Volume 287, number 1,2, 185–188 FEBS 10015 August 1991

ADONIS 001457939100725Q

Uniform labeling of a recombinant antibody Fv-fragment with ^{15}N and ^{13}C for heteronuclear NMR spectroscopy

Lutz Riechmann[1], John Cavanagh[2] and Siobhan McManus[1]

[1]*MRC Laboratory of Molecular Biology, Hills Road, Cambridge CB2 2QH, UK* and [2]*University of Cambridge, Department of Chemistry, Lensfield Road, Cambridge, CB2 1EW, UK*

Received 3 June 1991

The expression of functional antibody fragments in *Escherichia coli* enables a detailed analysis by NMR spectroscopy. This is demonstrated with the uniform labeling of an Fv-fragment (25 kDa) comprising the antigen binding site of an anitbody against 2-phenyloxazolone with ^{15}N and ^{13}C. The antigen-complexed Fv-fragment was analysed for a potential assignment by heteronuclear multi-dimensional NMR spectroscopy. For almost all backbone amides $^{15}N/^{1}H$ crosspeaks and for 80% of them TOCSY crosspeaks were observed. In a ^{13}C-edited-HCCH-2D experiment 17 out of 18 threonine spin-systems were identified. Thus detailed assignments are possible, but some amino acid specific labeling in addition to uniform labeling will be required for complete assignments of Fv-fragments.

Antibody; Fv-fragment; Protein-engineering; NMR; ^{15}N; ^{13}C

1. INTRODUCTION

High field NMR spectroscopy has now become a viable alternative to X-ray crystallography for high-resolution structural studies of macromolecules. Estimates of the size limit for detailed NMR studies of proteins lie between 20 and 30 kDa. In complex systems, resonance overlap in homonuclear proton spectra can be resolved by the use of heteronuclear experiments performed with ^{15}N- and ^{13}C-enriched samples, relying on the greater chemical shift dispersion of the heteronucleus [1]. Concurrently, progress in protein engineering has enabled the production of functional recombinant antibody-subunits in *E. coli* [2,3] and eukaryotic cells [4]. The smallest antibody fragment which still retains the natural antigen binding site is the so-called Fv-fragment. This consists of a pair of non-covalently associated heavy and light chain variable domains, each of which contains three hypervariable loops forming the antigen binding site.

An Fv-fragment has a size of about 25 kDa which formally places it within reach of a detailed structural analysis in solution by NMR spectroscopy. To date, attempts in this direction have been restricted to the labeling of specific amino acids in proteolytically obtained Fab-fragments ([5] and references therein), a proteolytically produced Fv-fragment [6] and a recombinant Fv-fragment expressed in myeloma cells [7] or to site-specific identification of amino acid side chains by mutagenesis of an Fv-fragment expressed in *E. coli* [8].

Correspondence address: L. Riechmann, MRC Laboratory of Molecular Biology, Hills Road, Cambridge, CB2 2QH, UK. Fax: (44) (223) 412178.

Understandably, in none of these cases were more detailed assignments attempted. We report here the first step of such an attempt with the complete ^{15}N- and ^{13}C-labeling of a recombinant antibody Fv-fragment purified from *E. coli* cultures.

2. EXPERIMENTAL

2.1. *Protein expression and purification*

For expression of the Fv-fragment of the anti-2-phenyloxazolone antibody NQ11.7.22, a *Hin*dIII/*Eco*RI-DNA fragment containing the light and heavy chain variable regions [8], was cloned into a modified version of the expression vector pET11 [9]. The *Hin*dIII/*Eco*RI fragment contains ribosome binding sites, the secretory leader sequences of the pectate lyase gene and termination codons for the genes of both chains. The pET11 vector had been modified previously (L. Farrell, unpublished) by insertion of an M13-origin downstream of the transcription terminator to allow rescue of single stranded plasmid DNA with helper phage. A *Hin*dIII site was introduced upstream of the Shine-Delgarno sequence in the original pET11 vector with the mutagenic oligonucleotide 5′-CTA GAA ATA AGC TTG TTT AAC-3′ to facilitate the insertion of the Fv-fragment expression cassette.

For expression, the plasmid was transformed into the *E. coli* strain BL21-DE3 [9]. Isotopically enriched (98%) protein – both ^{15}N singly labeled and $^{15}N/^{13}C$ doubly labeled Fv-fragment – was produced in commercial rich medium (CELTONE, Martek Corp., USA), diluted 3-fold with nitrogen-depleted M9-salts in the presence of 100 mg ampicillin per litre. In case of the ^{15}N-enriched medium 1% glycerol was added as an additional carbon source. 6 ml-cultures, grown at 37°C in 20 ml tubes, were induced with 1 mM isopropyl β-D-thiogalactopyranoside added together with fresh ampicillin (50 mg/l) 12–15 hours after inoculation with fresh bacterial colonies. After incubation at 37°C for a further 24 h, Fv-fragment was purified from the culture medium by antigen-affinity chromatography using 2-phenyloxazolone derivatised EAH-Sepharose [8].

2.2. *NMR spectroscopy*

NMR samples were prepared from 8 mg ^{15}N- and from 8 mg $^{15}N/^{13}C$-labeled Fv-fragment resulting in a protein concentration of

about 0.7 mM. The Fv-fragment/antigen complex was dissolved in 5 mM phosphate, 200 mM NaCl, pH_{app}6.2 in 8% D_2O (^{15}N sample) or 99.9% D_2O ($^{15}N/^{13}C$ sample). NMR spectra were recorded at 313K on a Bruker AMX-500 spectrometer using a broadband 5 mm probe and analysed with UXNMR (Bruker) or FELIX (Hare Research) software. Homonuclear two-dimensional double quantum and TOCSY spectra were recorded as described elsewhere [8]. $^{15}N/^{1}H$ and $^{13}C/^{1}H$ two-dimensional heteronuclear single quantum coherence (HSQC) correlation experiments [10] were recorded along with ^{15}N-filtered-TOCSY-2D and 3D experiments (recorded with an HMQC-sequence [11] after the TOCSY-spin lock) and a ^{13}C-filtered-HCCH-2D-experiment [12], omitting the indirectly obtained ^{1}H-dimension and carbonyl decoupling.

3. RESULTS AND DISCUSSION

The expression of Fv-fragments in a functional, secreted form has been demonstrated for various antibodies [3,4,8,13] using different expression systems. Here, for the production of an isotopically enriched Fv-fragment in *E. coli*, a T7 RNA polymerase expression system [9] was used. Expression of the Fv-fragment is regulated by a T7 gene 10 promotor. The T7 RNA polymerase is provided by the host strain (BL21-DE3) on induction. In this system no metabolic repression (e.g. addition of glucose) of the polymerase gene prior to induction was necessary for optimum yields. Addition of a metabolic repressor to prevent leakage required for many expression systems is not economical in the production of labeled protein. Yields here were comparable (4–6 mg/l in rich medium) to the previously used lacZ-expression system [8], in which 1% glucose was added for metabolic repression prior to induction. In minimal medium yields were generally much lower (less than 0.5 mg/l).

With both the ^{15}N- and the $^{13}C/^{15}N$-enriched medium, 8 mg functional Fv-fragment were purified from 1.5 litres (see Section 2 for details) of commercial rich medium derived from algae-culture, enough for 0.7 mM samples. Although this concentration is extremely low for NMR experiments, it is only slightly below the maximum of about 1 mM at which the Fv-fragment/antigen complex starts to aggregate. The solubility of the free Fv-fragment is even lower than 1 mM. Other Fv-fragments are similarly or even less soluble (unpublished). For the NMR experiments the Fv-fragment was complexed with a 4-fold excess of the ligand 4-glycyl-2-phenyl-oxazolone [8].

In Fig. 1 the spectrum from an $^{15}N/^{1}H$-correlated HSQC experiment recorded with the ^{15}N singly labeled Fv-fragment/phenyloxazolone complex is shown. 245 possible peptide backbone $^{15}N/^{1}H$ crosspeaks are reasonably well resolved. The Fv-fragment has 229 non-proline residues, 8 asparagines and 14 glutamines, thus almost all backbone amides are observed in the simple $^{15}N/^{1}H$-correlation experiment. In ^{15}N-edited-2D and 3D-TOCSY experiments (isotropic mixing time = 26 ms (2D) or 22 ms (3D), not shown) crosspeaks were detected for 183 backbone amides (80%). However,

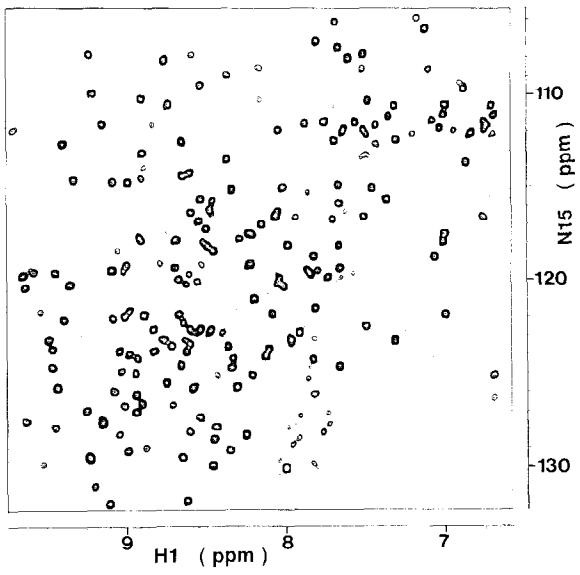

Fig. 1. Spectrum from a $^{15}N/^{1}H$-HSQC experiment recorded with 0.7 mM ^{15}N-labeled Fv-fragment complexed with 2.8 mM ligand in 92% H_2O/8% D_2O.

some α-protons are very close to the H_2O signal (see Fig. 2a) and may have been saturated in our ^{15}N-edited TOCSY experiments in H_2O by employing water suppression.

Fig. 2a originates from a $^{13}C/^{1}H$-correlated HSQC-experiment recorded with the $^{15}N/^{13}C$-labeled sample in D_2O. The problem of overlap in the α-carbon region shown here is very obvious. However, assignments are not impossible, as 17 out of 18 threonine side chains were identified using a 2D-HCCH experiment (Fig. 2c) in combination with homonuclear double quantum (not shown) and TOCSY-spectra (Fig. 2b) recorded with the unlabeled Fv-fragment. The general overlap in the α region made it impossible to distinguish many threonine from alanine spin systems in the homonuclear experiments. In the HCCH experiment the difference in the ^{13}C chemical shift of alanine-α-carbons ($\sim$ 50 ppm) and threonine-β-carbons ($\sim$ 20 ppm), whose attached protons lie in the same chemical shift range, enabled assignment. Also ^{1}H-chemical shift degeneracies of different threonines (e.g. Thr-12 and Thr-13 in Fig. 2a) were resolved in the ^{13}C-dimension.

However, low sensitivity due to fast relaxation of the Fv-fragment (T_1 = 1.1 s) will make assignment of many spin systems very difficult or impossible, when no help like the strong γ/β crosspeak in case of the threonines is available. Therefore amino acid specific labeling is likely to be necessary for sequence specific assignments even when triple resonance experiments (HCACO, HNCA, HNCO, [1]) for the assignment of the peptide backbone are used.

Based on these results we can now formulate a strategy for the assignment of antibody Fv-fragments.

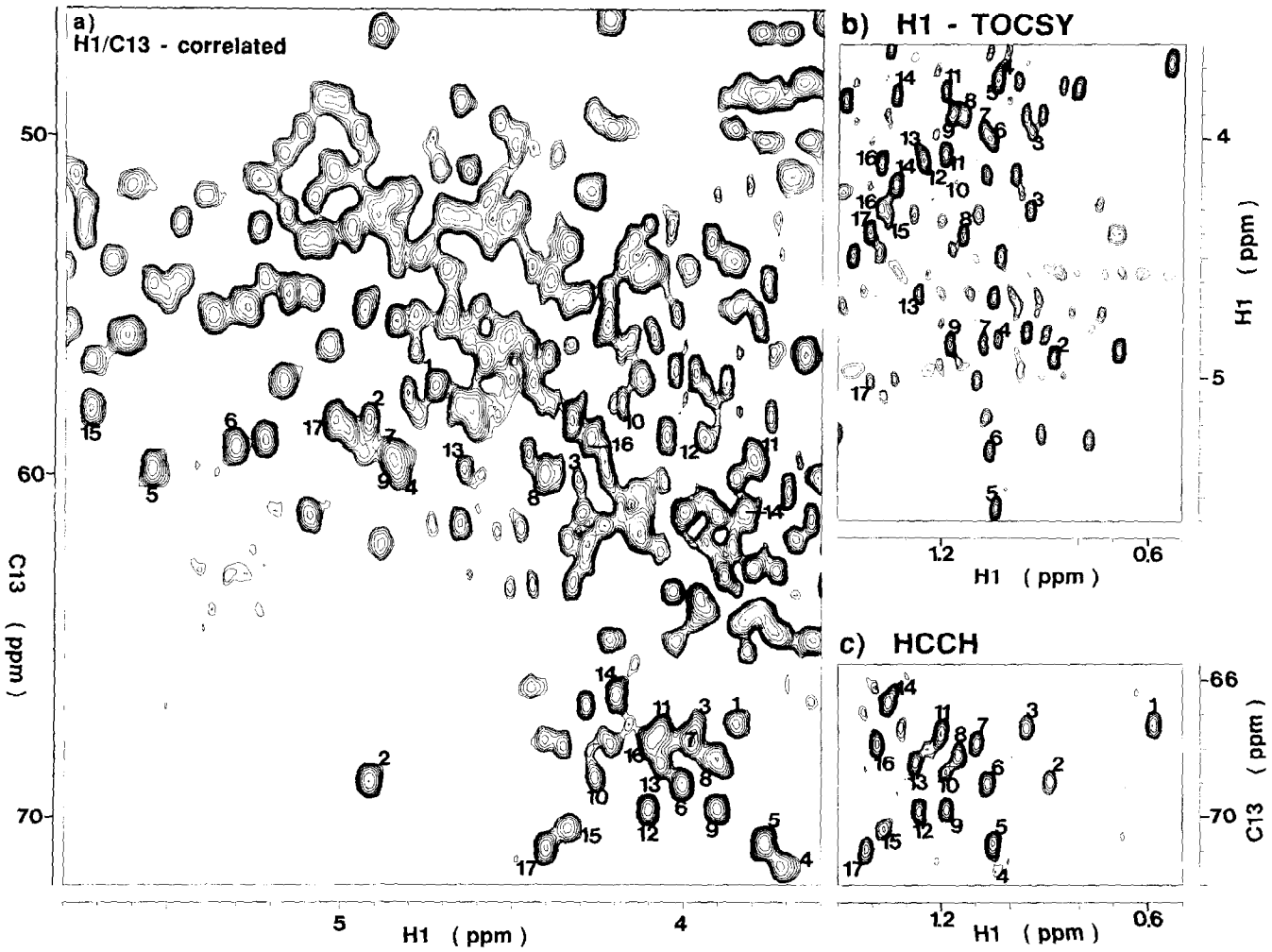

Fig. 2. Sections from, (a) a $^{13}C/^{1}H$-HSQC-2D and (c) a HCCH-2D experiment recorded with 0.7 mM $^{13}C/^{15}N$-labeled Fv-fragment complexed with 2.8 mM ligand in D_2O, and (b) a ^{1}H-TOCSY-2D experiment recorded with 1 mM unlabeled Fv-fragment complexed with 4 mM ligand. The threonine spin systems are emphasized by numbering.

Most of the protons of the Fv-fragment are observed and may in principle be identified through sufficient effort in, for example, amino acid-type-specific isotopic labeling. This is certainly a good investment in the case of a 'prototype' Fv-fragment if its β-sheet frameworks (comprising more than 80% of an Fv-fragment) are later used in combination with 'binding-site loops' from other antibodies, as in the case of reshaped human antibodies, when murine binding sites were grafted on to a human β-sheet framework [14]. In this situation, subsequent assignments may be based largely upon the signals of the prototype Fv-fragment.

However, complete assignments may not be necessary in each case, given the number of antibody structures available from crystallographic studies [15]. A more economical approach will often be the use of the heteronuclear NMR experiments for assignments in specific regions such as the antigen binding site. Identification of NMR-signals from key residues can be initiated by site-directed mutagenesis [8]. When assignments are then extended to the backbone, modeling of the binding site in combination with an assumed framework structure seems a much more feasible task than analysis of the whole Fv-fragment.

Acknowledgements: L.R. is a 'Leukemia Society of America Special Fellow', J.C. a research fellow of the Royal Society, and S.M. a recipient of an MRC studentship. We thank the IRC for Protein Engineering, Cambridge, for use of its NMR facilities.

REFERENCES

[1] Kay, L.E., Ikura, M., Tschudin, R. and Bax, A. (1990) J. Magn. Reson. 89, 496–514.
[2] Better, M., Chang, C.P., Robinson, R.R. and Horwitz, A.H. (1988) Science 240, 1041–1044.
[3] Skerra, A. and Plueckthun, A. (1988) Science 240, 1038–1041.
[4] Riechmann, L., Foote, J. and Winter, G. (1988) J. Mol. Biol. 203, 825–828.
[5] Anglister, J. and Zilber, B. (1990) Biochemistry 29, 921–928.

[6] Takahashi, H., Igarashi, T., Schimada, I. and Arata, Y. (1991) Biochemistry 30, 2840–2847.
[7] Wright, P.E., Dyson, H.J., Lerner, R.A., Riechmann, L. and Tsang, P. (1990) Biochem. Pharmacol. 40, 83–88.
[8] McManus, S. and Riechmann, L. (1991) Biochemistry, in press.
[9] Studier, F.W., Rosenberg, A.H., Dunn, J.J. and Dubendorff, J.W. (1990) Methods Enzymol. 185, 60–89.
[10] Bodenhausen, G. and Ruben, D.J. (1980) Chem. Phys. Lett. 69, 185–189.
[11] Müller, L. (1979) J. Am. Chem. Soc. 101, 4481–4484.
[12] Bax, A., Clore, G.M. and Gronenborn, A.M. (1990) J. Magn. Reson. 88, 425–433.
[13] Ward, S.E., Guessow, D., Griffiths, A.D., Jones, P.T. and Winter, G. (1989) Nature 341, 544–546.
[14] Riechmann, L., Clark, M., Waldmann, H. and Winter, G. (1988) Nature 332, 323–327.
[15] Alarzi, P.M., Lascombe, M.-B. and Poljak, R.J. (1988) Annu. Rev. Immunol. 6, 555–580.