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HRMAS NMR Observation of β-Sheet Secondary Structure on a Water Swollen Solid Support

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Abstract: In this paper HRMAS NMR was used to investigate whether peptides on a peptidyl resin swollen in aqueous solution can adopt an intramolecular β-sheet structure. A model peptide YQNPDGSQA, that was previously shown to adopt such a secondary structure in solution, (Blanco *et al.*, J. Am. Chem. Soc., 1993) was grafted onto three different solid supports that swell in aqueous solution to examine the influence of the resin on the structure. Both parameters of resin loading and pH inside the swollen peptidyl resin proved to be important for the physicochemical behaviour of the peptide on the support.

Keywords: HRMAS NMR; peptide; solid phase peptide synthesis; secondary structure; intramolecular β-sheet

INTRODUCTION

The rules that underlie protein folding have been extensively studied by structural examination of small model peptides. Especially for the α-helix structure, these attempts have resulted in a number of rules leading to the reliable prediction of this secondary structure element [1]. For the β sheet, however, the non-local nature of the interaction increases the difficulty of the problem. Moreover, the intermolecular aggregation that is often encountered at the concentration needed for a detailed structural study has ruled out the study of many sequences. This latter phenomenon equally occurs *in vivo*, where partially unfolded polypeptides tend to aggregate to form fibres with an extensive β-sheet content, and leads to various molecular diseases known collectively under the name of amyloid diseases [2].

Preventing the aggregation of peptides can be done by various parameters such as temperature, pH, addition of co-solvent, but the simplest way is to decrease the concentration. *In vivo*, some chaperone proteins prevent the aggregation of partially unfolded peptides by sequestering them in a hydrophobic environment [3]. Chemical immobilization of the molecules can also be used to prevent aggregation, and has led to improved refolding of recombinant proteins [4] or the observation of the random coil to α-helix transition for a polystyrene resin tethered poly-alanine peptide in DMF [5]. This latter study showed the potential of resin anchoring combined with high resolution magic angle spinning (HRMAS) NMR [6] to study aggregating systems. Further experimental evidence of α-helical peptide structure on different supports that swell in aqueous solution was reported for a viral peptide [7] and, very recently, for peptides corresponding to a transmembrane helix [8].

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Dilution of peptides on a resin to prevent aggregation would be of special interest for intramolecular β-sheet structures grafted onto water-soluble resins, but at this moment, it not clear at all whether the solid support is compatible with this secondary structure element. We report here the results of a structural study by HRMAS NMR of a model β-hairpin peptide on three different water soluble polymer supports, and investigate the criteria that lead to the formation of a similar structure to that in solution.

MATERIALS AND METHODS

Resin Description

The Pega resin (Novabiochem, Switzerland) consists of a polymer matrix made of dimethyl acryl-amide and mono-acrylamidoprop-1yl[2-aminopropyl-1yl]-polyethylene glycol_{800} cross-linked with bis 2-acrylamidoprop-1yl polyethylene glycol_{800}. The support is derivatized with Sheppard's base labile 4-hydroxymethylbenzoic acid linker. The initial loading of the resin was 0.4 mmole/g.

The PEPSYN resin (Dr W. M. M. Schaaper, Lelystad, The Netherlands) is a copolymer of *N*-acrylyl sarcosine methylester, *N*,*N*′-bisacrylyethylene diamine and *N*,*N*-dimethylacrylamide. The initial load of the resin was 1 mmole/g.

The POEPOP resin (Dr A. Bianco, Strasbourg, France) is a copolymer of poly-oxyethylene and poly-oxypropylene. The polyethylene glycol used for the resin in our study is characterized by a molecular weight of 1000.

Peptide Synthesis

Loading of the first amino acid on the HMBA resin: Using the method of the symmetric anhydride in order to load the first amino acid on the HMBA, 10 eq of the first amino acid was dissolved in dry DCM, and then 5 eq of DIC was added. The mixture was stirred for 20 min at 0 °C. The DCM was removed under vacuum, and DMF was added. Upon applying the mixture to the wet resin, 1 eq of DMAP was added.

Loading of the first amino acid on PEPSYN: 10 eq of ethylene diamine was added to the resin swollen in DMF. The mixture was stirred for 24 h, after which the excess of ethylene diamine was removed by washing the resin with a large amount of DMF. After this treatment the first amino acid was attached using the standard protocol.

Functionalization of POEPOP: phtalimide (0.12 mmole) was added under argon to a suspension of POEPOP resin in 1 : 1 dry DCM/THF containing PPh_3 (0.12 mmole). The mixture was cooled to 0 °C, and a solution of DEAD (0.12 mmole) in 1 : 1 dry DCM/THF was added over 1 min. The reaction was stirred for 26 h at room temperature, and the resin was washed with DCM, DMF, DCM, diethyl ether and dried under vacuum. The phtalimide-protecting group was removed by treating the resin with hydrazine hydrate (0.024 mmole) in NMP. After stirring for 20 h at room temperature, the resin was triply washed with DMF, water and again DMF. After this treatment, the standard protocol was applied. After loading the first amino acid a UV test was performed to determine the load of the resin after functionalization. A value of 0.45 mmole/g was found.

The full sequence of our model peptide was synthetized manually on the three resins, using a standard protocol for Fmoc chemistry: 5 equivalents (relative to the resin loading) of the Fmoc amino acid was dissolved in DMF, then HBTU (5 eq) and HOBt (5 eq) were added together with 10 equivalents of DIEA. The mixture was shaken for 45 min. Fmoc protecting groups were removed by 20% piperidine. The side chain deprotection was done by applying a mixture of 95% TFA 2.5% H_2O 2.5% TIS over 2 h.

NMR Spectroscopy

Before each NMR experiment, 5 mg of the different resins was swollen in a phosphate buffer. The pH of the buffer was adjusted to a value of 5 with NaOD and DCl, preliminary to the swelling of the resin. In a second experiment, we adjusted the pH of the resin slurry was adjusted directly to the same value with NaOD. Swelling of the different resins was determined by the syringe method. A known amount of each resin was loaded in a syringe and the solvent at the right pH was added in order to wash the resin [9].

All NMR experiments were performed on a Bruker DMX 600 MHz spectrometer (Bruker Spectrospin, Germany) equipped with a 4 mm ^{1}H-^{13}C HRMAS probe using a 6 kHz spinning rate. Chemical shifts are expressed in ppm using TMSP as an internal reference. ^{13}C referencing was performed using the indirect referencing method [10]. The NOESY experiments were recorded on 5 mg of resin with a mixing time of 300 ms with 64 scans per increment using the WANOESY [11]. Further acquisition parameters were 256 complex points in

t_2 dimension and 1024 complex points in t_1. The ^{1}H-^{13}C correlation spectrum was recorded with the refocused HSQC sequence as a reference spectrum for further relaxation studies [12]. Spectral offset in the ^{13}C direction was set at 42.6 ppm. Spectral processing and integration were performed with XWINNMR from Bruker.

RESULTS AND DISCUSSION

In the present work, the compatibility was examined of different resins that swell in aqueous solution with β-sheet formation. Our system corresponds to a model peptide (YQNPDGSQA), previously shown to adopt at least partially the β-sheet conformation in solution [13], grafted onto three different resins, Pega-HMBA [14], PEPSYN [15] and POEPOP [16]. After synthesis following the Fmoc/Tbu strategy of the peptide onto each of the resins, the side chain protection groups were cleaved by a 95% TFA solution, and the resins were thoroughly washed and dried before lyophilization. For the HRMAS NMR analysis, 5 mg of resin was swollen in 100 µl aqueous phosphate buffer at pH 5 directly into the rotor. In order to stay as close as possible to the conditions described for the solution study [13], the sample was brought to 277 K and was spun at 6 kHz inside the 14.1T spectrometer. The 1D spectrum of the Pega-HMBA peptidyl resin showed a good line width (Figure 1), and a TOCSY spectrum allowed the full assignment of the peptide.

As the chemical shift is a very sensitive probe of 3D environment, we compared our values with

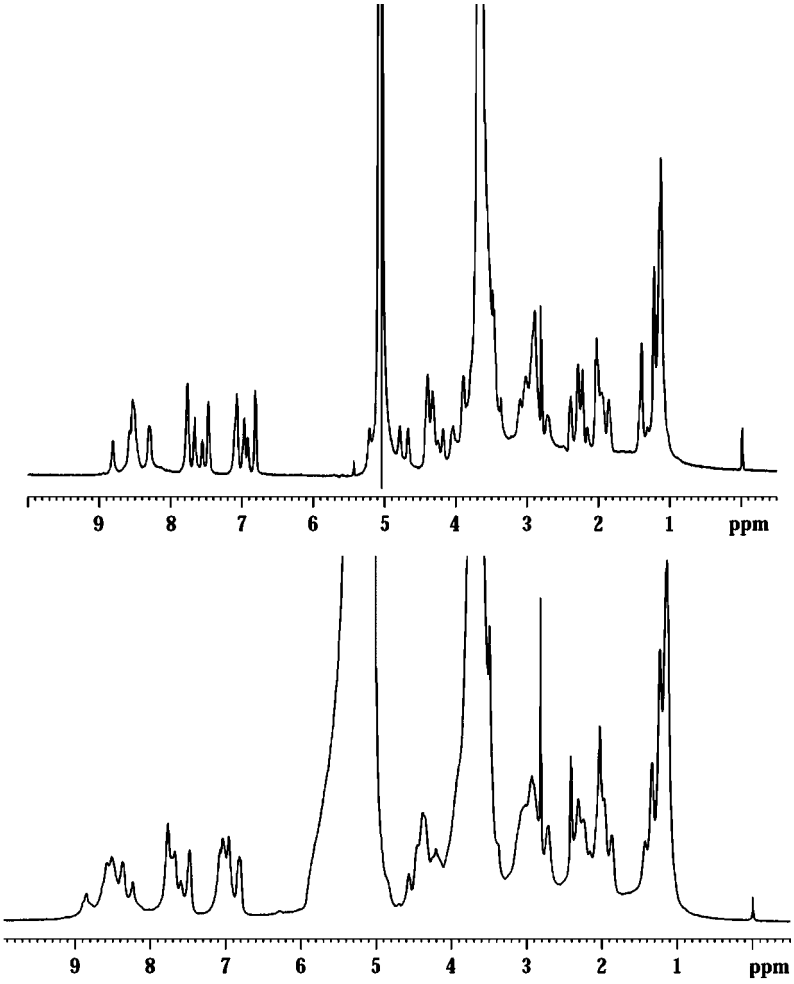

Figure 1 (Top) 1D ^{1}H HRMAS NMR spectrum of the peptide linked to the resin Pega-HMBA swollen in a phosphate buffer at pH 5.2 (real 3.5) at 277K. (Bottom) ^{1}H HRMAS NMR spectrum of the same resin swollen in a phosphate buffer at pH 5.2 (5.2) at 277K.

those reported for the peptide in solution [13]. The excellent agreement (Figure 2a) confirmed that the peptide on the resin adopts a conformation that is very close to that in solution. Further confirmation of the β-structure came from the assignment of the $^{13}C\alpha$ chemical shifts, determined from a ^{1}H-^{13}C HSQC experiment recorded at natural abundance. The resulting pattern of Cα shifts interpreted with the chemical shift index method [17] is equally indicative of an extended structure (Figure 2b).

The NOESY spectrum showed many strong sequential $H\alpha_i$-HN_{i+1} contacts in both the *N*- and *C*-terminal stretches of the peptide. The predominance of these contacts over the $H_N(i)$–$H_N(i+1)$ contacts is in agreement with a β-sheet formation [18]. The strong sequential H_N–H_N contact between the central residues Asp5 and Gly6 that form the turn region in solution, indicates the chain reversal of the model peptide on the resin. As for the side chains, we observed a weak long range contact between the *N*-terminal Tyr aromatic side chain and the *C*-terminal Ala methyl group, showing unambiguously that the anchored chain spends at least a fraction of its time in the β-sheet conformation (Figure 3). *J* coupling values have equally been used to characterize the extended nature of a peptide strand [18]. However, at the low temperature used, the linewidth of all amide resonances of grafted peptide was superior to 17 Hz. Because this exceeds the expected coupling constants, these latter could not be determined accurately.

Because the HRMAS spectra of moieties grafted onto a POEPOP resin have demonstrated a far superior spectral quality [19], we synthetized the same peptide on a POEPOP resin. Contrary to expectations, the spectral quality was not as good as for the peptide on the Pega-HMBA resin. Moreover, both TOCSY and NOESY spectra on this sample gave very little contact (Figure 4a). The difference was traced back to the influence of the pH on the sample: we indeed had adjusted the pH of the POEPOP peptidyl resin sample to a value of 5 by measuring it on the supernatant in the presence of the resin, whereas the previous Pega-HMBA sample was swollen in a phosphate buffer initially at pH 5. A subsequent measurement of the pH on the Pega-HMBA sample showed that the buffer used was not sufficiently strong to maintain the initial pH of 5, due to TFA counter-ions trapped in the resin after the deprotection step (the pH first dropped to a value of 3.5). The pH proved to be an important parameter, as spectra on the POEPOP resin at pH 3.5 yielded not only excellent resolution but also showed the same chemical shifts as the peptide in solution (Figure 2) and the characteristic NOE contacts as described for the Pega-HMBA system (Figure 4). Finally, when adjusting the pH of the Pega-HMBA sample to 5, we lost resolution (Figure 1b) and almost all of the NOE contacts.

A possible interpretation for these spectral quality differences came from a measurement of the swelling properties of the resins without and with grafted peptides. For both Pega HMBA and POEPOP resins, the swelling as measured by the syringe method [9] was superior at low pH when we consider the peptidyl resin, whereas it was mainly pH independent when considering the naked resin. For the POEPOP resin, the swollen volume for 1 g of peptidyl resin increased from 8.7 ml to 11.6 ml upon lowering the pH from 5 to 3. For the Pega-HMBA, a similar increase from 8.4 ml to 9.24 ml was observed for the resin with peptide grafted, whereas the resin without peptide swelled to a volume of 8.0 ml irrespective of pH. A similar correlation between decreased resin swelling both macroscopically and at the level of the individual bead was previously observed during the synthesis

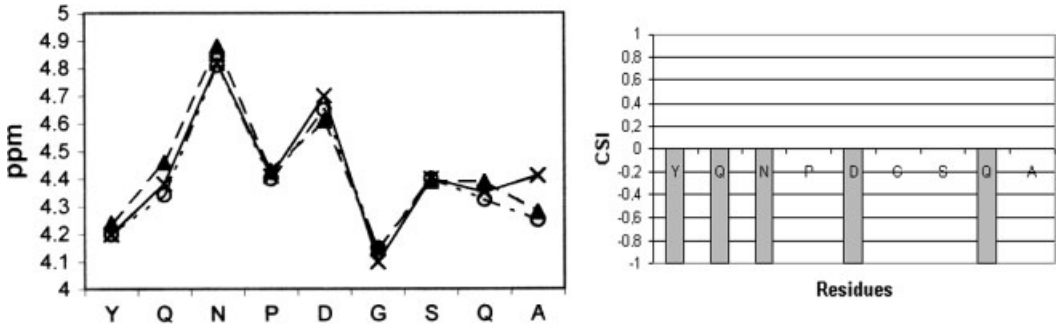

Figure 2 (a) Hα chemical shift values for the peptide tethered to the Pega HMBA (×), POEPOP (O) and in solution (▲). (b) ^{13}C Chemical Shift Index pattern derived from the ^{1}H-^{13}C HSQC spectrum of the peptide grafted on the Pega HMBA resin.

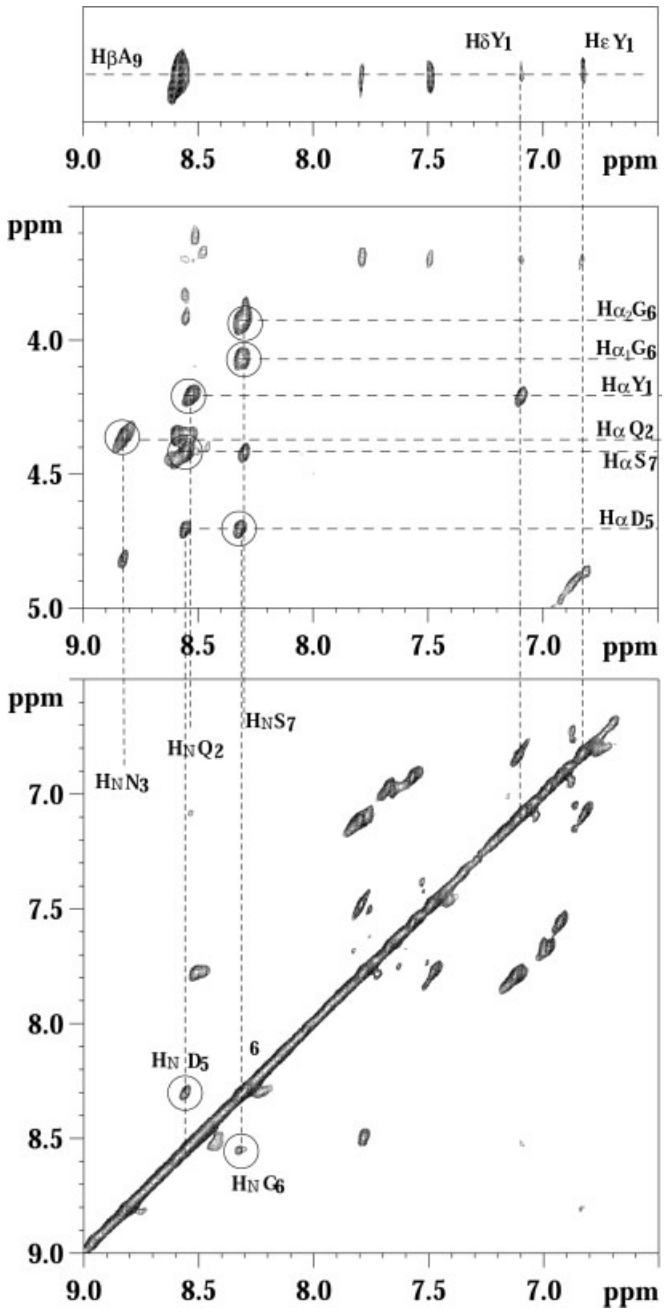

Figure 3 (a) 2D HRMAS NMR NOESY spectrum showing three regions of interest of the peptide linked to the resin Pega HMBA swollen in a phosphate buffer at pH 5.2 (real 3.5) and 277 K. Circled cross peaks are the sequential H_α (i)-H_N(i+1) contacts. In the lower panel the symmetrical H_ND5-H_NG6 peak indicates the turn position.

of the aggrecan peptide [20]. In this latter case, the addition of a polar aprotic solvent such as DMSO had improved both swelling and spectral quality, indicating that transient peptide aggregation leads to a form of non-covalent cross-linking. In our case of the β-hairpin peptide, the theoretical pH of the peptide moiety is 5.1 [21]. This, combined with the high density of peptide inside the resin bead is most probably the reason for the spectral improvement when the experimental conditions are further away from the peptide pI.

Finally, we synthetized the peptide on the PEPSYN resin. For this resin, however, spectral quality was poor independent of the pH used, and we were never

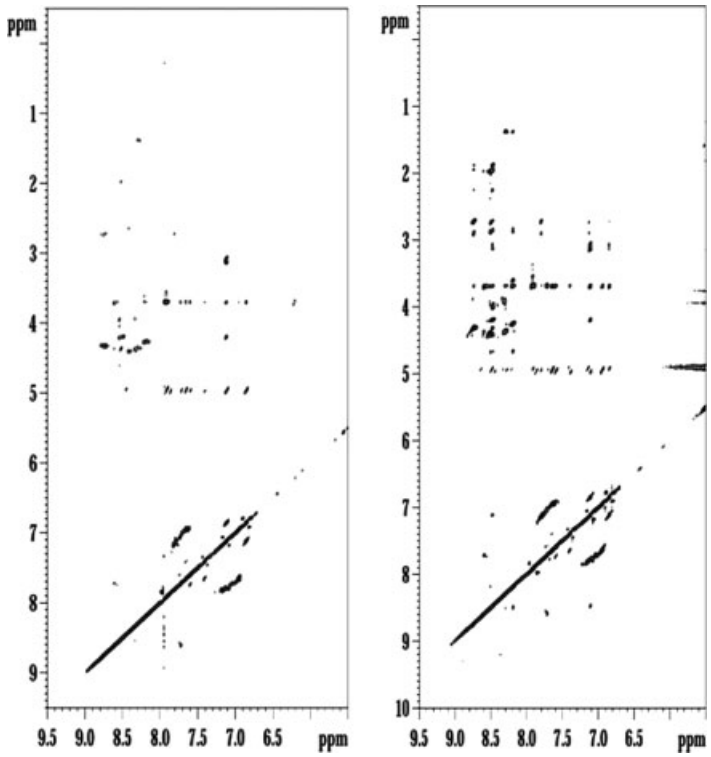

Figure 4 (left) 2D HRMAS NMR NOESY spectrum of the peptide linked to the POEPOP resin at pH 5.2 (right) NOESY spectrum of the same resin at pH 3.5.

able to find evidence of any peptide structuration. We attributed this failure to the high loading of the resin (1 mmole/g), which represents a concentration close to 1 molar at the interior of the beads. This high concentration led to excessive intermolecular interaction and hence to the aggregation of the peptide on the resin.

CONCLUSION

We conclude from these combined results that intramolecular β-sheet formation on different solid supports is possible, but that the conditions established in solution do not necessarily apply on the solid support. The high concentration of peptide chains inside the beads clearly can lead to potential intermolecular aggregation even if the peptide chains have lost their translational mobility. The possibility to decrease the initial load of the resin by using the orthogonal synthetic strategy Fmoc/Boc in order to overcome this drawback is currently under study in our laboratory.

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21. ur sequence was submitted to the following web site in order to compute the pI: of the peptide http://www.embl-heidelberg.de/cgi/pi-wrapper.plO

