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Peptides and proteins in a confined environment: NMR spectra at natural isotopic abundance

ANNALISA PASTORE,[a] SEVERO SALVADORI[b] and PIERO ANDREA TEMUSSI[a,c]*

[a] National Institute for Medical Research, Medical Research Council, London, UK
[b] Department of Pharmaceutical Sciences, University of Ferrara, Italy
[c] Department of Chemistry, Università di Napoli Federico II, via Cinthia, 80126 Napoli, Italy

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Abstract: Confinement of proteins and peptides in a small inert space mimics the natural environment of the cell, allowing structural studies in conditions that stabilize folded conformations. We have previously shown that confinement in polyacrylamide gels (PAGs) is sufficient to induce a change in the viscosity of the aqueous solution without changing the composition and temperature of the solvent. The main limitation of a PAG to run NMR experiments in a confined environment is the need for labelling the peptides. Here we report the use of the agarose gel to run the NMR spectra of proteins and peptides. We show that agarose gels are completely transparent in NMR experiments, relieving the need for labelling. Although it is necessary to expose biomolecules to fairly high temperatures during sample preparation, we believe that this is not generally an obstacle to the study of peptides, and found that the method is also compatible with temperature-resistant proteins. The mesh of agarose gels is too wide for direct effects of confinement on the stability of proteins but confinement can be easily exploited to interact the proteins with other reagents, including crowding macromolecules that can eventually lead to fold stabilization. The use of these gels is ideally suited for low-temperature studies; we show that a very flexible peptide at subzero temperatures is stabilized into a well-folded conformation. Copyright © 2007 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: NMR; confinement; agarose gel; lysozyme; low temperature

INTRODUCTION

Structural studies of biological molecules are generally performed in dilute solutions in which all interactions are diffusion-limited. On the contrary, solutions found in living systems contain very high concentrations of macromolecules that can influence interactions [1]. In the cytoplasm of *E. coli*, mixtures of macromolecules can occupy as much as 30–40% of the available volume [2]. Accordingly, such media are referred to as 'crowded' [3] and/or 'confining' [4].

Crowding and confinement may change some physicochemical properties of the environment, particularly local viscosity. These changes are not only important in cells whose dimensions (10–100 μm) usually correspond to a volume of the order of 10^{-13}–10^{-10} l, but may have even more dramatic consequences for organelles (10^{-20} l) or for the synaptic cleft whose linear dimension is of the order of 10–30 nm. The influence of viscosity on protein stability is well known [5–7] and was explicitly introduced by Karplus and Weaver [8] in a diffusion-collision model of the folding mechanism. It is very difficult to verify experimentally whether stability and/or the rate of folding should depend on medium viscosity since the co-solvents that are added to water to increase its viscosity may alter also the stability of the folded conformation by direct solvation [5,9]. All experimental studies on the influence of viscosity have made use of viscogenic additives, generally polyols, such as ethylene glycol, glycerol or carbohydrates [6,] and the concomitant effects of the viscogenic agents on solvent viscosity and protein stability are difficult to separate. Another important problem is furnished by the conformation–activity relationship of bioactive peptide hormones. Since most of these hormones are small, linear peptides with high conformational flexibility, the probability of finding them in solution in the so-called 'bioactive conformation', is very low. Some conformation pre-selection can be obtained by the use of mixtures of water with organic solvents, often called cryoprotective mixtures. We have shown that, mainly owing to their increased viscosity, they can favour compact conformations over extended, disordered ones, thereby acting as conformational sieves [10].

A reasonable mimic of confined environments and in particular of their influence on local viscosity, is furnished by the pores of polyacrylamide gels (PAGs) that are routinely used to measure residual dipolar couplings in proteins [11,12]. We have shown that the mere confinement in this type of material is sufficient to induce a change in the viscosity of an aqueous solution without changing the composition and the temperature of the solvent [13,14]. NOESY spectra of ^{15}N uniformly labelled [Leu]enkephalin are consistent with either a conformational selection or a slowing down of internal motions, thereby favouring the interaction of the peptide with the receptor. Embedding proteins in

*Correspondence to: P. A. Temussi, Dipartimento di Chimica, Universita' di Napoli, via Cinthia, 80126 Napoli, Italy; e-mail: temussi@unina.it

PAGs with average dimensions of the pores in the range 12–8 nm induced in all cases a stabilization of the native conformation, as measured by the temperature of unfolding [14].

Yet another interesting advantage offered by protein or peptide solutions embedded in gels is the possibility of recording NMR spectra at very low temperatures. NMR spectra at subzero temperatures are recorded either by increasing the pressure to values of the order of kilobars [15] or by using bundles of capillaries instead of regular NMR tubes [16]. The mesh of intertwining fibres that flanks nanopore cavities in gels is reminiscent of the intricate mesh of the cytoskeleton present in living systems [1], but it may also be viewed as a complicated structure of very thin capillaries, thus hinting at the possibility of extending the conformational study of peptides to unusual temperature ranges. The use of supercooled water allows recording high-resolution NMR spectra at subzero temperatures, without adding cosolvents like polyols to lower the freezing point of water, and may induce interesting conformational preferences.

The main limitation to the use of PAG to run NMR spectra of peptides resides is the need for labelling them. While most of the chemical groups in PAG are connected in a rigid mesh, there are also flexible side chains containing amino groups that would obscure a large part of the ^{1}H spectrum but do not interfere with the recording of ^{15}N edited NMR spectra. ^{15}N labelling is routinely done for proteins by overexpression in suitable enriched media but is more difficult for peptides, and resorting to the synthesis of N^{15} uniformly labelled peptides is prohibitively expensive. We have explored alternatives that can allow the measurement of natural abudance NMR spectra of linear peptides in confined environments. Here we report the use of agarose gel to run NMR spectra of proteins and peptides. Agarose gels are routinely used for the separation of biological molecules, particularly nucleic acids, by electrophoresis and have been proposed already as a confinement medium to study the effect of molecular confinement on the conformational dynamics of apomyoglobin by frequency domain fluorometry [17]. In addition, agarose gel has been successfully used as a confinement medium for the crystallization of proteins in the absence of gravity [18]. Preliminary exploratory spectra showed us that all agarose resonances are broadened beyond recognition, effectively disappearing in the base line.

In order to examine the compatibility of this medium for NMR spectroscopy studies of proteins and peptides, we have run spectra of natural abundance hen egg white (HEW) lysozyme as an example of heat-resistant protein and of the neuropeptide [Ala^{20}]NPS as an example of a very flexible bioactive peptide.

MATERIALS AND METHODS

Peptide Synthesis

Human [Ala^{20}]NPS was synthesized according to published methods [19] using Fmoc/*t*-butyl chemistry with a Syro XP multiple peptide synthesizer (MultiSynTech GmbH, Witten Germany). Pre-loaded Fmoc 4 benzyloxybenzyl ester resin (Fluka, Buchs, Switzerland) was used for the synthesis of all peptides. The peptide was cleaved from the resin using reagent B [20]; after filtration of the exhausted resin, the solvent was concentrated *in vacuo*, and the residue was triturated with ether. Crude peptides were purified by preparative reversed-phase HPLC, and the purity grade was checked by analytical HPLC analyses and mass spectrometry using a matrix-assisted laser desorption ionization time-of-flight mass spectrometer (Bruker BioScience Corp., Billerica, MA) and an electrospray ionization Micromass ZMD-2000 mass spectrometer (Waters).

Sample Preparation

Lysozyme was purchased from Fluka and used without further purification by dissolving the required amounts in phosphate buffer at pH 6.5. For the sample at pH 1.9, the pH of the solution was adjusted by small additions of 0.1 M HCl. Low-gelling agarose VII-A was purchased from Sigma. The NMR samples were prepared directly in NMR tubes by adding first the required amount of agarose, then the protein/peptide solution and heating at 65 °C for 5–10 min.

NMR Spectroscopy

NMR spectra were recorded on a Varian INOVA spectrometer operating at 600 MHz ^{1}H frequency. Typically, 0.3–0.5 mM protein samples were used. The peptide samples were 1.5 mM. Water suppression was achieved by the WATERGATE pulse sequence [21]. The nuclear Overhauser enhancement spectroscopy (NOESY) spectra [22] were recorded with mixing times of 70 and of 400 ms for lysozyme and [Ala20]NPS, respectively. The spectra were processed and zero-filled to the next power of two using the NMRPipe program [23]. Baseline correction was applied when necessary.

RESULTS

NMR Samples in Agarose

In commonly used protocols, the gel is prepared before the actual electrophoresis experiment by dissolving the agarose powder in water at high temperature (in the range 60–90 °C) and the biological molecules are diffused under the influence of the electric field. Such a procedure would be impractical for the preparation of gels embedded with solutions at the high concentrations needed for NMR experiments.

The main problems in sample preparation stem from the fact that addition of the protein/peptide solution to the high-temperature sol (*ca* 65 °C for the low-gelling agarose VII-A that was used) might lead to a premature gelification, and from the difficulty of transferring the

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(very viscous) sample to the NMR tube. We overcame these difficulties by dissolving the required amount of agarose directly inside the NMR tube and then adding a peptide/protein solution. Since this procedure requires the heating of the peptide/protein for a few minutes at 65 °C, it was feared that it could be applied only to very heat-resistant molecules. In practice, the heating at 65 °C lasts only for a few minutes; thus the sample preparation is compatible with many peptides/proteins that are reasonably heat resistant, including proteins with unfolding temperatures lower than 65 °C but that can reversibly refold when cooled to room temperature.

Lysozyme

Owing to the rigidity of the gel matrix, all resonances coming from the agarose protons are too broad to be observed in a 1D NMR spectrum. Therefore it is possible to record 1D spectra of unlabelled peptides/proteins. The first example we chose is HEW lysozyme. As it is well known, this protein is a small enzyme, exceptionally abundant in egg whites, that catalyses the hydrolysis of polysaccharides comprising the cell walls of bacteria. Lysozyme is also one of the best characterized proteins from a structural point of view, both in solution [24] and in the crystal state [25]. An additional reason to choose it as an example for the use of agarose gel as a confinement medium is that its thermal unfolding temperature (T_m) is fairly high, in the range between 70 and 80 °C, depending on pH and ionic strength, well above the melting temperature of agarose VII-A.

Figure 1 shows the comparisons of the 1D spectra of HEW lysozyme in a 1.5% agarose gel at pH 6.5 (Figure 1(A)) and at pH 1.9 (Figure 1(B)). It can be seen that both spectra are of very high resolution, a clear indication that encapsulation of the protein in the agarose gel has not altered the 3D structure of the protein. Since the unfolding temperature of HEW lysozyme is higher than the melting point of agarose VII-A, it is not possible to check the effect of confinement on protein stability, but it is possible to run spectra at conditions (e.g. low pH) compatible with lower unfolding temperatures and aggregation phenomena [26]. Figure 1(C) shows the NOESY spectrum of HEW lysozyme at pH 1.9 in agarose gel.

(Ala^{20})NPS: a Flexible Peptide

Linear peptides have generally little or no structure in water. Therefore, their conformation is not expected to be influenced by the (high-temperature) sample preparation in agarose gel, but it is hoped that confinement can induce some degree of structuring, as we observed for enkephalin in PAG [13]. As an example

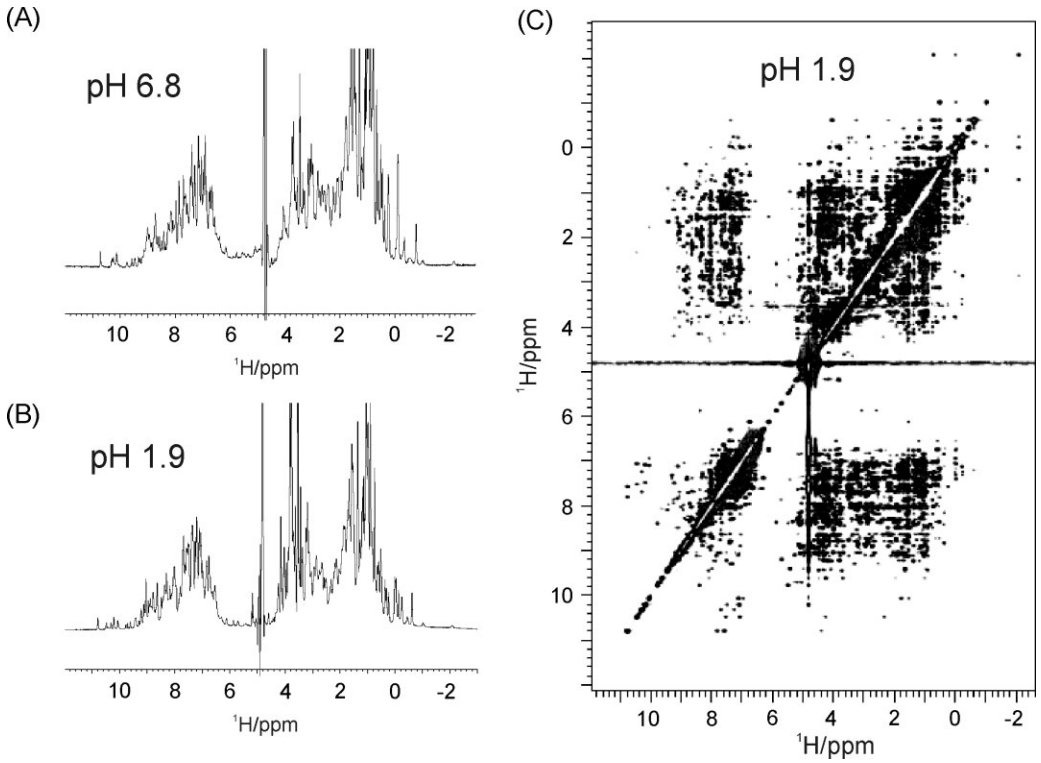

Figure 1 NMR spectra of HEW lysozyme in 1.5% agarose gel at 25 °C. (A) 600 MHz ^{1}H 1D spectrum of 1.5 mM HEW lysozyme in phosphate buffer at pH 6.8. (B) 600 MHz ^{1}H 1D spectrum of 1.7 mM HEW lysozyme at pH 1.9. (C) 600 MHz ^{1}H NOESY spectrum of 1.7 mM HEW lysozyme at pH 1.9.

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of a linear bioactive peptide we chose [Ala^{20}]NPS, an analog of human NPS. Human NPS is a 20-residue peptide with the following primary structure SFRNGVGTGMKKTSFQRAKS. [Ala^{20}]NPS is an analog of hNPS that has exactly the same activity of the parent peptide but a slightly simpler spectrum. NPS selectively binds and activates a previously orphan G-protein coupled receptor now known as NPSR [27,28]. The profile of behavioural effects evoked by administration of NPS (stimulation of arousal and wakefulness together with anxiolytic-like effects) appears to be quite unique. In fact, stimulants such as amphetamine and cocaine potently induce arousal but appear to be anxiogenic at the same time, while anxiolytics such as benzodiazepines produce sedative effects and are also widely used as hypnotics. While the main requirements for biological activity in the primary structure of NPS have been identified by exhaustive structure–activity relationship studies [29,30] the role of conformational preferences is not clear. Bernier *et al.* [30], on the basis of a preliminary NMR and CD structural characterization, proposed a 'nascent helix' in the region spanning residues 5 through 13 and hypothesized that such a helix may be necessary for a proper interaction of NPS with its receptor. Since we observed that NPS is completely flexible not only in water but also in mixtures of water with 2,2,2-trifluoro ethanol (9 : 1 TFE : water; at 300 K) and hexafluoro acetone trihydrate (HFA : H_2O 1 : 1 at 300 K), which are known to be strong helix inducers (T.Tancredi, personal communication), we chose to investigate the influence of confinement and of low temperatures. We recorded 1D and homonuclear ^{1}H NOESY spectra of [Ala^{20}]NPS in bulk and in agarose gels of different mesh and with the addition of a crowding agent. Once again the gel is completely transparent in NMR measurements. However, the spectra in bulk, in 1.5% agarose and in 4% agarose are virtually identical, hinting that the mesh of agarose gels is too large to induce substantial ordering effects (data not shown).

We tried to limit the dimensions of the cavities by the addition of Ficoll 400, a known crowding agent [31], but the spectrum in 4% agarose, even in the presence of Ficoll 400 did not show any sign of structuring. On the other hand, as mentioned in the introduction, embedding a peptide in the agarose gel facilitates the use of low temperatures, offering a further possibility of studying conformational preferences. In fact, NMR spectra of peptides and proteins remain of high quality at temperatures well below 0 °C.

Figure 2 shows the comparison of the NOESY spectrum of [Ala^{20}]NPS in 40 mM phosphate buffer at 25 °C with the corresponding spectrum of the same solution embedded in a 1.5% agarose gel at −8 °C. It is easy to appreciate that the NOESY spectrum at the low temperature has improved enormously. It is important to note that we used a mixing time (400 ms) that can be considered short for a linear peptide, since the use of very long mixing times may lead to severe spin diffusion. The different pH values (6.8 vs 5.0) may account for the fact that, in the room temperature spectrum, we did not observe the seven NH–NH cross peaks reported by Bernier *et al.* [30] (2006). On the other hand, in the spectrum at −8 °C and pH 6.8 there are as many cross peaks as in a small folded protein. A full structure characterization of NPS at low temperature is presently being carried out in our laboratory.

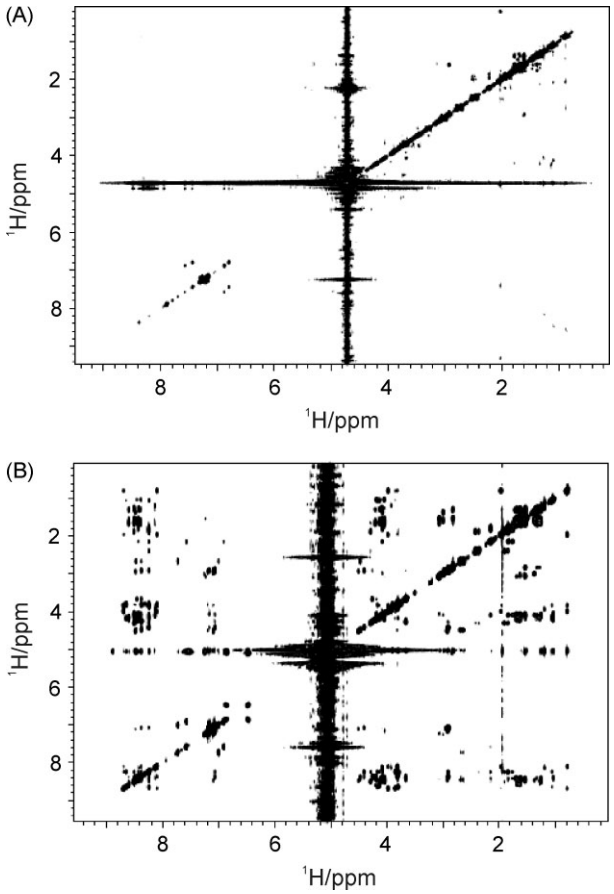

Figure 2 Comparison of 600 MHz ^{1}H NOESY spectra of a natural abundance sample of [Ala^{20}]NPS dissolved in 40 mM phosphate buffer at pH 6.8 and embedded in a 1.5% agarose gel. (A) Homonuclear ^{1}H NOESY spectrum at 25 °C with a mixing time of 400 ms. (B) Homonuclear ^{1}H NOESY spectrum at −8 °C with a mixing time of 400 msec.

DISCUSSION

As indicated also by the preliminary exploratory spectra of agarose gels embedding just buffer solutions, this medium is completely transparent in NMR experiments, relieving the need for labelling both peptides and proteins. The most problematic aspect in

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the preparation of samples of gel containing solutions of peptides or proteins is the need for exposing the biological specimens to fairly high temperatures. Although heating for a short time at temperatures above room temperature is not generally a problem for most peptides, it was feared that such a procedure could damage proteins beyond recognition. We showed here that embedding in agarose gels is also compatible with temperature-resistant proteins.

Contrary to what we observed with PAG [14] there was no significant stabilization in proteins, nor a structure-inducing effect on the linear peptide. It is clear that the mesh of agarose gels is too wide for direct effects of confinement on the stability of proteins and/or peptides, but it is ideally suited for a combination of confinement and crowding. Although the only attempt made with [Ala^{20}]NPS by adding Ficoll 400 as a crowding agent [31] to the 4% gel did not induce structural stabilization, it is easy to envisage that the addition of a variety of crowding agents makes the use of these gels potentially very versatile.

The most important result of the present study is the possibility of using agarose gels to perform NMR measurements at subzero temperatures. The spectacular structure induction of [Ala^{20}]NPS at $-8\,^{\circ}C$ is full of promises for future studies on many peptide hormones; but, in general, structural biology in gel-embedded protein solutions has the unique potential for obtaining novel insights into biomolecular dynamics, hydration, and cold denaturation also because the use of supercooled water allows reaching temperatures below $0\,^{\circ}C$ without adding chemicals or applying very high pressure for lowering the freezing point of water [16]. It is possible to foresee that gels may be useful in experiments of great relevance for biology, e.g. studies of hydrogen/deuterium exchange rates, kinetics of fast exchanging protons in enzymes and in exploring by NMR cold denaturation processes. The latter process, although suggested theoretically long ago [32], has been rarely observed experimentally without the addition, to protein solutions, of destabilizing agents. Full access to the cold denatured state is normally limited by the obvious reason that cold denaturation occurs for most proteins well below the freezing point of water. The use of gels promises to be more practical than the mentioned method based on bundles of capillaries [16], not only because of the ease of keeping solutions in the subzero condition but also of the possibility of studying unlabelled proteins.

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