JOURNAL OF MAGNETIC RESONANCE **125,** 185–187 (1997)
ARTICLE NO. MN961099

Two-Dimensional NMR Spectroscopy of Peptides on Beads

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Received November 27, 1996; revised December 17, 1996

There is currently considerable interest in utilizing combinatorial chemistry for identification and extraction of novel compounds as pharmacological and immunological agents (*1*). Large libraries of peptides can be generated with both chemical and biological approaches and subsequently screened for biological activity, making possible the identification of vital amino acid sequences (*2*). One of the most widely used techniques for construction of combinatorial peptide libraries is the synthesis of peptides covalently attached to solid supports, usually polymeric resin beads, followed by cleavage of the peptides and biological screening with various ligand binding assays (*3*). Significantly, synthesized peptides can exhibit biological activity while still attached to the solid supports (*4–6*). This suggests that peptides covalently bonded to polymers can adopt biologically relevant conformations. In this Communication, we describe NMR experimental results which suggest that an immunogenic peptide adopts a folded structure while bound to polystyrene-based tentagel [TG] resin. These results indicate that there may be a role for three-dimensional structure determination by NMR spectroscopy in combinatorial peptide chemistry. This could be important for drug design as well as structural biology of small peptide domains attached to beads.

The conserved sequence Gly–Pro–Gly–Arg–Ala–Phe [GPGRAF, single letter code] on the tip of the V3 loop of the gp120 glycoprotein of HIV-1 is believed to be the principal neutralizing determinant of the virus (*7, 8*). A stable conformation of the six-residue sequence embedded in longer peptide sequences has been detected in solution (*9–11*) and in the sequence displayed on the major coat protein of filamentous bacteriophage solubilized in detergent micelles (*12*).

Figure 1 consists of a schematic representation of the peptide bonded to polymer resin and of experimental one-dimensional ^{1}H NMR spectra of the complex. Conventional solid-phase synthesis (*13*) using amino acids protected by Fmoc groups, with NovaSyn TG resin (Nova Biochem) as the solid support, was utilized. Removal of the protecting groups and several cycles of washing/drying were carried out prior to the NMR experiments. The samples for the NMR experiments were prepared by swelling the peptide–resin complex in DMSO-d_6. The integrity and purity of the peptides were verified by mass spectrometry and conventional two-dimensional ^{1}H/^{15}N HMQC NMR spectra obtained after cleavage from the solid support.

Figures 1B and 1C contain ^{1}H NMR spectra of the peptide–resin complex swelled in DMSO-d_6. The stationary ^{1}H NMR spectrum in Fig. 1B is very broad because of the limited molecular motion of the polymer resin and the attached peptide. Magic-angle spinning (MAS) is successful in reducing broadening caused by chemical-shift anisotropy and homonuclear ^{1}H–^{1}H dipolar broadening, as well as inhomogeneity due to differences in the magnetic-field susceptibility at the bead interface (*14, 15*), resulting in much narrower ^{1}H resonances, as shown in Fig. 1C. The ^{1}H resonances observed in the one-dimensional NMR spectrum in Fig. 1C arise from the peptide, the polystyrene-based TG resin, and the polyethylene-glycol linker (peptide on beads).

Resonance assignments and structural analysis of the peptide can be accomplished with two-dimensional NMR experiments on swollen peptide–resin samples in the presence of magic-angle spinning (*14*). Figure 2A contains the two-dimensional ^{1}H/^{15}N-HMQC spectrum of the GPGRAF bead complex synthesized with ^{15}N-Gly, ^{15}N-Ala, and ^{15}N-Phe at positions 3, 5, and 6, respectively. Each resonance in the HMQC spectrum is associated with a specific residue, as indicated in Fig. 2A and was assigned by conducting separate HMQC experiments with single ^{15}N-labeled amino acids incorporated in the peptide.

Figure 2B contains the amide resonance region of the two-dimensional ^{1}H NOE spectrum of GPGRAF coupled to polymer resin. Remarkably, approximately 20 intra- and interresidue NOE cross peaks are observed from the six-residue peptide on beads. In general, NOE cross peaks are observable only if the molecules exhibit a stable conformation, which allows magnetization transfer between nearby ^{1}H nuclei (*16*). In addition, short peptides in solution usually do not yield any NOE cross peaks because of their fast molecular motion and conformational flexibility. The relatively large number of NOE cross peaks in the spectrum in Fig. 2B suggests that a significant part of the population of the peptide on beads is folded into a defined, stable conformation with less molecular motion than for the same peptide in solution. Assignments of the resonances to the respective

1090-7807/97 $25.00

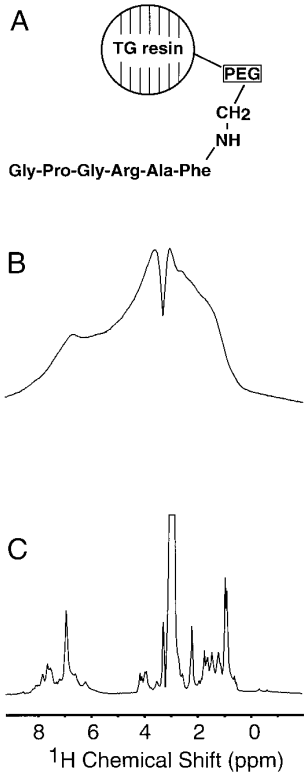

FIG. 1. (A) Schematic diagram of the peptide–tentagel [TG] resin complex. The GPGRAF peptide is covalently bonded to the polyethyleneglycol [PEG] linker through an amide bond. (B, C) ^{1}H NMR spectra of the GPGRAF-resin complex swelled in DMSO-d_6. (B) Stationary sample. (C) Magic-angle spinning (MAS) at 5 kHz. The spectra were taken on a Bruker DMX 750 NMR spectrometer using a Bruker VT-MAS probe. The spectra were referenced externally to DMSO at 2.5 ppm.

amino acids are based on the ^{15}N/^{1}H HMQC spectrum in Fig. 2A and NOE cross peaks between adjacent residues. In particular, a number of cross peaks associated with important interresidue NOEs identified in the previously characterized turn conformation of the GPGRAF sequence (*9–12, 17, 18*) are present. Among these are sequential $NN_{i,i+1}$ NOEs between Gly-3 and Arg-4 and between Ala-5 and Phe-6, respectively, as marked in Fig. 2B. Other diagnostic NOEs include $\beta N_{i,i+1}$ between Arg-4 and Ala-5 and between Ala-5 and Phe-6, as well as an interresidue NOE between Pro-2 and Gly-3.

Several two-dimensional ^{1}H NOE spectra that serve as control spectra are shown in Fig. 3. The spectrum of GPGRAF on beads acquired at a temperature of 50°C is shown in Fig. 3B. The positions of the ^{1}H resonances are shifted, and the intensities of various NOE cross peaks are substantially reduced compared to those observed in the spectrum obtained at 25°C. The cross peaks apparent in the spectrum in Fig. 3B are most likely due to intraresidue NOEs. The spectral changes that accompany the increase in temperature can be explained by the increased molecular motion and a reduction in the number of peptides having folded conformation.

NMR spectra were also obtained for other six-residue peptide sequences chosen because of their having little likelihood of folded conformations. Figure 3C contains the two-dimensional ^{1}H NOE spectrum of GAGAGA on beads, which shows no interresidue NOE cross peaks, indicating the absence of a stable conformation. In addition, the spread of the amide chemical-shift frequencies, which is only 0.4 ppm in GAGAGA, is smaller than observed in the spectrum of GPGRAF (approximately 1 ppm) and is also indicative of a random or flexible conformation (*16*). Another sequence, GGGGGG, which exhibits a propensity for helix formation in solution but aggregates when attached to polymer resins (*19*), also exhibits small chemical-shift dispersion, Fig. 3D, and only ^{1}H NOEs which are most likely intraresidue in the

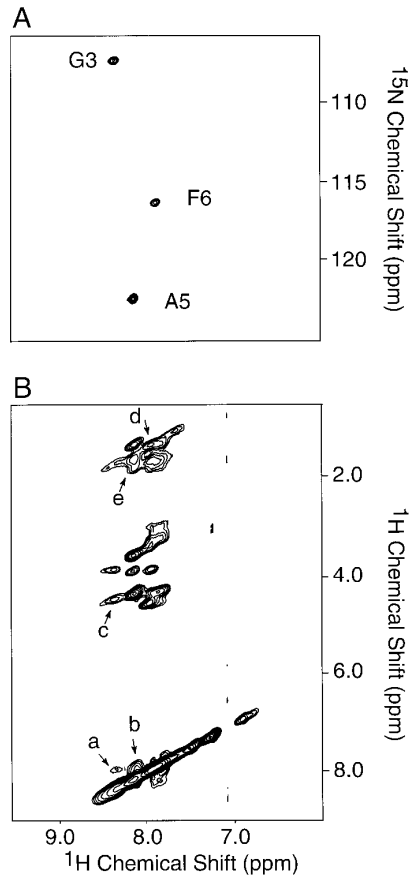

FIG. 2. Two-dimensional NMR spectra of the GPGRAF–resin complex swelled in DMSO-d_6. (A) ^{15}N/^{1}H HMQC spectrum of the peptide synthesized using ^{15}N-Gly, ^{15}N-Ala, and ^{15}N-Phe at positions 3, 5, and 6, respectively. ^{15}N was externally referenced to *N*-acetylglycine at 118.67. (B) Two-dimensional ^{1}H NOE spectrum. The mixing time of 200 ms was not in the spin-diffusion region, as determined by a buildup curve (*16*). NOE cross peaks marked in the spectrum: (a) Gly3–Arg4 $NN_{i,i+1}$, (b) Ala5–Phe6 $NN_{i,i+1}$, (c) Pro2–Gly3 $\alpha N_{i,i+1}$, (d) Ala5–Phe6 $\beta N_{i,i+1}$, (e) Arg4–Ala5 $\beta N_{i,i+1}$.

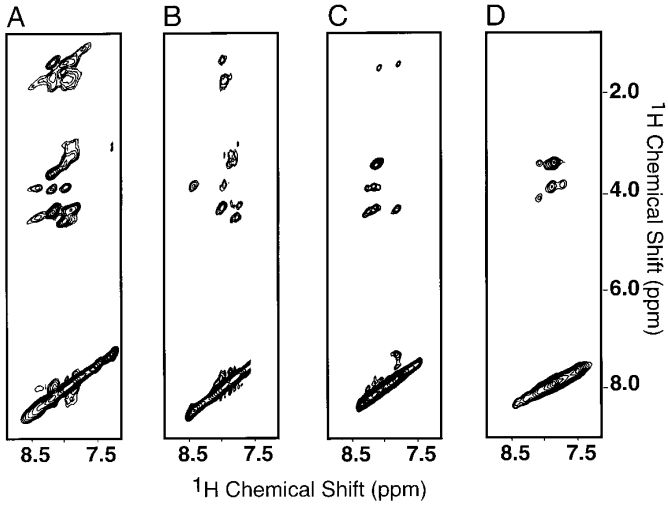

FIG. 3. Two-dimensional ^{1}H NOE spectra of peptide–resin complexes swelled in DMSO-d_6. Identical NMR parameters were used for all of the spectra, which have similar signal-to-noise ratios: (A) GPGRAF at 25°C; (B) GPGRAF at 50°C; (C) GAGAGA at 25°C; (D) GGGGGG at 25°C.

alpha-carbon resonance region (around 4 ppm). The reduced number of NOEs in the spectra of GAGAGA and GGGGGG may be attributable to increased motion, as well as to decreased folding of these sequences when they are attached to beads, compared to GPGRAF.

The NMR evidence for a folded conformation of the peptide GPGRAF attached to beads is consistent with findings of higher levels of biological activities and immunogenic properties when peptide sequences are parts of larger molecular entities. It has been shown, for example, that GPGRAF binds strongly to anti-HIV antibodies when it is displayed on the surface of filamentous phage, as detected in ELISA assays (*20*). A multidimensional NMR study has characterized a conformation of the hexapeptide displayed on the major coat protein of the filamentous phage that is more well defined than the conformation of the free peptide in solution (*12*). Previous studies have also shown formation of helices in short polypeptides linked to an organic ''template'' (*21*). It is hypothesized that stable conformations of very short sequences are associated with steric constraints imposed by binding of the peptides to much larger biological or chemical compounds.

Two-dimensional ^{1}H MAS NMR experiments suggest that the GPGRAF sequence populates a folded conformation when attached to a polymer resin. Since peptides in libraries exist in this form and have biological activities, this NMR approach has the potential to contribute to biochemical and biotechnological research and development of novel peptide compounds originating in combinatorial chemistry approaches.

ACKNOWLEDGMENTS

This research was supported by Grant R37 GM24266 from the National Institutes of Health and utilized the Resource for Solid State NMR of Proteins at the University of Pennsylvania, supported by Grant P41 RR09731 from the Biomedical Technology Program Division of Research Resources, National Institutes of Health. R.J. was supported by a postdoctoral fellowship from the Cancer Research Institute, and A.P.V. was supported by a postdoctoral fellowship from FAPESP—Brazil (Fundação de Amparo a Pesquisa do Estado de São Paulo).

REFERENCES

1. G. P. Smith, *Science* **228,** 1315 (1985).
2. M. A. Gallop, R. W. Barrett, R. W. Dower, S. P. A., and E. M. Fodor, *J. Med. Chem.* **37,** 1233 (1996).
3. R. A. Houghten, C. Pinilla, S. E. Blondelle, J. R. Appel, C. T. Dooley, and J. H. Cuervo, *Nature* **354,** 84 (1994).
4. H. M. Geysen, R. H. Meloen, and S. J. Barteling, *Proc. Natl. Acad. Sci. USA* **81,** 3998 (1984).
5. K. S. Lam, S. E. Salmon, E. M. Hersh, V. J. Hruby, W. M. Kazmierski, and R. J. Knapp, *Nature* **354,** 82 (1991).
6. J. D. McBride, N. Freeman, G. J. Domingo, and R. J. Leatherbarrow, *J. Mol. Biol.* **259,** 819 (1996).
7. G. J. LaRosa, J. P. Davide, and K. Weinhold, *Science* **249,** 932 (1990).
8. K. Javaherian, A. J. Langlois, and G. J. LaRosa, *Science* **250,** 1590 (1990).
9. K. Chandrasekhar, A. T. Profy, and H. J. Dyson, *Biochemistry* **30,** 9187 (1991).
10. A. Zvi, R. Hiller, and J. Anglister, *Biochemistry* **31,** 6972 (1992).
11. R. de Lorimier, M. A. Moody, B. F. Haynes, and L. D. Spicer, *Biochemistry* **2055,** 33 (1994).
12. R. Jelinek, T. Terry, J. J. Gesell, P. Malik, R. N. Perham, and S. J. Opella, *J. Mol. Biol.,* in press.
13. R. B. Merrifield, *J. Am. Chem. Soc.* **85,** 2149 (1963).
14. S. K. Sarkar, R. S. Garigipati, J. L. Adams, and P. A. Keifer, *J. Am. Chem. Soc.* **118,** 2305 (1996); R. C. Anderson, J. P. Stokes, and M. J. Shapiro, *Tetrahedron Lett.* **36,** 5311 (1995); R. C. Anderson, M. A. Jarema, M. J. Shapiro, J. P. Stokes, and M. Ziliox, *J. Org. Chem.* **60,** 2650 (1995).
15. P. A. Keifer, L. Baltusis, D. M. Rice, A. A. Tymiak, and J. N. Shoolery, *J. Magn. Reson. A* **119,** 65 (1996).
16. K. Wuthrich, ''NMR of Proteins and Nucleic Acids,'' Wiley, New York, 1986.
17. J. B. Ghiara, E. A. Stura, R. L. Stanfield, A. T. Profy, and I. A. Wilson, *Science* **264,** 82 (1994).
18. H. J. Dyson and P. E. Wright, *Annu. Rev. Biophys. Biophys. Chem.* **20,** 519 (1991).
19. A. G. Ludwick, L. W. Jelinski, D. Live, A. Kintanar, and J. J. Dumais, *J. Am. Chem. Soc.* **108,** 6493 (1986).
20. T. Terry, A. E. Willis, F. M. Veronese, and R. N. Perham, unpublished results.
21. D. S. Kemp, J. G. Boyd, and C. C. Muendel, *Nature* **352,** 451 (1991).