures it is not easy to discriminate between solvation effects and the influence of viscosity, but if the main cause of the changes observed with the two cryomixtures is the confinement in the gel pores, it may be possible to investigate the behaviour in pure water. NOESY spectra of enkephalin in bulk water, in the temperature range 273–293 K (Fig. 2a), show practically no cross peaks, as expected from literature data [14], whereas the spectrum in aqueous solution inside the gel is similar to the corresponding one in the cryomixture (Fig. 2b). The absence of NOEs in bulk water can be attributed to a rotational correlation time close to the zero crossing point (350 ps at 500 MHz), to an unfavourable com-

[1] Standard IUPAC single- and triple-letter codes for amino acids are used throughout.

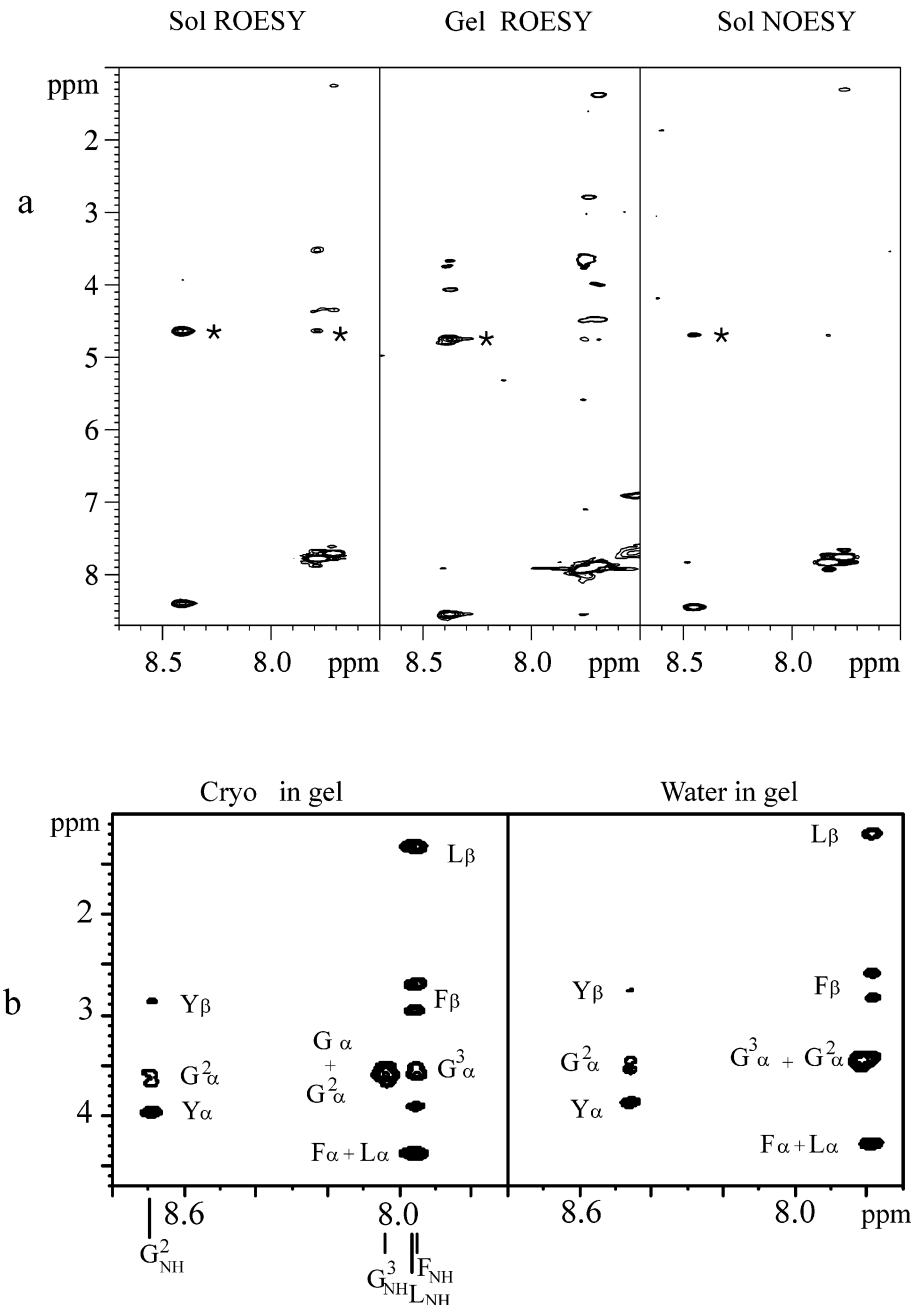

Fig. 2. a: Comparison of partial ^{15}N-edited ROESY spectra of ^{15}N-labelled [Leu]enkephalin in bulk water (left) with the corresponding spectrum in water hosted by gel pores (centre) and with the ^{15}N-edited NOESY spectrum in bulk water (right). All spectra are recorded at 283 K. Mixing times were 80 ms for the ROESY experiments and 200 ms for the NOESY experiment, typical acquisition time 12 h. Starred cross peaks originate from chemical exchange. b: Comparison of the expanded regions of the ^{15}N-edited NOESY spectra of ^{15}N-labelled [Leu]enkephalin in the gel pores using DMSO/water cryomixture (20/80 v/v) (left) and water (right) solutions showing also the cross peak assignments. The temperature of both spectra is 273 K. Typical acquisition time 3 h. Concentration of ^{15}N-labelled enkephalin in 20 mM Na phosphate, pH 5.4, was approximately 1 mM in all cases. All spectra were recorded on a Bruker DRX-500 spectrometer equipped with XYZ pulsed field gradients ^{1}H/^{15}N probehead.

bination of tumbling and internal motions and/or the presence of a complex mixture of extended conformers in equilibrium.

A way to discriminate between the effect of tumbling and other causes is to resort to a ROESY experiment that is essentially insensitive to the value of the rotational correlation time. We have performed several ROESY experiments (at 283 K) in gel with different mixing times, 30, 80, 120, 200 and 400 ms, and much longer acquisition times. Although all spectra are less rich in cross peaks than the corresponding NOESY spectra, the ROESY spectra at 80 ms are a good compromise between $T_1\rho$ decay and global decrease in intensity connected to high viscosity. The ROESY spectra proved indeed diagnostic to discriminate between the influence on rotational correlation time τ_c (influencing NOESY experiments) and the decrease of segmental mobility induced by viscosity. Cross peaks in gel are definitely more intense than the corresponding peaks in bulk solvent, showing that viscosity has an influence distinct from simple increase of rotational correlation time τ_c.

Fig. 2a shows the comparison of partial ^{15}N-edited ROESY spectra of ^{15}N-labelled [Leu]enkephalin in bulk water (left) with the corresponding spectrum in water hosted by gel pores (centre) and with the ^{15}N-edited NOESY spectrum in bulk water (right). The intensity of cross peaks in gel, measured with respect to diagonal peaks, is always greater than that of the corresponding peaks in bulk water, the increase ranging from 20% to over 100%.

The increase in the intensities of NOE crosspeaks is not, per se, an indication of an induction of structure. It can also be due to the increase in rotational correlation time τ_c induced by the increase in viscosity, to the change of τ_e, the angular component of the correlation time related to internal motions, to the increase of the order parameter S^2, or to a combination of these factors. It is fair to say however, that the side chains of [Leu]enkephalin appear much stiffer in gel than in bulk water.

The NH resonances are not spread over a sizeable range, as expected for aqueous solutions of enkephalin, reflecting the disordered nature of most conformers present in the equilibrium mixture but it is easy to assign cross peaks on the basis of previous literature data [10]. Fig. 2b shows the comparison of the gel phase ^{15}N-edited NOESY spectra for the water/DMSO ($x = 0.050$) and neat water solutions of ^{15}N-labelled [Leu]enkephalin at 273 K. All cross peaks labelled in Fig. 2b are intraresidue or sequential, with little diagnostic value for structure determination, but their number and intensity (both very similar and in cryomixture and in water) are sufficient to show the powerful influence of viscosity on the composition of the mixture of conformers in equilibrium.

We can conclude by saying that: (i) it is possible to run NMR spectra in a confined environment mimicking the synaptic cleft, (ii) the mere confinement is sufficient to induce a change in viscosity of the aqueous solution without changing the composition *and* the temperature of the solvent and (iii) NOESY spectra hint either a conformational selection or a slowing down of internal motions: both phenomena can favour the interaction of the peptide with the receptor.

The finding that the very geometry of the site is sufficient to induce a substantial increase in viscosity and the ensuing ordering effect on flexible neurotransmitters shows the importance of performing structural studies in environments that mimic the natural ones as closely as possible. The main differences between the actual biological system (the cleft) and our mimic (gel pores) rest in the composition of the intersynaptic fluid and in the nature of the walls. In the real system the walls are membrane surfaces, i.e. charged heads of membrane lipids, possibly functionalised with carbohydrates, and membrane proteins. These surface components reduce the accessible volume, i.e. they change the actual short dimension of the cleft, and can increase viscosity even further thanks to their chemical constitution. Functionalisation of acrylamide to match these requirements is currently under way in our laboratory.

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