4130 J. Am. Chem. Soc. 2001, 123, 4130−4138

Evidence of Secondary Structure by High-Resolution Magic Angle Spinning NMR Spectroscopy of a Bioactive Peptide Bound to Different Solid Supports

Julien Furrer,[†] Martial Piotto,[‡] Maryse Bourdonneau,[‡] David Limal,[§] Gilles Guichard,[||] Karim Elbayed,[†] Jésus Raya,[†] Jean-Paul Briand,[||] and Alberto Bianco*,[||]

Contribution from the Institut de Chimie, UMR 7510 CNRS-Bruker, Université Louis Pasteur, 67084 Strasbourg, France, UMR 7510 CNRS-Bruker, 67160 Wissembourg, France, Laboratoire de Chimie Macromoléculaire, ENSCMu, 68093 Mulhouse, France, and Immunologie et chimie thérapeutiques, Institut de Biologie Moléculaire et Cellulaire, UPR 9021 CNRS, 67084 Strasbourg, France

Received October 2, 2000. Revised Manuscript Received February 20, 2001

Abstract: The structure of the 19-amino acid peptide epitope, corresponding to the 141−159 sequence of capsid viral protein VP1 of foot-and-mouth disease virus (FMDV), bound to three different resins, namely, polystyrene-MBHA, PEGA, and POEPOP, has been determined by high-resolution magic angle spinning (HRMAS) NMR spectroscopy. A combination of homonuclear and heteronuclear bidimensional experiments was used for the complete peptide resonance assignment and the qualitative characterization of the peptide folding. The influence of the chemicophysical nature of the different polymers on the secondary structure of the covalently attached FMDV peptide was studied in detail. In the case of polystyrene−MBHA and polyacrylamide−PEGA resins, the analysis of the 2D spectra was hampered by missing signals and extensive overlaps, and only a propensity toward a peptide secondary structure could be derived from the assigned NOE correlations. When the FMDV peptide was linked to the polyoxyethylene-based POEPOP resin, it was found to adopt in dimethylformamide a helical conformation encompassing the C-terminal domain from residues 152 to 159. This conformation is very close to that of the free peptide previously analyzed in 2,2,2-trifluoroethanol. Our study clearly demonstrates that a regular helical structure can be adopted by a resin-bound bioactive peptide. Moreover, a change in the folding was observed when the same peptide−POEPOP conjugate was swollen in aqueous solution, displaying the same conformational features as the free peptide in water. The possibility of studying solid-supported ordered secondary structures by the HRMAS NMR technique in a wide range of solvents can be extended either to other biologically relevant peptides and proteins or to new synthetic oligomers.

Introduction

High-resolution magic angle spinning (HRMAS) NMR spectroscopy is becoming a powerful tool in the field of solid-phase organic synthesis for the characterization of resin-bound compounds, including small molecules and peptides.[1] This technique has allowed us to monitor solid-phase multistep organic synthesis and to analyze the synthetic difficulties of peptide sequences growing on a solid support.[2,3] In particular, a detailed study of the correlation between the difficulty of coupling amino acids and the aggregation state of the peptide chains was performed by HRMAS.[3a] The aggregation process of resin-bound polyalanine sequences was recently investigated, as a model system, to disclose the factors that influence the coupling yield in solid-phase peptide chemistry.[3b] High-resolution MAS NMR technology also allowed the total assignment of single bead-linked short peptides.[4]

Another fundamental aspect is related to the study of the conformation adopted by a peptide bound to a solid support.[3,5] Indeed, it has been shown that synthetic epitopes exhibit a relevant biological activity while attached to a polymeric matrix and that peptide−resin conjugates, in which the resin is used

[†] Institut de Chimie, Université Louis Pasteur.
[‡] Bruker.
[§] Laboratoire de Chimie Macromoléculaire, ENSCMu.
[||] Institut de Biologie Moléculaire et Cellulaire.

(1) (a) Keifer, P. A. *Drug Discovery Today* **1997**, *2*, 468−478. (b) Shapiro, M. J.; Wareing, J. R. *Curr. Opin. Chem. Biol.* **1998**, *2*, 372−375. (c) Lippens, G.; Bourdonneau, M.; Dhalluin, C.; Warrass, R.; Richert, T.; Seetharaman, C.; Boutillon, C.; Piotto, M. *Curr. Org. Chem.* **1999**, *3*, 147−169.

(2) (a) Bianco, A.; Furrer, J.; Limal, D.; Guichard, G.; Elbayed, K.; Raya, J.; Piotto, M.; Briand, J.-P. *J. Comb. Chem.* **2000**, *2*, 681−690. (b) Warrass, R.; Lippens, G. *J. Org. Chem.* **2000**, *65*, 2946−2950. (c) Sefler, A. M.; Gerritz, S. W. *J. Comb. Chem.* **2000**, *2*, 127−133. (d) Graven, A.; Grøtli, M.; Meldal, M. *J. Chem. Soc., Perkin Trans. 1* **2000**, 955−962. (e) Riedl, R.; Tappe, R.; Berkessel, A. *J. Am. Chem. Soc.* **1998**, *120*, 8994−9000. (f) Ruhland, T. Andersen, K.; Pedersen, H. *J. Org. Chem.* **1998**, *63*, 9204−9211. (g) Luo, Y.; Ouyang, X.; Armstrong, R. W.; Murphy, M. M. *J. Org. Chem.* **1998**, *63*, 8719−8722. (h) Chin, J.; Fell, B.; Shapiro, M. J.; Tomesch, J.; Wareing, J. R.; Bray, A. M. *J. Org. Chem.* **1997**, *62*, 538−539. (i) Sarkar, S. K.; Garigipati, R. S.; Adams, J. L.; Keifer, P. A. *J. Am. Chem. Soc.* **1996**, *118*, 2305−2306. (j) Pop, I. E.; Dhalluin, C. F.; Déprez, B. P.; Melnyk, P. C.; Lippens, G. M.; Tartar, A. L. *Tetrahedron* **1996**, *52*, 12209−12222. (k) Wehler, T.; Westman, J. *Tetrahedron Lett.* **1996**, *37*, 4771−4774. (l) Fitch, W. L.; Detre, G.; Holmes, C. P.; Shoorlery, J. N.; Keifer, P. A. *J. Org. Chem.* **1994**, *59*, 7955−7956.

(3) (a) Dhalluin, C.; Boutillon, C.; Tartar, A.; Lippens, G. *J. Am. Chem. Soc.* **1997**, *119*, 10494−10500. (b) Warrass, R.; Wieruszeski, J.-M.; Boutillon, C.; Lippens, G. *J. Am. Chem. Soc.* **2000**, *122*, 1789−1795.

(4) Gotfresden, C. H.; Grøtli, M.; Willert, M.; Meldal, M.; Duus, J. Ø. *J. Chem. Soc., Perkin Trans. 1* **2000**, 1167−1171.

(5) Jelinek, R.; Valente, A. P.; Valentine, K. G.; Opella, S. J. *J. Magn. Reson.* **1997**, *125*, 185−187.

10.1021/ja003566w CCC: $20.00 © 2001 American Chemical Society
Published on Web 04/17/2001

as a carrier instead of classical carrier proteins, can be used for the generation or the purification of specific antibodies.[6,7] This suggests that these peptides are probably able to adopt the structure that is necessary for the interaction with the target molecule when bound to the resin bead. The first example of a peptide structural study by HRMAS reported in the literature describes the folding of an antigenic hexapeptide bound to a Tentagel-like resin.[5]

The conformational analysis of peptides bound to a solid support by HRMAS NMR spectroscopy is however rendered difficult by the nature of the resin. The quality of the spectra is directly influenced by the swelling properties of the resin, the dynamics of the system, and the chemicophysical features of the solid support.[8,9] Moreover, the different resins, containing either aromatic moieties or based on a polyacrylamide–polyoxyethylene core, may influence the conformation adopted by the covalently attached peptide.

In this paper, we report a detailed conformational characterization of an immunogenic foot-and-mouth disease virus (FMDV) peptide epitope bound to three different solid supports, namely, polystyrene–methylbenzydrylamine (MBHA), acrylamidopropyl–PEG-*N*,*N*-dimethylacrylamide (PEGA),[10] and polyoxyethylene–polyoxypropylene (POEPOP)[2a,11] resins. Foot-and-mouth disease virus affects both domestic and wild ruminants and swine. Although vaccines obtained by inactivation of the virus are generally effective, the use of a synthetic peptide vaccine based on the identification of the major immunogenic site of the virus particle has attracted much attention.[12] In particular, a 20-amino acid peptide, corresponding to the G–H loop of viral protein VP1, one of the four envelope proteins of the virus, was shown to elicit neutralizing antibody levels sufficient to afford complete protection in guinea pigs and, to a limited extent, in cattle.[13,14] Several antigenic peptide variants of the 141–160 region of VP1 from virus of serotype A have been identified and submitted to conformational studies in solution to correlate their secondary structure with the biological activity.[15] In particular, the peptide variant containing a phenylalanine in position 148 and a proline in position 153, also called peptide USA, has been analyzed by 2D and 3D NMR spectroscopy in 2,2,2-trifluoroethanol and aqueous solution.[15d,16] In the present work, we have studied the structure of this peptide bound to three different resins by homonuclear and heteronuclear bidimensional HRMAS NMR experiments. The conformation adopted by the peptide on the different solid supports is described, and the influence of the polymer and of the swelling solvents on the structure of the peptide is discussed in detail.

Table 1. ^{1}H and ^{13}C Chemical Shifts (ppm) of 141–159 FMDV Peptide Bound to MBHA, PEGA, and POEPOP Resins, Swollen in DMF-d_7 at 300 K

	MBHA[a]			PEGA[a]			POEPOP[a]		
residue	NH	αH	αC	NH	αH	αC	NH	αH	αC
Gly1	8.35	3.94	42.4	8.25	3.85/4.02	42.5	8.32	3.97	42.8
Ser2	8.44	4.44	56.2	8.17	4.38	56.5	8.37	4.41	56.6
Gly3	8.60	3.96	42.4	8.51	3.84/4.01	42.5	8.56	3.93	42.8
Val4	7.93	4.30	58.9	7.86	4.08	60.3	7.84	4.30	59.1
Arg5	8.21	4.44	53.0	8.09	4.40	53.5	8.18	4.42	53.5
Gly6	8.25	4.00	42.4	8.09	3.85	42.5	8.32	3.97	42.8
Asp7	8.34	4.76	50.1	8.26	4.67	50.7	8.38	4.67	50.7
Phe8	8.31	4.60	54.9	8.12	4.50	55.8	8.32	4.51	55.9
Gly9	8.39	4.00	42.4	8.25	3.85/4.02	42.5	8.46	3.85/3.97	42.8
Ser10	8.44	4.44	56.2	8.17	4.38	56.5	8.10	4.42	56.6
Leu11	8.13	4.52	51.5	7.98	4.41	51.7	8.15	4.46	52.2
Ala12	8.18	4.55	51.6	8.07	4.52	48.6	8.17	4.53	49.1
Pro13								4.41	62.1
Arg14				8.03	4.42	53.5	8.30	4.25	55.4
Val15				7.86	4.08	60.3	7.83	4.03	61.1
Ala16				8.14	4.21	54.0	8.21	4.19	51.2
Arg17				7.93	4.14	54.5	7.93	4.18	55.0
Gln18				7.83	4.22	56.6	7.87	4.24	54.5
Leu19				7.60	4.32	51.8	7.63	4.35	52.1

[a] FMDV peptide is attached to the different resins through the C-terminal Leu19 residue.

Results

Resonance Assignment and Secondary Structure of FMDV Peptide Bound to MBHA Resin. The 141–159 FMDV peptide epitope attached to the polystyrene-based MBHA resin through the C-terminal Leu159 residue was swollen in DMF-d_7 and analyzed by HRMAS NMR. Only 12 amino acids from the N-terminal part of the sequence could be detected (Table 1).[17] The last seven C-terminal residues remained invisible presumably because of a lack of mobility. To test this hypothesis, a second resin-bound peptide analogue was synthesized introducing 6-aminohexanoic acid residue (Ahx) as a spacer between the peptide C-terminus and the resin. However, even in the presence of the flexible pentamethylene chain of Ahx, the residue resonances from Pro13 to Leu19 were missing, thus preventing the complete structural analysis of the whole 19-mer. Dilution of the peptide on the solid support by a factor of 8 and 20 did not permit the detection of the missing amino acids. Interestingly, the N-acetylated short peptide made up of the last seven C-terminal residues bound directly to MBHA resin displayed already poor mobility and the complete identification of its spin systems was difficult. The assignment of the N-terminal fragment of the full peptide was done using homonuclear spectra and confirmed by the analysis of the ^{1}H–^{13}C and amide ^{1}H–^{15}N direct correlations (Figure 1A). For the acquisition of the spectra, a 50-μL HRMAS rotor was filled with ~5 mg of peptide–resin conjugate, thus allowing performance of the heteronuclear measurements even on a nonlabeled peptide, within a reasonable experimental time (see Experimental Procedures). The first glycine residue was identified from the dipolar interaction between the protons of the acetyl capping

(6) (a) McBride, J. D.; Freeman, N.; Domingo, G. J.; Leatherbarrow, R. J. *J. Mol. Biol.* **1996**, *259*, 819–827. (b) Lam, K. S.; Salmon, S. E.; Hersh, E. M.; Hruby, V. J.; Kazmierski, W. M.; Knapp, R. J. *Nature* **1991**, *354*, 82–84. (c) Geysen, H. M.; Meloen, R. H.; Barteling, S. J. *Proc. Natl. Acad. Sci. U.S.A.* **1984**, *81*, 3998–4002.

(7) McMurray, J. S. *Biopolymers* **1998**, *47*, 405–411.

(8) (a) Elbayed, K.; Bourdonneau, M.; Furrer, J.; Richert, T.; Raya, J.; Hirshinger, J.; Piotto, M. *J. Magn. Reson.* **1999**, *136*, 127–129. (b) Keifer, P. A. *J. Org. Chem.* **1996**, *61*, 1558–1559.

(9) Vaino, A. R.; Janda, K. D. *J. Comb. Chem.* **2000**, *2*, 579–596.

(10) Meldal, M. *Tetrahedron Lett.* **1992**, *33*, 3077–3080.

(11) (a) Renil, M.; Meldal, M. *Tetrahedron Lett.* **1996**, *37*, 6185–6188. (b) Grøtli, M.; Gotfresden, C. H.; Rademann, J.; Buchardt, J.; Clark, A. J.; Duus, J. Ø.; Meldal, M. *J. Comb. Chem.* **2000**, *2*, 108–119.

(12) Murdin, A. D. *Vaccine* **1986**, *4*, 210–211.

(13) Bittle, J. L.; Houghten, R. A.; Alexander, H.; Shinnick, T. M.; Sutcliffe, J. G.; Lerner, R. A.; Rowlands, D. J.; Brown, F. *Nature* **1982**, *298*, 30–33.

(14) DiMarchi, R.; Brooke, G.; Gale, C.; Cracknell, V.; Doel, T.; Mowat, N. *Science* **1986**, *232*, 639–641.

(15) (a) Rowlands, D. J.; Clarke, B. E.; Carroll, A. R.; Brown, F.; Nicholson, B. H.; Bittle, J. L.; Houghten, R. A.; Lerner, R. A. *Nature* **1983**, *306*, 694–697. (b) France, L. L.; Piatti, P. G.; Newman, J. F.; Toth, I.; Gibbons, W. A.; Brown, F. *Proc. Natl. Acad. Sci. U.S.A.* **1994**, *91*, 8442–8446. (c) Pegna, M.; Molinari, H.; Zetta, L.; Gibbons, W. A.; Brown, F.; Rowland, D.; Siligardi, G.; Mascagni, P. *J. Pept. Sci.* **1996**, *2*, 75–90. (d) Pegna, M.; Molinari, H.; Zetta, L.; Melacini, G.; Gibbons, W. A.; Brown, F.; Rowland, D.; Chan, E.; Mascagni, P. *J. Pept. Sci.* **1996**, *2*, 91–105. (e) Siligardi, G.; Drake, A. F.; Mascagni, P.; Rowlands, D.; Brown, F.; Gibbons, W. A. *Eur. J. Biochem.* **1991**, *199*, 545–551.

(16) Petit, M.-C.; Benkirane, N.; Guichard, G.; Phan Chan Du, A.; Marraud, M.; Cung, M. T.; Briand, J.-P.; Muller, S. *J. Biol. Chem.* **1999**, *274*, 3686–3692.

(17) In the text, FMDV residues are numbered from 1 to 19.

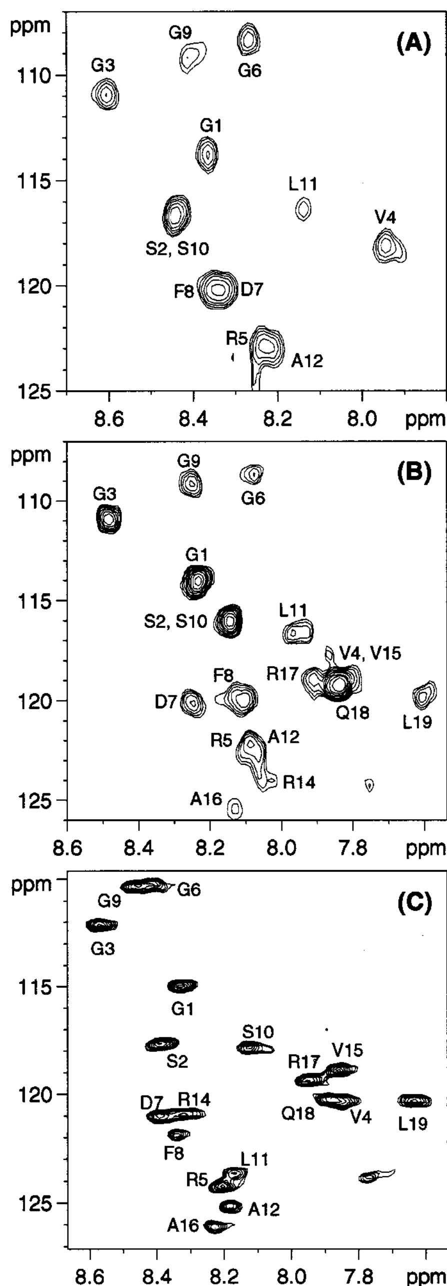

Figure 1. 2D HRMAS $^{1}H-^{15}N$ HSQC spectra for the resin-bound FMDV epitope, swollen in DMF-d_7: (A) MBHA resin, (B) PEGA resin, and (C) POEPOP resin.

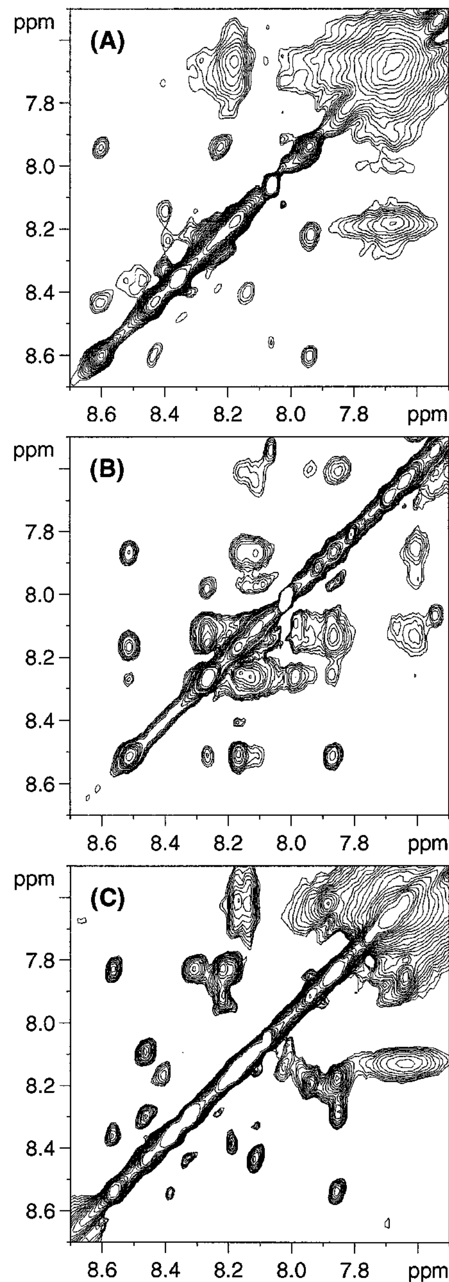

Figure 2. Amide region of the HRMAS 2D NOESY spectrum for the resin-bound FMDV epitope, swollen in DMF-d_7: (A) MBHA resin (τ_m = 300 ms), (B) PEGA resin (τ_m = 250 ms), and (C) POEPOP resin (τ_m = 300 ms).

group and the first NH of the sequence. Figures 2A and 3A display the HRMAS 2D NOESY spectra of the amide and the fingerprint region, respectively. A qualitative analysis of the secondary structure was made on the basis of the backbone short- and medium-range NOE cross-peaks, which are summarized in Figure 4A. The series of strong sequential NH−NH NOEs from Ser2 to Arg5 residues and between Phe8 and Gly9 indicate a propensity of the resin-bound peptide to adopt a helical conformation. Few medium-range nuclear Overhauser effects, typical of secondary helical structures, were also visible: cross-peaks NH3−NH5, NH9−NH11, αH2−NH4, αH5−NH7, αH4−NH7, αH8−NH11, and αH7-βH10.[18] In the region comprising Ser2 to Gly6 and between Phe8 and Gly9 strong sequential αH_i-NH_{i+1} NOE connectivities, typical of extended structures, were also observed. The simultaneous presence of these two types of spatial interactions suggests that the peptide is in fast exchange between different conformations. The dipolar interactions αH5−NH6 and αH5−NH7, which characterize the folding of the RGD peptide region, were also observed in the same sequence of the free FMDV peptide in 2,2,2-trifluoroethanol (TFE).[15d] However, the strong NH6−NH7 cross-peak, which allowed identification of a type-II β-turn in solution, was not detected in the resin-bound peptide.[15d,19] An unexpected very weak cross-peak between sequential αH7 and αH8 was observed in the NOESY spectrum. The same spatial correlation was still present in the spectrum of the FMDV peptide that was diluted 8 times on the MBHA solid support. This latter finding allows us to exclude the possibility that this

(18) Wüthrich, K. *NMR of Protein and Nucleic Acids*; Wiley & Sons: New York, 1986.

(19) Johnson, W. C.; Pagano, T. G.; Basson, C. T.; Madri, J. A.; Gooley, P.; Armitage, I. M. *Biochemistry* **1993**, *32*, 268−273.

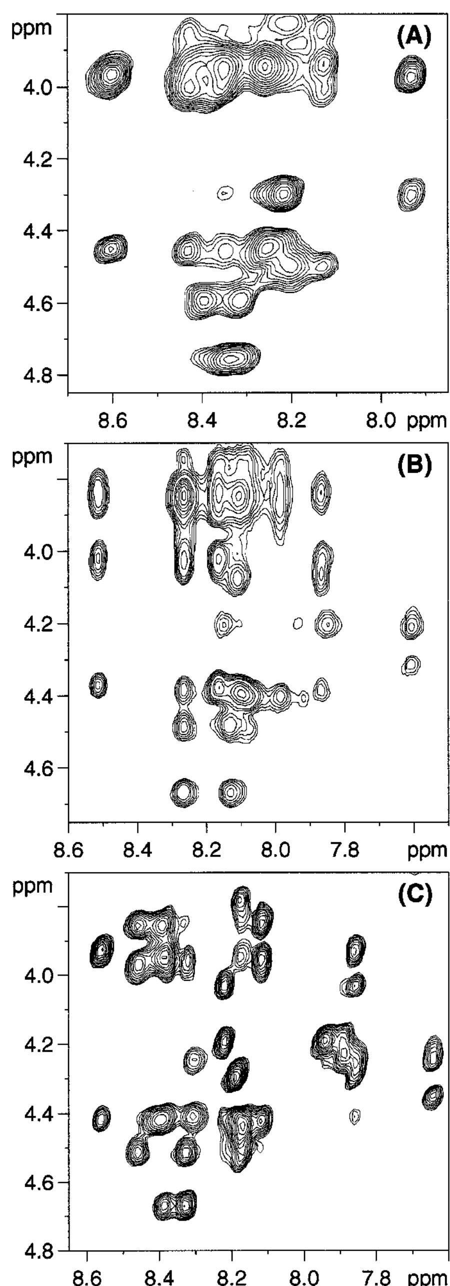

Figure 3. Fingerprint region of the HRMAS 2D NOESY spectrum for the resin-bound FMDV epitope, swollen in DMF-d_7: (A) MBHA resin ($\tau_m = 300$ ms), (B) PEGA resin ($\tau_m = 250$ ms), and (C) POEPOP resin ($\tau_m = 300$ ms).

NOE correlation could derive from peptide−peptide intermolecular interactions. The contribution to the αH−αH correlation is likely related to a conformational population that might be induced by unspecific interactions of the peptide with the resin. It is worth noting that almost all the anchoring sites (more than 98%) of a compound attached to a solid support are located in the inner surface of the bead. Consequently, a linked molecule will be surrounded not only by the swelling solvents but also by the polymeric matrix. The aromatic groups of the resin could interact with the amino acid residues and influence the distribution of the different peptide conformational populations in equilibrium.

Resonance Assignment and Secondary Structure of FMDV Peptide Bound to PEGA Resin. The FMDV peptide was covalently linked to the PEGA resin through the Ahx spacer and swollen again in DMF-d_7. In contrast to the peptide bound to MBHA resin, all 19 amino acid spin systems, except Pro13, could be observed (Table 1 and Figure 1B). The N-terminal Gly1 residue was identified by the spatial correlation between its NH proton and the methyl of acetyl N-capping group. The main short-range and medium-range backbone NOE correlations are reported in Figure 4B. Although some connectivities could not be detected because of resonance overlap, the strong sequential NH−NH cross-peaks from residue 1 to residue 12 suggests that the peptide has the tendency to adopt a helical structure in this region, as observed for MBHA resin (Figure 2B). This conclusion is partially reinforced by the presence of medium-range NH1−NH3, NH2−NH4, NH9−NH11, αH2−NH4, and αH5−NH7 dipolar interactions. Some other NOEs characteristic for the α-helix are present between αH8 and NH11 and between the αH−βH of G1/V4, D7/S10, and G9/L12. As in the case of the MBHA resin-bound peptide, the secondary structure of the N-terminal part of the FMDV epitope is an average of helical and extended conformations as evidenced by the presence of short-range αH_i-NH_{i+1} dipolar correlations (Figure 3B).[18] The poor dispersion of the resonances and the numerous signal overlaps did not allow the identification of secondary structure elements at the C-terminal part of the peptide−PEGA conjugate. Again, a weak cross-peak due to a short αH7−αH8 distance was observed. The population of the conformers that displays the αH−αH NOE could be induced by the interaction of the peptide with the *N*,*N*-dimethylamide and amide groups which constitute the core of the resin and which are potential donor and/or acceptor of H-bonds. In the RGD region, only the spatial vicinity between αH of Arg5 and NH of Asp7 was detected, while the turn characteristic αH5−NH6 and NH6−NH7 dipolar correlations were obscured by spectral overlap.

Resonance Assignment and Secondary Structure of FMDV Peptide Bound to POEPOP Resin. The 141−159 FMDV variant was assembled on the POEPOP resin, derivatized with an amino function, again through the 6-aminohexanoic acid spacer (Table 1). Figure 1C shows the $^1H-^{15}N$ direct correlations of the 19 amino acids, except proline. The spin system identification was confirmed by the analysis of the $^1H-^{13}C$ HSQC−TOCSY spectrum (Figure 5). This experiment is extremely powerful and would have permitted alone the assignment of all the peptide residues. A number of intense sequential, medium-range amide−amide and αH_i-NH_{i+1} NOE correlations throughout the POEPOP−peptide was observed from Ser2 to Leu19 (Figures 2C and 3C). The conformation adopted by the N-terminal part of the peptide linked to POEPOP is similar to that of the other two peptide−resin conjugates (Figure 4). The close distance between αH and NH protons of residues S2−V4, D7−G9, V4−D7, and D7−S10 are consistent with this conclusion (Figure 4C). The presence of the correlation between αH12 and δH13 and between NH12 and δH13 permits assignment of a trans conformation to the tertiary Ala−Pro amide bond. A cross-peak between the alanine side chain and the δ protons of proline further confirms this spatial orientation. A NOE between the NH of Arg14 and δCH_2 of Pro13 suggests that the proline is also involved in the helical folding. Indeed, it has been reported that Ala−Pro segments can be accommodated in the conformational space relative to the right-handed α-helical region.[20] In comparison with the other two resin-bound peptides, the NOEs around the RGD motif that should be diagnostic for a β-turn conformation are completely overlapped by other signals. Most interesting is the structure of the peptide

(20) Sankararamakrishnan, R.; Vishveshwara, S. *Biopolymers* **1990**, *30*, 287−298.

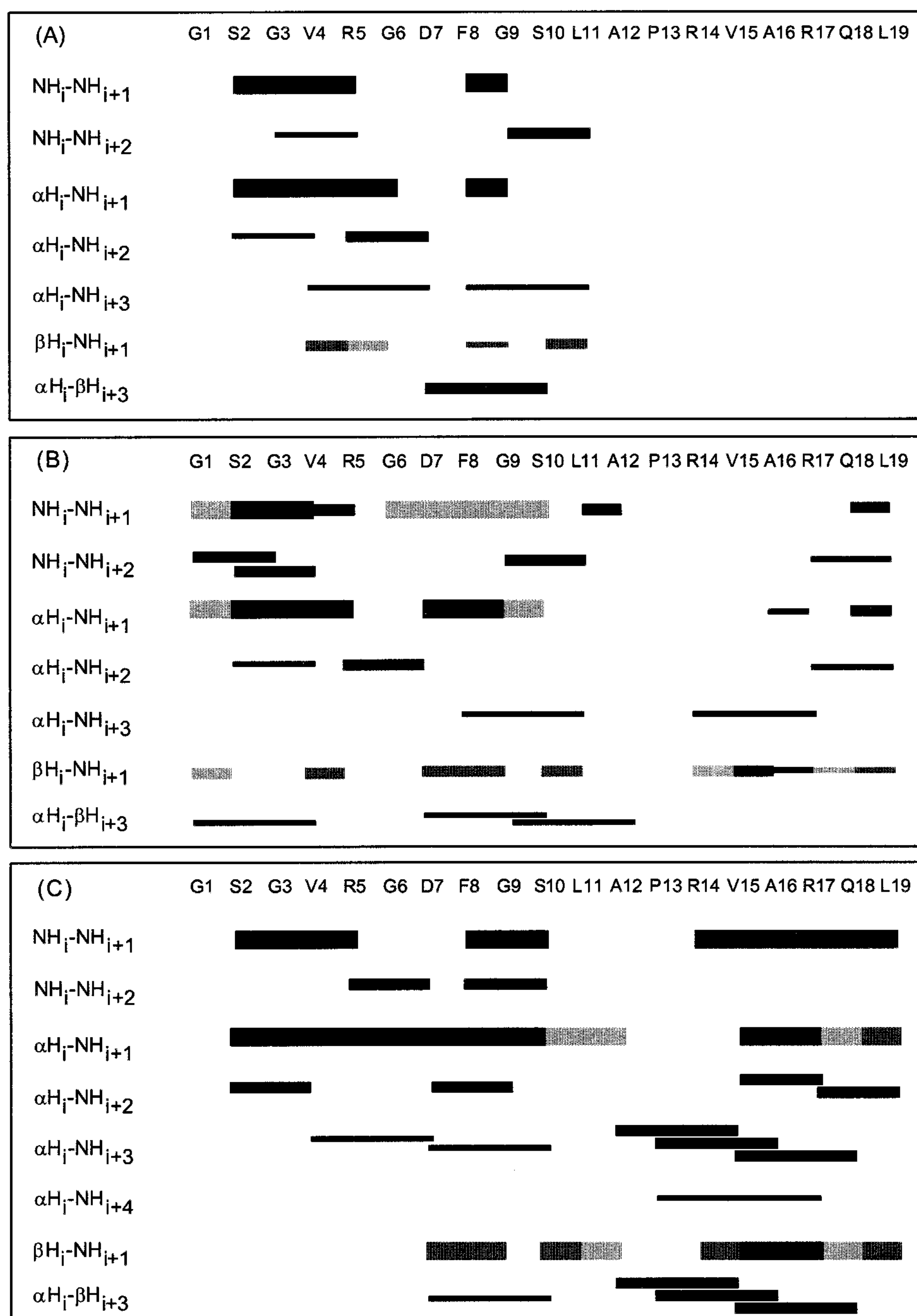

Figure 4. Summary of the backbone NOE connectivities observed for the resin-bound FMDV epitope, swollen in DMF-d_7: (A) MBHA resin, (B) PEGA resin, and (C) POEPOP resin. The strong, medium, and weak intensities are represented by line of different thickness. Gray areas indicate the presence of more than one overlapped cross-peak.

at the C-terminus. A series of consecutive αH_i-NH_{i+3}, αH_i-NH_{i+4}, and $\alpha H_i-\beta H_{i+3}$ spatial correlations of medium intensity from residues 12 to 18 allows the conclusion that the peptide folds into an α-helix (Figure 4C).[18] The presence of αH_i-NH_{i+2} NOEs in correspondence to the last four residues from Val15 to Leu19 suggests that the polypeptide chain ends as a 3_{10}-helix. This is not surprising as the 3_{10}-helix occurs as an N- or C-terminal extension to α-helical structures.[21] The helical folding of the C-terminal domain of the FMDV peptide linked to POEPOP was confirmed by the application of the chemical shift index method very recently reported for the resin-bound peptides (Figure 6).[22]

(21) Barlow, D. J.; Thornton, J. M. *J. Mol. Biol.* **1988**, *201*, 601−619.
(22) Fruchart, J.-S.; Lippens, G.; Warrass, R.; Seetharaman, C.; Dhalluin, C.; Boutillon, C. *J. Pept. Res.* **2000**, *56*, 346−351.

Folding of FMDV Peptide Bound to POEPOP Swollen in Different Solvents. The epitope of FMDV covalently linked to POEPOP resin was also studied in deuterated dimethyl sulfoxide (DMSO) and water. In DMSO-d_6, all 19 amino acid spin systems were assigned, although the 2D dispersion of the signals was limited and many overlapped cross-peaks were present. The observation of few sequential NH−NH distances and only three clear medium-range NH_i-NH_{i+2} (V4/G6) and αH_i-NH_{i+2} (P13/V15, R17/L19) NOEs suggests that the conformational pattern is different from that found for the resin-bound peptide swollen in dimethylformamide. This is not surprising since DMSO is a solvent acceptor of H-bonding and is potentially able to disrupt a folded peptide structure. Finally, we have acquired a series of 1D and 2D HRMAS spectra of

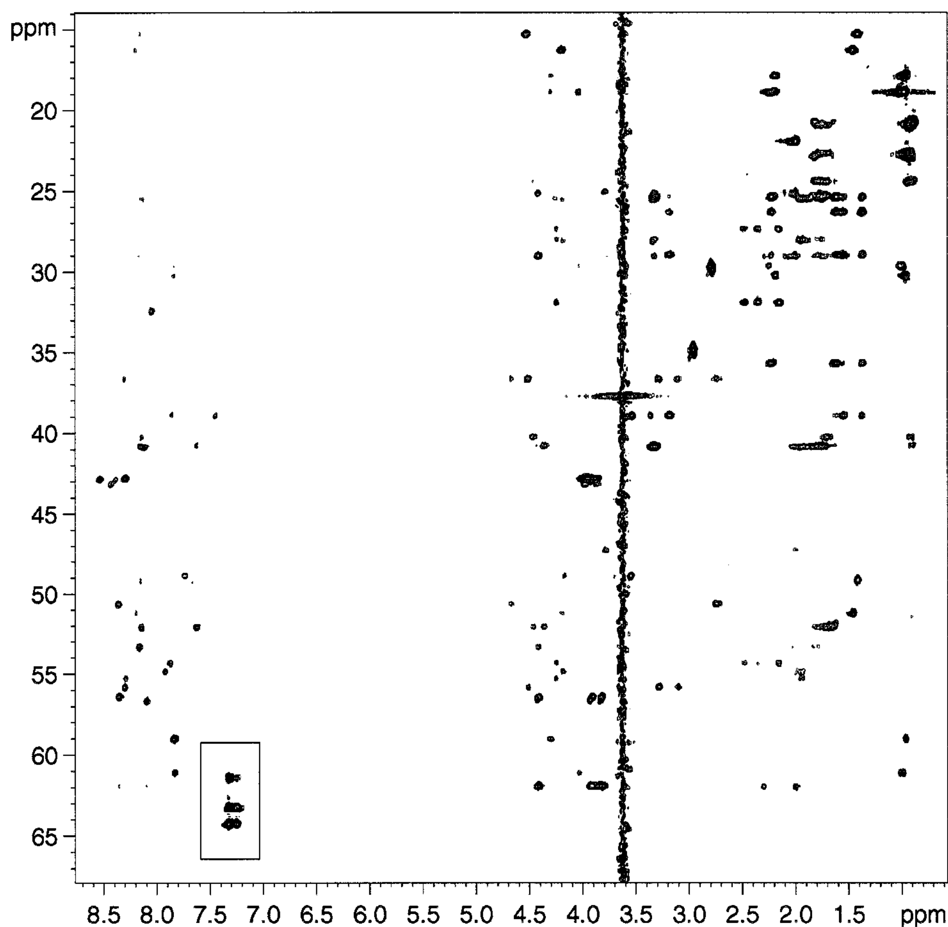

Figure 5. 2D HRMAS $^{1}H-^{13}C$ HSQC-TOCSY spectrum of FMDV epitope bound to POEPOP resin, swollen in DMF-d_7. Cross-peaks inserted in the box are relative to the folded aromatic signals.

the peptide−resin conjugate in an aqueous phosphate buffer. It must be noted that the dispersion of the NH resonances was lower than the one observed in the two aprotic organic solvents. However, it was possible to perform the total amino acid assignment and a qualitative characterization of the peptide conformation in this medium (Figure 7). Compared to the free peptide previously analyzed in water, all the amide protons are shifted upfield by ~0.20 ppm (Table 2). This tendency is in agreement with the different temperatures of the two studies (285 K in solution and 300 K on the resin).[16] The chemical shifts of the α-protons of the two systems differ by less than 0.05 ppm.

A series of sequential αH−NH strong NOEs all along the backbone accompanied by a certain number of less intense amide−amide proton correlations was indicative of a conformational averaging (Figure 7B). The lack of medium- and long-range correlations suggests that the solid-supported peptide is flexible in aqueous solution. Some other side chain−main chain sequential and short-range NOEs, namely, γH4−NH5, γH4−NH6, γH13−NH14, γH15−NH16, and γH15−NH17, were detected and found to characterize also the free peptide in solution.[16] In full agreement with the NOE pattern, the application of the chemical shift index method did not indicate a regular folding.[23]

Discussion

Besides its application in solid-phase organic chemistry, HRMAS NMR spectroscopy is a very promising technique for the structural study of resin-bound peptides. HRMAS has been used to investigate the aggregation properties of peptides covalently linked to a polystyrene resin.[3] In addition, cross-linked polyoxyethylene−polyoxypropylene resins have allowed the HRMAS characterization of short peptides on a single resin bead, opening the doors to the possible identification of active compounds derived from one-bead, one-compound combinatorial libraries.[4] Another field of great interest is the analysis of the conformation adopted by biologically relevant peptides on a solid support.[5,6] Opella and co-workers showed some years

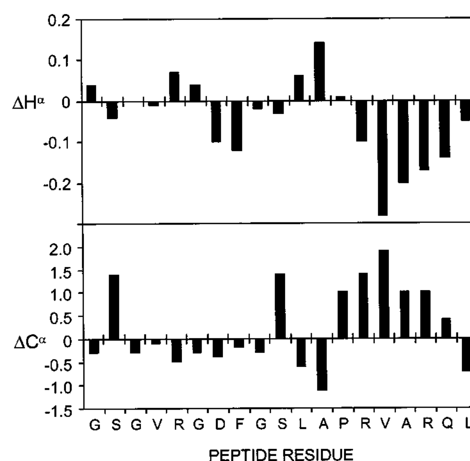

Figure 6. Chemical shift index of α-protons (A) and α-carbons (B) plotted for the 19 residues of the FMDV peptide bound to POEPOP resin.

(23) Wishart, D. S.; Sykes, B. D.; Richards, F. M. *Biochemistry* **1992**, *31*, 1647−1651.

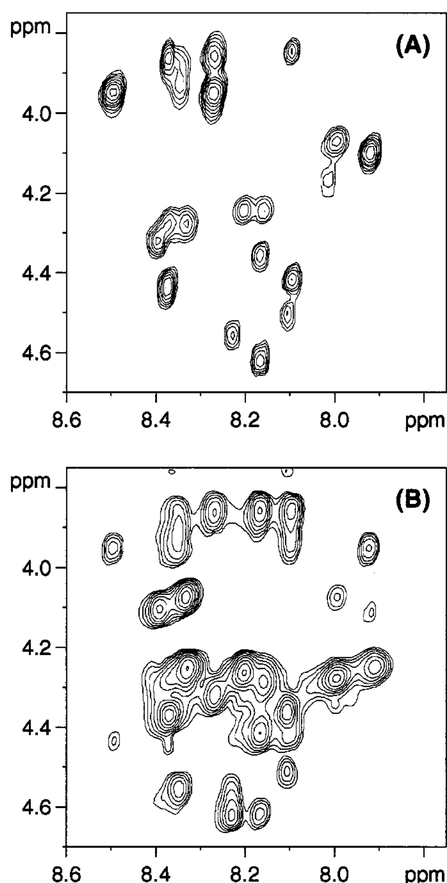

Figure 7. Fingerprint region of the HRMAS 2D TOCSY (A) and NOESY ($\tau_m = 250$ ms) (B) spectra for the resin-bound FMDV epitope, swollen in 50 mM aqueous phosphate buffer (pH 5.7).

Table 2. ^{1}H and ^{13}C Chemical Shifts (ppm) of 141−159 FMDV Peptide Bound to POEPOP Resin Swollen in 50 mM Phosphate Buffer at 300 K

residue	NH	αH	αC	residue	NH	αH	αC
Gly 1	8.34	3.98	45.2	Leu 11	8.16	4.36	54.8
Ser 2	8.37	4.43	58.4	Ala 12	8.10	4.50	50.5
Gly 3	8.49	3.95	45.2	Pro 13		4.37	63.2
Val 4	7.92	4.10	62.2	Arg 14	8.37	4.28	56.0[a]
Arg 5	8.39	4.32	56.1	Val 15	8.00	4.07	62.0
Gly 6	8.26	3.86	45.2	Ala 16	8.33	4.27	52.5
Asp 7	8.16	4.62	53.3	Arg 17	8.20	4.24	56.2
Phe 8	8.23	4.56	58.0	Gln 18	8.33	4.29	56.0[a]
Gly 9	8.34	3.93	45.2	Leu 19	8.15	4.24	55.4
Ser 10	8.09	4.42	58.4				

[a] Overlapped.

ago that an immunogenic peptide of six residues in length is able to adopt a stable conformation when linked to a Tentagel-like resin.[5]

To investigate further this aspect, we have compared the propensity of the 141−159 FMDV antigenic peptide (USA variant) to fold into a helical structure when bound to different solid supports, namely, MBHA, PEGA, and POEPOP resins.[10,11] Indeed, correlations between the antigenic properties and conformational features of different FMDV variants have been studied.[15]

The combination of the swelling and solvation properties of resin-bound peptides with the chemicophysical nature of the solid supports plays a fundamental role in the characterization of such conjugates by HRMAS NMR spectroscopy. For the conformational studies of the solid-supported peptides, we have initially chosen deuterated dimethylformamide as the best common solvent for the swelling of the three different resins. The three resin-linked peptides display similar conformational behavior, although the polymer matrix influences the quality of the spectra and makes, in the case of MBHA and PEGA resins, their interpretation more difficult. The FMDV peptide attached to MBHA is characterized, on the only visible N-terminal part, by the presence of an equilibrium between different conformations in fast exchange. The complete absence of signals from the C-terminal sequence certainly arises from a strong lack of mobility in this region, even when a spacer is inserted between the peptide and the solid support. A decrease of the peptide concentration on the MBHA resin by a factor of 8 and 20 did not allow detection of the C-terminal residues. The complete spin system assignment was also very difficult in the case of the short [13−19] sequence, due to the lack of many intraresidue correlations. In view of these results, we rule out the phenomenon of aggregation. Previous work has shown that this process is generally propagated all along the resin-bound peptide, thus hampering the identification of any NMR signal.[3] We believe that the rigidity of the C-terminal part of the peptide should be ascribed to the interaction of this particular sequence, in a folded or an unfolded structure, with the aromatic core of the resin. In addition, a decrease of the signal intensities of the residues following Gly9 is indicative of a gradient of mobility along the peptide backbone (Figure 1A). As the choice of the swelling solvent is related to the hydrophilic or hydrophobic nature of the polymer and to that of the attached peptide,[1a] we tentatively tried to use a solvent that disrupt ordered structures, such as dimethyl sulfoxide, to induce more flexibility at the C-terminal part of FMDV. Again, only the first 12 N-terminal residues were detected on the 2D HRMAS spectra of the peptide−resin conjugate swollen in DMSO-d_6. In both DMF and DMSO, the signals of the methyl groups of Val15, Ala16, and Leu19 were the sole visible at the C-terminus in the ^{1}H−^{13}C edited spectrum (see Supporting Information). These methyl are more mobile than the peptide backbone and can therefore be detected even if the backbone is not visible.

A higher resolution was achieved with PEGA and POEPOP resins swollen in dimethylformamide. All chemical shifts of the peptide attached to PEGA have been assigned, and the NMR data show a conformational averaging. Few elements of secondary structure could be identified at the N-terminal part, suggesting only a propensity of the peptide to adopt a folded conformation in this region. PEGA resin is constituted of copolymerized bis-2-acrylamidoprop-1-yl-PEG, *N*,*N*-dimethyl-acrylamide, and 2-acrylamidoprop-1-yl-(2-aminoprop-1-yl)-PEG, which introduce in the core of the resin an array of amide groups.[10] These potential donors and acceptors of H-bonding may give rise to the possibility of intermolecular interactions between the resin core and the bound peptide, in competition with the peptide self-folding into a regular helical structure. The best results were obtained in the case of the peptide−POEPOP conjugate, with a quality and a dispersion of the signals comparable to that of the spectra of the free peptide in solution. The FMDV peptide adopted a N-terminal conformation very close to that of the other two peptide−resin conjugates. More interestingly, a defined and regular helical structure, which encompasses C-terminal residues 152−159, beginning as an α-helix and terminating with a 3_{10}-helical type from Val15 to Leu19, could be determined. This helix mixture is not surprising since it has been found in several proteins as well as at the C-terminal part of the free FMDV peptide studied in a solution of TFE.[15d,21] In particular, the 3_{10}-helix may be present at the N- and C-termini of α-helices, involving less than four residues.

The helix of FMDV peptide–resin system is probably distorted by the presence of Pro13 within the helical conformation.[20] It has been shown that the USA peptide variant adopts in TFE a helical structure in the region 151–158 (Leu11/Gln18). A loop, not stabilized by H-bonds, was also identified around the RGD sequence, which is used by the virus to bind the cell receptor in the initial stage of the infection. The helical folding characterizes the C-terminal part of several FMDV peptide analogues and would be induced by TFE.[24] TFE is a weak H-bonding solvent with a dielectric constant lower than that of water ($\epsilon_{25} = 26$). Aprotic dimethylformamide could be also considered as a solvent with a low capacity to compete with the formation of intra- and intermolecular H-bonds. Moreover, the value of its dielectric constant ($\epsilon_{25} = 36.7$) is close to the ϵ of TFE. Although DMF is not an usual solvent for the conformational analysis of peptides in solution, we may tentatively conclude that these two solvents are able to induce similar conformations. We have undertaken the recording of a series of bidimensional spectra of the FMDV peptide in a solution of DMF-d_7 at different concentrations and temperatures. It appears that the secondary structure of the peptide in this solvent is less well-defined in comparison with that of the POEPOP-linked peptide. Short-range NH–NH and αH–NH cross-peaks are present all along the peptide backbone. However, a lower amount of NOE correlations typical of α- and 3_{10}-helices are present (see Supporting Information).

Finally, we have acquired 1D and 2D HRMAS spectra of the POEPOP–FMDV peptide swollen in water in order to evaluate its conformational behavior in this medium. The lower dispersion of the proton signals and the reduced number of NOE correlations suggest that the peptide structure is flexible. This has also been shown for the same conjugate swollen in the H-bonding acceptor solvent dimethyl sulfoxide. It has been found very recently that, in aqueous solution, the free peptide is characterized by two β-turns at the C-terminus, rather than a helical arrangement.[16] Interestingly, the values of the α-proton chemical shifts of the peptide bound to POEPOP were found to be the same as those of the free peptide. Moreover, similar spatial correlations were observed, thus indicating identical folding features for the two systems in aqueous solution. The conformational change of the FMDV antigenic peptide from a helix to a more flexible structure was demonstrated to be important for the recognition by antibodies.[16]

Conclusion

We have analyzed the conformation of a highly immunogenic peptide directly attached to three different solid supports using the emergent high-resolution magic angle spinning NMR technique. In the case of MBHA- and PEGA-bound peptide, the interpretation of the NOE parameters, which define the molecule conformation, was not straightforward, suggesting only a propensity toward a folded structure. However, a well-defined and regular helical structure, which is very close to the secondary structure adopted by the peptide in a solution of 2,2,2-trifluoroethanol, could be identified for the FMDV peptide linked to POEPOP resin in dimethylformamide. Indeed, this study clearly shows that a bioactive peptide bound to a solid support folds into an ordered conformation. Moreover, the folding of this peptide changes as the peptide–POEPOP is swollen in aqueous solution, showing a conformation similar to that adopted by the free peptide in water. Most importantly, this result opens the doors to the structural analysis of peptides without removing the molecules from the resin. The possibility of studying ordered secondary and tertiary structures directly on the solid support will certainly be extended to other biologically relevant peptides and proteins and to new synthetic oligomers.

Experimental Procedures

General Information. All reagents and solvents were obtained from commercial suppliers and used without further purification. Tetrahydrofuran (THF) and dichloromethane (DCM) were carefully distilled prior to use. MBHA and PEGA resins were purchased from Applied Biosystems (Foster City, CA) and Novabiochem (Läufelfingen, Switzerland), respectively. POEPOP resin (substitution, 0.82 mmol/g) was prepared as described by Renil and Meldal,[11a] using poly(ethylene glycol) 1000 (PEG_{1000}) furnished by Fluka. RP-HPLC analysis was done on a C_{18} column (5 μm, 150 × 4.6 mm) using a linear gradient of (A) 0.1% TFA in water and (B) 0.08% TFA in acetonitrile, 5–65% B in 20 min at 1.2 mL/min flow rate. Chromatograms were recorded at 210-nm wavelength. MALDI-TOF mass analysis was performed on a linear MALDI-TOF Bruker instrument using α-cyano-4-hydroxycinnamic acid as matrix. Amino acid analysis was performed on an Applied Biosystem 130A separation system coupled to an Applied Biosystem 420A derivatizer.

Abbreviations. Symbols and abbreviations for amino acids and peptides are in accord with the recommendations of the IUPAC–IUB Commission on Nomenclature (*J. Biol. Chem.* **1972**, *247*, 977). Other abbreviations: Boc, *tert*-butyloxycarbonyl; *t*Bu, *tert*-butyl; Bzl, benzyl; DEAD, diethylazodicarboxylate; DSS, 2,2-dimethyl-2-silapentane-5-sulfonate sodium salt; Fmoc, fluorenylmethyloxycarbonyl; OSu, hydroxysuccinimidyl; TFA, trifluoracetic acid, TFE, 2,2,2-trifluoroethanol; TMSTf, trimethylsilyl trifluoromethanesulfonate; MALDI, matrix-assisted laser desorption/ionization; NOE, nuclear Overhauser effect.

Peptide Synthesis. The sequence of the peptide, corresponding to the VP1 region 141–159 of FMDV (variant USA), used in this study is ^{141}GSGVRGDFGSLAPRVARQL^{159}. An additional 6-aminohexanoic acid residue (Ahx) was added to the C-terminal part of the peptide in order to have a spacer between the peptide and the resins. The N-terminus of the peptide was acetylated. Two peptides were synthesized on MBHA resin (0.8 mmol/g): one without Ahx residue and the other containing Ahx at the C-terminus. The short N-terminal acetylated heptapeptide ^{153}PRVARQL^{159} was synthesized on MBHA resin (0.8 mmol/g). Two peptides, without Ahx spacer, were synthesized on MBHA resin decreasing the loading to 0.11 and 0.043 mmol/g, respectively. The peptides on PEGA (0.4 mmol/g) and POEPOP-NH_2 (0.58 mmol/g) resins were both prepared with Ahx as C-terminal spacer. The synthesis of the MBHA resin-bound peptides were performed on a multichannel peptide synthesizer[25] using standard Fmoc/*t*Bu strategy, while Boc/Bzl chemistry was used for the automatic syntheses of the peptides linked to PEGA[10] and amino-POEPOP resins. The side-chain protecting groups were removed using reagent K and HF, respectively.[26] The peptides were characterized by amino acid analysis after hydrolysis in 6 N HCl for 24 h at 110 °C. Peptide on MBHA resin was also cleaved by treatment with a mixture of TFA and TMSOTf[27] and analyzed by HPLC and MALDI mass spectrometry. Peptide for the 2D NMR study in solution was prepared as previously described.[16]

Synthesis of POEPOP-NH_2 resin. Phthalimide (1.766 g, 12 mmol) was added under Ar to a suspension of $POEPOP_{1000}$ resin (0.82 mmol/g) (2.93 g, 2.4 mmol) in 1:1 dry DCM/THF (100 mL) containing PPh_3 (3.147 g, 12 mmol).[28] The mixture was cooled to 0 °C, and a solution

(24) (a) Luo, P.; Baldwin, R. L. *Biochemistry* **1997**, *36*, 8413–8421. (b) Sönnichsen, F. D.; Van Eyk, J. E.; Hodges, R. S.; Sykes B. D. *Biochemistry* **1992**, *31*, 8790–8798. (c) Khandelwal, P.; Seth, S.; Hosur, R. V. *Eur. J. Biochem.* **1999**, *264*, 468–478. (d) Luidens, M. K.; Figge, J.; Breese, K.; Vajda, S. *Biopolymers* **1996**, *39*, 367–376.

(25) Neimark, J.; Briand, J.-P. *Pept. Res.* **1993**, *6*, 219–228.

(26) King, D.; Fields, C.; Fields, G. *Int. J. Pept. Protein Res.* **1990**, *36*, 255–266.

(27) Limal, D.; Briand, J.-P.; Dalbon, P.; Jolivet, M. *J. Pept. Res.* **1998**, *52*, 121–129.

(28) (a) Mitsunobu, O. *Synthesis* **1981**, 1–28. (b) Cho, C. Y.; Youngquist, R. S.; Paikoff, S. J.; Beresini, M. H.; Hebert, A. R.; Berleau, L. T.; Liu, C. W.; Wemmer, D. E.; Keough, T.; Schultz, P. G. *J. Am. Chem. Soc.* **1998**, *120*, 7706–7718.

of DEAD (1.890 mL, 12 mmol) in 1:1 dry DCM/THF (20 mL) was added over 1 min, followed by an additional 20 mL of solvent. The reaction mixture was stirred for 26 h at room temperature, and the resin was filtered, washed with DCM, DMF, NMP, methanol, DCM, and diethyl ether, and dried under high vacuum. The phthalimide protecting group was removed treating the resin, swollen in NMP (20 mL), with hydrazine hydrate (20 mL). After stirring 20 h at room temperature, the resin was extensively washed with methanol, DMF, DMSO, water, DMF, DCM, and diethyl ether and dried under vacuum. The loading was calculated by reaction of a weighted amount of resin with Fmoc-Osu (10 equiv) in DCM. After repeating the reaction twice, Fmoc was removed with 25% piperidine in DMF and recovered for the determination of the UV absorbance at 306 nm. Loading 0.58 mmol/g.

NMR Spectroscopy. The identification of amino acid spin systems and sequential assignment were made using a combination of HRMAS TOCSY,[29] NOESY,[30] and HSQC[31] experiments. 1D and 2D liquid-state NMR spectra were recorded on a Bruker ARX 500-MHz spectrometer. The sample was dissolved in DMF-d_7. HRMAS 1D and 2D NMR spectra were obtained on Bruker Avance 400- and 500-MHz spectrometers equipped with for 4-mm ^{1}H/^{15}N/^{2}H and ^{1}H/^{13}C/^{2}H HRMAS gradient probes. The samples were packed into a 4-mm HRMAS rotor, and solvents were added to the resin directly inside the rotor. In all experiments, samples were spun between 4 and 8 kHz. The spectra were acquired at a temperature of 300 K and referenced to the peak of the solvent. DSS was used as external reference to calibrate the spectra in aqueous solution. All 2D spectra were recorded in pure phase mode using a time proportional phase incrementation method. Homonuclear spectra were recorded with 2048 data points in t2 and 256 increments in t1. Typically 8 or 16 scans per increment were accumulated. A spectral width of 4629.63 Hz was used for the proton. TOCSY data were recorded with a MLEV-17 sequence of 50 ms. Through-space dipolar connectivities were obtained from NOESY spectra using mixing times from 250 to 300 ms. ^{1}H−^{13}C and ^{1}H−^{15}N HSQC spectra were recorded with 2048 data points in t2 and 128 or 256 increments in t1. The number of scans accumulated for ^{1}H−^{13}C HSQC were 16, 64, and 64 for POEPOP (expt 1 h 20 min), PEGA (expt 6 h 50 min), and MBHA resin (expt 4 h 30 min), respectively. The number of scans accumulated for ^{1}H−^{15}N HSQC were 32, 64, and 96 for POEPOP (expt 2 h 15 min), PEGA (expt 4 h 30 min), and MBHA resin (expt 6 h 50 min), respectively. Sweep widths for ^{1}H and ^{13}C dimensions were 4629.63 and 35 461 Hz, respectively, while spectral widths for ^{1}H and ^{15}N dimensions were 4807.69 and 8110.42 Hz, respectively. A 2D ^{1}H−^{13}C HSQC-TOCSY spectrum was acquired, limiting the spectral region of the carbon atoms to the aliphatic resonances (∼50 ppm), using a sweep width of 5000 Hz for ^{1}H and 8174.97 Hz for ^{13}C.[32] The number of scans was 64, requiring an experimental time of 7 h 30 min. WATERGATE pulse sequence was applied for the suppression of water signal.[33] The samples were swollen in DMSO-d_6, DMF-d_7, and 50 mM phosphate buffer (pH 5.7) with 10% D_2O.

(29) Braunschweiler, L.; Ernst, R. R. *J. Magn. Reson.* **1983**, *53*, 521−528.

(30) Jeneer, J.; Meier, B. H.; Bachmann, P.; Ernst, R. R. *J. Chem. Phys.* **1979**, *71*, 4546−4553.

(31) Bodenhausen, G.; Ruben, D. J. *Chem. Phys. Lett.* **1980**, *69*, 185−189.

Acknowledgment. The authors thank Mrs C. Lichte and Dr J. Reinbolt for the amino acid analysis, and Dr R. Graff for recording 1D and 2D NMR data in solution. Dr. W. Saudek and Dr. S. Improta are also acknowledged for their critical review of the manuscript. This work was supported by a grant from EC (No. Fair 5-CT-3577).

Supporting Information Available: 500-MHz HRMAS TOCSY, NOESY, and HSQC spectra of 141−159 FMDV epitope bound to MBHA resin, PEGA resin, and POEPOP resin, swollen in different deuterated solvents. 500-MHz COSY, TOCSY, NOESY, and ROESY spectra of 141−159 FMDV epitope in DMF-d_7 solution at different concentrations and temperatures. This material is available free of charge via the Internet at http://pubs.acs.org.

JA003566W

(32) Willker, W.; Flögel, U.; Leibfritz, D. *J. Magn. Reson.* **1997**, *125*, 216−219.

(33) Piotto, M.; Saudek, V.; Sklenár, V. *J. Biomol. NMR* **1992**, *2*, 661−665.