FEBS Letters 350 (1994) 87–90

FEBS 14353

A pulsed field gradient isotope-filtered 3D ^{13}C HMQC-NOESY experiment for extracting intermolecular NOE contacts in molecular complexes

Woentae Lee[a,*], Matthew J. Revington[a], Cheryl Arrowsmith[a], Lewis E. Kay[b]

[a]*Division of Molecular and Structural Biology, Ontario Cancer Institute and Department of Medical Biophysics, University of Toronto, 500 Sherbourne St., Toronto, Ont., M4X 1K9, Canada*

[b]*Protein Engineering Network Centers of Excellence and Departments of Medical Genetics, Biochemistry and Chemistry, Medical Sciences Building, University of Toronto, Toronto, Ont., M5S 1A8, Canada*

Received 17 May 1994; revised version received 1 July 1994

Abstract

A pulsed field gradient three-dimensional isotope-filtered ^{13}C HMQC-NOESY experiment has been developed to characterize intermolecular contacts in a 37 kDa macromolecular ternary complex consisting of uniformly ^{13}C labeled *trp*-repressor, its natural abundance co-repressor, L-tryptophan, and natural abundance operator DNA. The pulse scheme makes use of pulsed field gradients for the removal of artifacts and dephasing of unwanted magnetization during isotope filtering, and employs a strategy to minimize the time that magnetization resides in the transverse plane. The experiment provides solely intermolecular NOE contacts between protons of the labeled protein and protons of the unlabeled species, and has proven to be especially useful in eliminating ambiguities between intra- and intermolecular NOEs in the isotope-edited 3D ^{13}C HMQC-NOESY spectrum of the complex.

Key words: Isotope-filtered NMR; Intermolecular NOE; *trp*-repressor DNA interaction; Pulsed field gradient NMR

1. Introduction

In the last several years, the development of multidimensional NMR in concert with isotopic labeling has allowed the structure determination of a large number of macromolecules with molecular weights less than approximately 20 kDa [1,2]. Several groups have presented NMR investigations of macromolecular complexes, such as protein–protein, protein–peptide, protein–ligand and protein–DNA complexes which contain mixtures of labeled and unlabeled species [3–8]. For such mixed systems, NOE constraints derived from isotope-edited experiments alone often proved difficult to assign due to ambiguities in distinguishing NOEs between isotopically labeled and unlabeled species.

Recently, heteronuclear isotope-filtered experiments have been developed to overcome these ambiguities [9–12]. Most versions of such pulse sequences require extensive phase cycling to suppress isotope-attached proton signals. A second limitation of such methods, especially for application to large complexes, is the loss in sensitivity as a result of the decay of magnetization during the requisite delays for filtering. Recently Bax and colleagues have addressed these limitations in the development of an isotope-filtered 2D HOHAHA experiment which makes use of heteronuclear dephasing during the Hartman–Hahn transfer period to eliminate signals from the isotopically enriched molecule [13]. This approach, therefore, minimizes the number of delays required for effective purging of proton signals from the labeled molecule. In addition, pulsed field gradients are employed to minimize the number of phase cycling steps. Here we describe a 3D ^{13}C F_1-edited,F_3-filtered NOE experiment for observing NOEs between protons attached to an isotopically labeled molecule and protons attached to an unlabeled molecule. Sensitivity losses are minimized through the use of an approach which combines ^{1}H chemical shift evolution and $^{1}H/^{13}C$ scalar transfer times in order to decrease the time during which ^{1}H magnetization is in the transverse plane [14–16]. Because the number of phase cycling steps must be kept to a minimum, pulsed field gradients are employed to assist in the elimination of experimental artifacts and to dephase magnetization arising from protons coupled to heteroatoms during the purging portion of the sequence. The experiment is applied to the study of a 37 kDa ternary *trp*-repressor, co-repressor, DNA complex.

*Corresponding author.

Abbreviations: NMR, nuclear magnetic resonance; NOE, nuclear Overhauser enhancement; 3D, three-dimensional; NOESY, nuclear Overhauser enhancement spectroscopy; HMQC, heteronuclear multiple quantum coherence; ppm, parts per million. Isotope-edited experiments select protons bound to ^{13}C or ^{15}N; isotope-filtered experiments suppress protons bound to ^{13}C or ^{15}N spins.

2. Experimental

Uniformly ^{13}C enriched *trp*-repressor was isolated from *E. coli* strain CY15070 grown on ^{13}C-labeled M9 medium as described previously [17,18]. The sample used for NMR consisted of 1.0 mM uniformly

SSDI 0014-5793(94)00740-3

^{13}C enriched *trp*-repressor dimer, 2.0 mM unlabeled tryptophan, 1.0 mM unlabeled operator DNA [6], 99.9% D_2O, pH 6.0, 50 mM potassium phosphate buffer, 37°C. The 3D ^{13}C F_1-edited,F_3-filtered HMQC-NOESY spectrum was recorded as an 84 × 26 × 512 complex matrix on a Varian UNITYplus 500 MHz spectrometer equipped with a gradient unit and an actively shielded triple resonance probehead. The acquisition times were: $t_1(^1H)$ = 20 ms, $t_2(^{13}C)$ = 8.7 ms and $t_3(^1H)$ = 64 ms. Spectral widths of 4200 Hz, 3000 Hz and 8000 Hz in F_1, F_2 and F_3, respectively, were employed. The value of τ_m was set to 110 ms. A relaxation delay of 1 s was used along with 128 scans for each complex (t_1,t_2) point to give a total measuring time of 87 h. The 3D ^{13}C edited NOESY spectrum was recorded on a UNITY 600 MHz spectrometer as an 84 × 26 × 512 complex matrix with acquisition times of $t_1(^1H)$ = 14 ms, $t_2(^{13}C)$ = 4.3 ms and $t_3(^1H)$ = 64 ms. A relaxation delay of 0.9 s was employed with 128 scans for each complex (t_1,t_2) point and a mixing time of 110 ms was used to give a total measuring time of 87 h.

3. Results and discussion

The pulse scheme for the 3D ^{13}C F_1-edited,F_3-filtered HMQC-NOESY experiment is illustrated in Fig. 1. The mechanism of the sequence can be described concisely as follows:

$$^1H_i(t_1/2) \xrightarrow{J_{HC}} {}^{13}C_i(t_2) \xrightarrow{J_{HC}} {}^1H_i(t_1/2) \xrightarrow{NOE} {}^1H_i + {}^1H_j \xrightarrow{PURGE} {}^1H_j$$

where ^1H_i is one-bond coupled to a ^{13}C spin ($^{13}C_i$), while ^1H_j is not, and protons i and j are close in space. Briefly, proton magnetization evolves due to both the one-bond ^{1}H–^{13}C scalar coupling and chemical shift between points a and b of the pulse scheme. Therefore, immediately prior to the ^{13}C 90° pulse of phase φ, evolution due to scalar coupling has proceeded for $\tau_a + \tau_c$ (slightly less than $1(2J_{HC})$ to minimize relaxation losses) while ^{1}H chemical shift has evolved for $\tau_a + 2\tau_b - \tau_c = t_1/2$. The values of τ_a, τ_b and τ_c are chosen according to:

$$\begin{aligned} \tau_a &= \tau_{CH} + n\zeta \\ \tau_b &= n/(4SW1) - n\zeta \\ \tau_c &= \tau_{CH} - n\zeta + pwc \\ \zeta &= (\tau_{CH} + pwc - g1)/(N - 1) \end{aligned} \quad (1)$$

where τ_{CH} is chosen to be slightly less than $1/(4J_{HC})$, pwc is the carbon 180° pulse width, SW1 and N are the spectral width and the number of complex points in the indirectly detected proton dimension, respectively, g1 is the duration of the pulsed field gradient applied during τ_c and n = 0,1,2,...(N−1) (n = 0 for the first complex t_1 point, n = 1 for the second t_1 point, etc.) [16].

Carbon chemical shift evolution occurs for a period, t_2, and subsequently magnetization is transferred back to protons where refocussing due to the one-bond ^{1}H–^{13}C coupling occurs and chemical shift evolution of ^{1}H magnetization proceeds for an additional $t_1/2$ period. The values of τ_a', τ_b' and τ_c' are given by Eq. (1) with the substitution of g2 for g1. Note that proton magnetization is present in the transverse plane for a duration, $t_1 + 4\tau_{CH} - 4n\zeta$ (from points a to c in the sequence of Fig. 1, neglecting the t_2 period), as opposed to a duration of $t_1 + 4\tau_{CH}$. During the mixing time magnetization is transferred between dipolar coupled spins, and only those NOEs corresponding to the transfer of magnetization from ^{13}C-bound protons to ^{12}C-bound protons are selected during the subsequent purging scheme. The approach for the elimination of ^{13}C-based protons that is

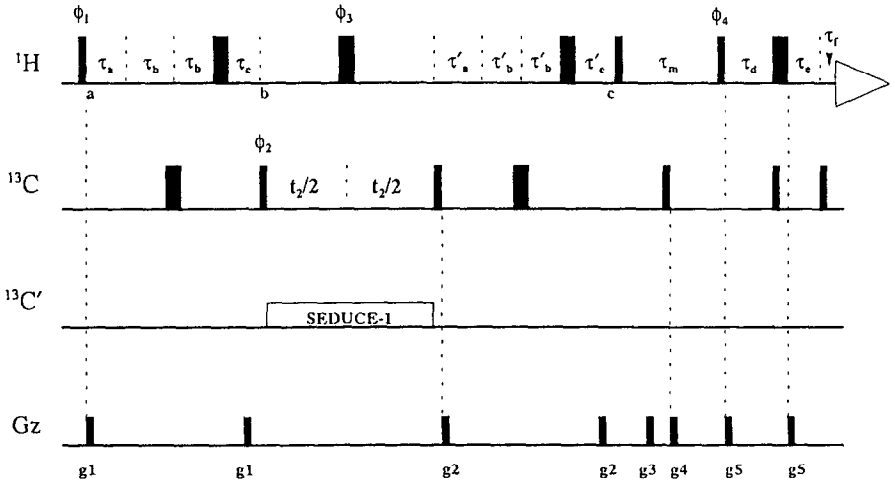

Fig. 1. Pulse scheme for the gradient 3D ^{13}C F_1-edited,F_3-filtered HMQC-NOESY experiment. All narrow (wide) pulses have flip angles of 90° (180°). Pulses for which phases are not indicated are applied along the x-axis. All carbon pulses are applied with an 18.5 kHz field centered at 43 ppm, while ^{1}H pulses are applied with a 23.5 kHz field centered at 3.0 ppm. The two ^{13}C 180° pulses are applied as composite $(90_x180_y90_x)$ pulses. A SEDUCE-1 decoupling field [22] (420 ms 90° pulses; 1.3 kHz field at peak height) centered at 177 ppm is employed for carbonyl decoupling during t_2. The values of τ_a, τ_b and τ_c are defined in the text, with τ_{CH} (see Eq. 1) set to 1.7 ms. The values of τ_d, τ_e and τ_f are 4.0 ms, 3.6 ms and 0.4 ms ($\tau_d = \tau_e + \tau_f$) with τ_m set to 110 ms. The values of τ_d and τ_e were selected in order to acheive suppression of aliphatic ^{13}C coupled ^{1}H resonances in F_3. The phase cycle employed is: $\varphi1 = (x,-x)$; $\varphi2 = 8(x),8(-x)$; $\varphi3 = 2(x),2(-x)$; $\varphi4 = 4(x),4(y)$; rec = $2(x,-x),2(-y,y),2(-x,x),2(y,-y)$. Quadrature in F_1 and F_2 is achieved via States-TPPI [23] of $\varphi1$ and $\varphi2$, respectively. The durations and strengths of the gradients are: g1 = (0.1 ms,10 G/cm); g2 = (0.1 ms,7 G/cm); g3 = (1 ms,5 G/cm); g4 = (0.4 ms,8 G/cm) and g5 = (1 ms,7.5 G/cm). All gradients employed are rectangular.

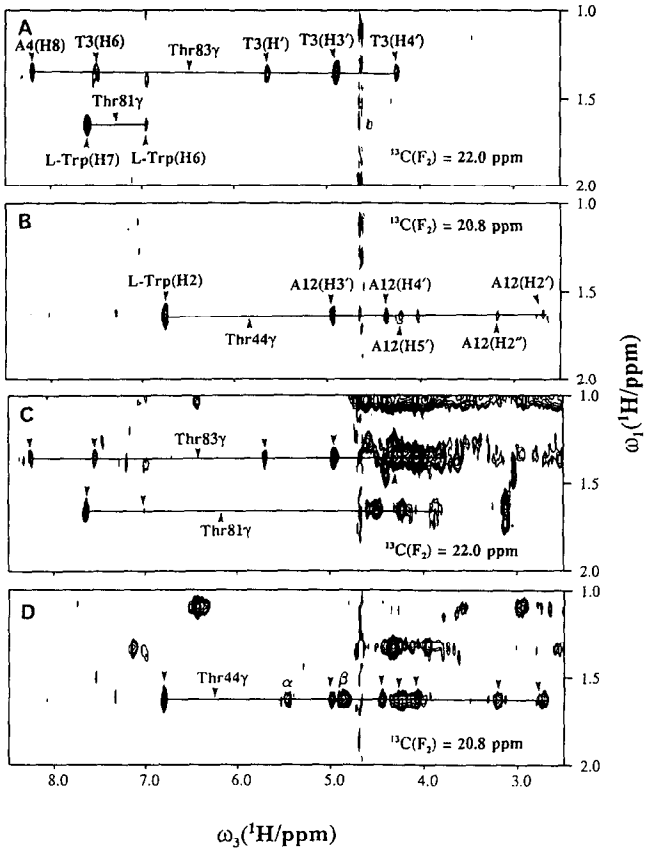

Fig. 2. (A and B) $F_1(^1H)$–$F_3(^1H)$ slices from the 3D ^{13}C F_1-edited,F_3-filtered HMQC-NOESY spectrum of a ternary complex consisting of uniformly ^{13}C enriched *trp*-repressor dimer, unlabeled tryptophan and unlabeled DNA. The corresponding regions of slices from the 3D ^{13}C edited HMQC-NOESY spectrum of the complex are illustrated for comparison in C and D. Intermolecular NOEs are labeled with arrowheads.

employed here is similar to the strategy developed by Ikura and Bax [10] and Fesik and co-workers [11]. Because of the difficulties in filtering out signals of protons coupled to heteroatoms displaying large variations in one-bond scalar couplings, a double purging scheme is employed with delays, τ_d and τ_e, optimized for different values of J_{HC}.

Pulsed field gradients are employed to minimize artifacts in a manner as described by Bax and Pochapsky [19]. Gradient pairs g1, g2 and g5 surround 180° pulses and eliminate potential artifacts arising due to imperfections in these pulses. Magnetization arising from ^{13}C-bound ^1H spins, and which has evolved due to the one-bond ^1H-^{13}C scalar coupling interaction during the τ_d period, is subsequently converted into ^1H–^{13}C zero and double quantum coherences by the first ^{13}C 90° purge pulse. This magnetization is then dephased by the second g5 gradient pulse. Since the use of gradients ensures against the possibility of the magnetization being converted into net observable signal through the action of the final ^{13}C 90° purge pulse, neither of these final two ^{13}C pulses needs to be phase cycled. It should be noted that the use of homospoil-type pulses to select for or against ^1H magnetization coupled to ^{13}C spins in protein applications dates back to the work of Bruhwiler and Wagner [20]. The gradient pulse g3 eliminates any magnetization which is not aligned along the *z*-axis during the mixing time. Incomplete refocusing of proton magnetization, which is antiphase with respect to the one-bond coupled carbon prior to the mixing period, can lead to the generation of ^1H–^{13}C 2-spin order at the time of application of the ^1H 90° pulse immediately preceding the mixing period. This magnetization is eliminated by the combined action of the ^{13}C 90° pulse and the gradient g4 during the mixing time.

Fig. 2 illustrates regions of two slices from each of the 3D ^{13}C F_1-edited,F_3-filtered HMQC-NOESY spectrum and the 3D ^{13}C-edited HMQC-NOESY spectrum [21] of a 37 kDa complex between uniformly ^{13}C-labeled *trp*-repressor (a homodimer of 107 amino acids per subunit), natural abundance L-tryptophan (the co-repressor necessary for sequence specific DNA binding) and a 20 basepair operator DNA sequence d(CGTAC-TAGTTAACTAGTACG), also natural abundance. Only intermolecular contacts are observed in Fig. 2A,B while both inter- and intramolecular NOEs are present in Fig. 2C,D. The intermolecular NOE crosspeaks observed in Fig. 2A,B can be classified into two distinct groups: those between protein and DNA and those between protein and the co-repressor, L-tryptophan. Because the spectra of bound DNA and co-repressor were assigned previously using $^{13}C/^{15}N$ and ^{13}C double filtered experiments, the NOEs from each class can be readily separated. Examples of each of the two types of NOEs are indicated in Fig. 2. Of particular note are the NOEs involving Thr-81 Hγ and Thr-44 Hγ. Because of the degeneracy of the Hγ resonances (1.62 ppm), separation of the NOEs via the $^{13}C\gamma$ shifts is necessary to make the unambiguous assignments indicated. The close proximity of these two residues to both the DNA and the co-repressor render these intermolecular NOEs crucial for a detailed structural description of the complex [6]. On the basis of the experiment described above, a total of 28 intermolecular NOEs were unambiguously identified. As a result of the symmetry of the complex, each proton–proton contact occurs twice. Most of the NOEs observed are between the consensus site of operator DNA and the helix-turn-helix motif of the protein. The NMR experiment presented has also been used to study the complex between an isotopically labeled C-terminal SH_2 domain of phospholipase-Cγ1 and a natural abundance 12-residue phosphopeptide, providing over 100 intermolecular NOEs [5].

In summary, in this letter we have presented a pulsed field gradient 3D ^{13}C F_1-edited,F_3-filtered HMQC-NOESY experiment for assigning intermolecular NOEs between isotope labeled and unlabeled components of a

molecular complex. While such techniques have been applied in the past for studying complexes of the order of 20–25 kDa, we show here that the methods can successfully be applied to systems as large as 37 kDa. In addition to providing intermolecular contacts, this experiment also complements the 3D ^{13}C edited HMQC-NOESY experiment by providing a straightforward means of identification of intermolecular NOEs in the early stages of the assignment procedure of the labeled species, thereby avoiding possible misassignments. Additionally, as demonstrated by Folkers et al. [12], this type of experiment is useful for identifying intersubunit NOEs in symmetric oligomeric proteins which contain a mixture of labeled and unlabeled subunits.

Acknowledgements: This research was supported by the NCI of Canada with funds from the Terry Fox Run (C.H.A, L.E.K.), the Human Frontier Science Programme (C.H.A) and the Natural Sciences and Engineering Research Council of Canada (L.E.K). M.J.R. was supported by a studentship from the Natural Sciences and Engineering Research Council of Canada.

References

[1] Bax, A. and Grzesiek, S. (1993) Acc. Chem. Res. 26, 131–138.
[2] Clore, G.M. and Gronenborn, A.M. (1991) Science 252, 1390–1399.
[3] Folkers, P.J.M., Nilges, M., Flomer, R.H.A., Konings, R.N.H. and Hilbers, C.W.J. (1994) Mol. Biol. 236, 229–246.
[4] Ikura, M., Clore, G.M., Gronenborn, A.M., Zhu, G., Klee, C.B. and Bax, A. (1992) Science 256, 632–638.
[5] Pascal, S.M., Singer, A.U., Gish, G., Yamazaki, T., Shoelson, S.E., Pawson, T., Kay, L.E. and Forman-Kay, J.D. (1994) Cell 77, 461–472.
[6] Zhang, H., Zhao, D., Revington, M., Lee, W., Jia, X., Arrowsmith, C. and Jardetzky, O. (1994) J. Mol. Biol. (in press).
[7] Omichinski, J.G., Clore, G.M., Schaad, O., Felsenfeld, G., Trainor, C., Appella, E., Stahl, S.J. and Gronenborn, A.M. (1993) Science 261, 438–446.
[8] Meadows, R.P., Nettesheim, D.G., Xu, R.X., Olejniczak, E.T., Petros, A.M., Holzman, T.F., Severin, J., Gubbins, E., Smith, H. and Fesik, S.W. (1993) Biochemistry 32 754–765.
[9] Wider, G., Weber, C., Traber, R., Widmer, H. and Wuthrich, K. (1990) J. Am. Chem. Soc. 112, 9015–9016.
[10] Ikura, M. and Bax, A. (1992) J. Am. Chem. Soc. 114, 2433–2440.
[11] Gemmecker, G., Olejniczak, E.T. and Fesik, S.W. (1992) J. Magn. Reson. 96, 199–204.
[12] Folkers, P.J.M., Rolmer, R.H.A., Konings, R.N.H. and Hilbers, C.W. (1993) J. Am. Chem. Soc. 115, 3798–3799.
[13] Bax, A., Grzeskiek, S., Gronenborn, A.M. and Clore, G.M. (1994) J. Magn. Reson. Series A 106, 269–273.
[14] Grzesiek, S. and Bax, A. (1993) J. Biomol. NMR 3, 185–204.
[15] Logan, T.M., Olejniczak, E.T., Xu, R.X. and Fesik, S.W. (1993) J. Biomol. NMR 3, 225–231.
[16] Muhandiram, D.R., Xu, G.Y. and Kay, L.E. (1993) J. Biomol. NMR 3, 463–470.
[17] Paulh, J.L. and Yanofsky, C. (1986) Nucleic Acids Res. 14, 7851–7861.
[18] Arrowsmith, C.H., Patcher, R.B., Altman, R.B., Iyer, S.B. and Jardetzky, O. (1990) Biochemistry 29, 6332–6341.
[19] Bax, A. and Pochapsky, S. (1992) J. Magn. Reson. 99, 638–643.
[20] Ikura, M., Kay, L.E. and Bax, A. (1990) J. Magn. Reson. 86, 204–209.
[21] Bruhwiler, D. and Wagner, G. (1986) J. Magn. Reson. 69, 546–551.
[22] McCoy, M. and Mueller, L. (1992) J. Am. Chem. Soc. 114, 2108–2112.
[23] Marion, D., Ikura, M., Tschudin, R. and Bax, A. (1989) J. Magn. Reson. 85, 393–399.