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Observation of intersubunit NOEs in a dimeric P22 Mnt repressor mutant by a time-shared [^{15}N,^{13}C] double half-filter technique

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SUMMARY

A time-shared [^{15}N,^{13}C] half-filter technique is presented, which can be used to study proton–proton NOEs between biomolecules. The filter is demonstrated in a 2D [^{15}N,^{13}C] double filtered NOESY experiment of a dimeric Mnt repressor mutant consisting of completely [^{15}N,^{13}C] labeled monomer and unlabeled monomer. The benefit of this combined [^{15}N,^{13}C] half-filter is that a single NMR experiment can be designed that yields all NOE interactions between labeled and unlabeled protons ((^{13}C,^{14}N/^{12}C), (^{15}N,^{14}N/^{12}C), (^{12}C,^{15}N/^{13}C) and (^{14}N,^{15}N/^{13}C)) in the protein, where conventional half-filters would require at least three separate NMR experiments to obtain these NOEs. The intermonomer NOEs of the Mnt mutant confirmed the secondary structure of the DNA-binding domain as an antiparallel β ribbon, formed from an N-terminal segment contributed by each monomer. Moreover, several intersubunit NOEs were characterized in the C-terminal part of the Mnt mutant for which no structural data is available yet.

NMR studies of proteins have progressed substantially by methodological developments that rely on uniform incorporation of the stable isotopes ^{15}N and ^{13}C in the protein (Clore and Gronenborn, 1991). Such methods facilitate the process of the assignment of resonances and resolve spectral overlap by spreading the proton resonances of proteins into a heteronuclear domain. For the structure determination of large (>20 kDa) proteins these NMR techniques become crucial.

Another interesting theme for the application of NMR spectroscopy is the study of molecular interaction. Particularly suited for such studies are isotope-editing techniques (Fesik, 1988; Otting and Wüthrich, 1990; Stockman and Markley, 1992). Thus, half-filter techniques were developed for the selective observation of the components of macromolecular complexes. With the aid of these methods NOE interactions could be observed that define short interproton distances in protein–DNA complexes (Otting et al., 1990), in a DNA–drug complex (Leupin et al., 1990), in

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enzyme–inhibitor complexes (Fesik et al., 1991; Weber et al., 1991), in protein–peptide complexes (Ikura and Bax, 1992) and between monomers in dimeric proteins (Burgering et al., 1993; Folkers et al., 1993).

Normally, such half-filters use the J-coupling either with a ^{15}N isotope or with a ^{13}C isotope, and in this way an NH or CH proton can be selected or rejected. For an unambiguous interpretation of the ^{1}H-^{1}H NOEs both protons need to be characterized in double half-filter experiments. Thus, for a complete analysis six different [^{15}N,^{13}C] double half-filtered NOESY experiments can be envisaged: [^{13}C,$^{12}C/^{14}N$], [^{15}N,$^{12}C/^{14}N$], [^{13}C,^{13}C], [^{15}N,^{15}N], [^{13}C,^{15}N] and [$^{12}C/^{14}N$,$^{12}C/^{14}N$], where each of these experiments can define a new set of proton–proton interactions. Thus far, this would require the recording of at least three separate double half-filtered NOESY experiments. Recently, however, following a suggestion from Sørensen (1990), it was shown that [^{15}N, ^{1}H] and [^{13}C,^{1}H] coherences can be selected simultaneously (Farmer, 1991; Boelens et al., 1993). Since such 2D heteronuclear experiments are closely related to those incorporating half-filters, it is also possible to design combined half-filters. Figure 1a shows a time-shared [^{15}N,^{13}C] half-filter which can be implemented in various multidimensional experiments. Since the delays for the polarization transfer of both nuclei are different, $\tau_n = (2\ ^{1}J_{H,N})^{-1}$ and $\tau_c = (2\ ^{1}J_{H,C})^{-1}$, the protons attached to ^{13}C should be decoupled for the period $\delta = \tau_n - \tau_c$. This is accomplished in the half-filter of Fig. 1a by applying the ^{13}C pulses at a time δ after (or before) the ^{1}H 180° pulse. The phases of ϕ_n and ϕ_c can be varied independently to select ^{13}C,^{15}N- or ^{12}C,^{14}N-attached proton magnetization. However, in many cases the separate selection of either ^{13}C or ^{15}N is not essential, since the attached protons can already be discriminated by their ^{1}H frequency. Therefore, it will be sufficient to select or reject ^{13}C and ^{15}N magnetization simultaneously.

This communication describes the combination of two of these combined [^{15}N,^{13}C] half-filters in a 2D NOESY experiment applied to a symmetrical dimeric protein. As pointed out earlier (Breg et al., 1990) the NMR structure determination of dimeric proteins is hampered by the intrinsic difficulty to distinguish the intra- from the intersubunit NOEs in a regular NOESY spectrum, since, due to symmetry, they have the same spectral properties. Therefore it is important to study all possible intersubunit NOEs to obtain a complete structural description of the native dimer. 2D $^{14}N/^{15}N$ and $^{12}C/^{13}C$ double filtered NOESY experiments of similar dimeric proteins yielded spectra with intersubunit NOEs between nitrogen-attached protons (Burgering et al., 1993) and between carbon-attached protons (Burgering et al., 1993; Folkers et al., 1993), respectively. The [^{13}C,^{15}N] half-filter described can be used in double filtered NOE experiments. In this way, not only intersubunit NOEs can be observed between protons attached to nuclei of the same type but also those between carbon-bound protons and nitrogen-bound protons, giving a complete survey of all possible intersubunit NOE interactions.

The pulse sequence of the 2D time-shared [^{13}C,^{15}N] double filtered NOESY is presented in Fig. 1b. The phase cycle of the pairs of 90° heteronuclear editing pulses ($\phi 2$) in the two half-filters is similar to the scheme proposed earlier (Burgering et al., 1993; Folkers et al., 1993). In order to efficiently suppress artifacts, pulsed field gradients were applied in a similar fashion as proposed by Vuister et al. (1993). The sets of gradients (G1,G4) of identical polarity and strength at either side of the 180° proton pulse in the half-filter remove the effect of the flip-angle imperfection of the refocussing pulse. The two gradients (G2,G3) in the NOE mixing time were included in order to remove all transverse magnetization components. Together with the two 90° heteronuclear pulses these gradients prohibit the transfer of unwanted coherences through the NOE mixing time.

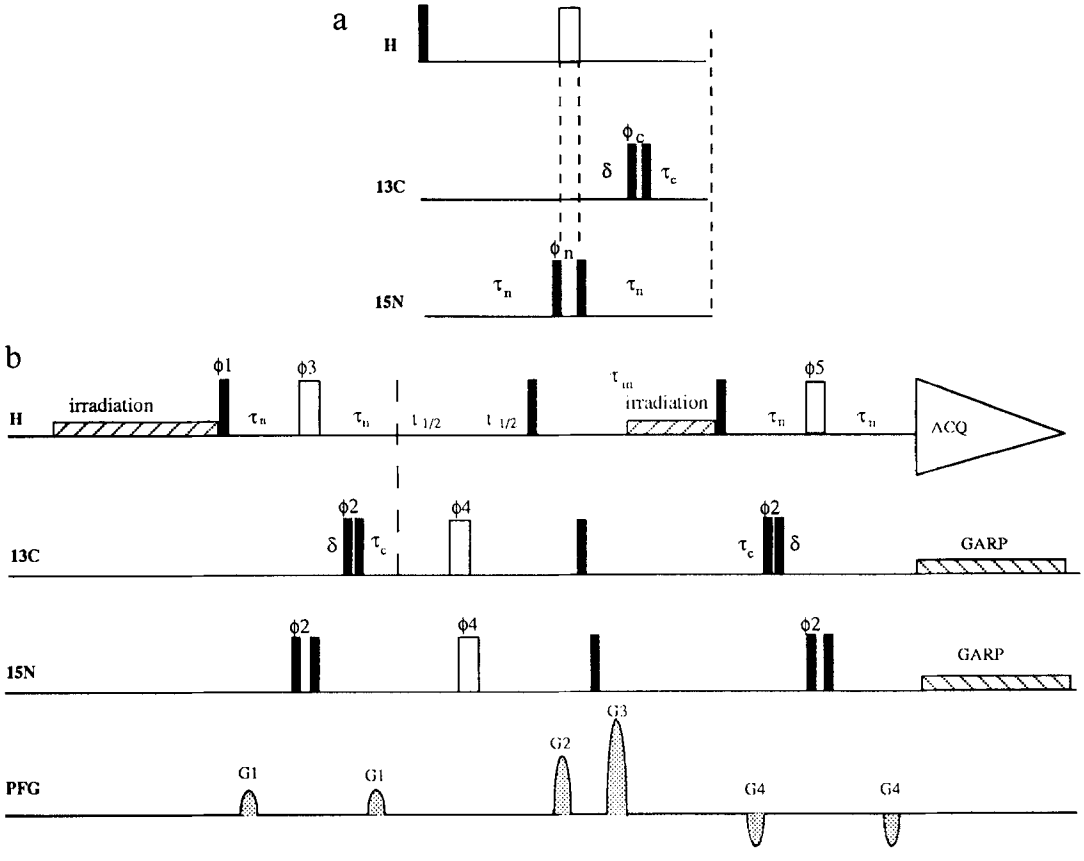

Fig. 1. (a) Pulse scheme of a time-shared [^{13}C,^{15}N] half-filter building block. Narrow (filled) and wide (open) rectangles represent 90° and 180° pulses, respectively. All pulses can be applied along the x-axis, except where phases ϕ_n and ϕ_c are indicated. These editing phases can be varied in an independent fashion by either x or −x, resulting in either an effective 180° heteronuclear pulse or an effectively omitted pulse. As an example the following phase cycle is considered: ϕ_n = x, −x,x,−x and ϕ_c = x,x,−x,−x. The coherences will have the following relative signs: ϕ1: ^{14}NH = −, ^{15}NH = +, ^{12}CH = −, ^{13}CH = +; ϕ2: ^{14}NH = −, ^{15}NH = −, ^{12}CH = −, ^{13}CH = +; ϕ3: ^{14}NH = −, ^{15}NH = +, ^{12}CH = −, ^{13}CH = −; ϕ4: ^{14}NH = −, ^{15}NH = −, ^{12}CH = −, ^{13}CH = −. By either addition or subtraction of the results the desired coherences can be selected: 1 + 2 + 3 + 4 = [^{14}N,^{12}C], 1 − 2 + 3 − 4 = [^{15}N] and 1 + 2 − 3 − 4 = [^{13}C]. (b) Pulse scheme for the 2D time-shared [^{13}C,^{15}N] double filtered NOESY spectrum. Narrow (filled) and wide (open) rectangles represent 90° and 180° pulses, respectively. Unless otherwise indicated all pulses are applied along the x-axis. The phase cycling was as follows: ϕ1 = 2(x),2(−x), ϕ2 = x,−x, ϕ3 = 8(x),8(−x), ϕ4 = 4(x),4(−x), ϕ5 = 16(x),16(−x), Acquisition = x,−x,−x,x.

During the recovery delay and the NOE mixing time low-power H_2O irradiation was applied. The heteronuclear decoupling during acquisition was performed by low-power GARP decoupling pulse sequences. Quadrature detection in the t_1 dimension was achieved by the States–TPPI method, applied to the phase of the second 90° proton pulse. The values of the delays are defined as follows: $\tau_n = (2\,{}^1J_{H,N})^{-1} = 5.55$ ms, $\tau_c = (2\,{}^1J_{H,C})^{-1} \approx 3.94$ ms and $\delta = \tau_n - \tau_c$. Sine-bell-shaped pulses (PFG) were applied along the z-axis. The gradients all had a duration of 500 μs, followed by a 500 μs recovery delay. The strengths of the gradients were as follows: G1 = 7.5 G/cm, G2 = 16.5 G/cm, G3 = 33.5 G/cm and G4 = −10 G/cm.

The proposed pulse sequence has a possible drawback in that loss of sensitivity by relaxation may be expected for carbon-attached proton magnetization, due to the additional period 2δ in the sequence compared to the 2D $^{12}C/^{13}C$ double filtered NOESY. Another point is the simultaneous ^{13}C and ^{15}N decoupling during the acquisition, which can lead to sample heating.

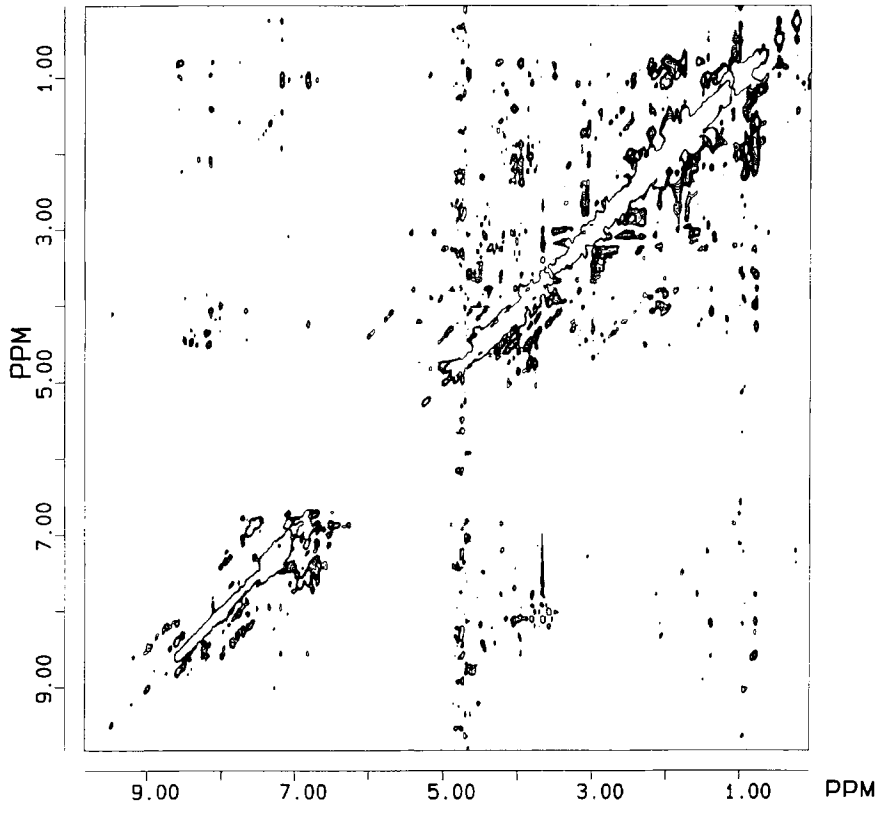

Fig. 2. Complete 2D $^{12}C/^{13}C,^{14}N/^{15}N$ double filtered NOESY spectrum of a 1:1 mixture of ($^{13}C,^{15}N$) labeled and unlabeled mutant Mnt (1–76) repressor. A total of 12 mg protein was dissolved in 500 µl 95% $^{1}H_2O$/5% $^{2}H_2O$ at pH 4.65. The spectrum was recorded on a standard Bruker AMX600 spectrometer equipped with a shielded-gradient ($^{13}C,^{15}N$) triple resonance probe. In the 2D spectrum some small artifacts are present which stem from small-molecular-weight contaminants. The spectrum was processed on a Silicon Graphics workstation using the TRITON software package. The dataset, 400 and 1024 points in the t_1 and t_2 dimensions, respectively, was recorded with 196 scans and a NOE mixing time of 150 ms. Separate baseline corrections were applied after Fourier transformation and appropriate window-function multiplications, to obtain a 1K × 1K spectral matrix. The spectrum was analysed on a workstation using the program REGINE written in our laboratory.

The proposed experiment was applied to the mutant Mnt repressor (1–76), a β-sheet DNA-binding protein (Knight et al., 1989; Phillips, 1991) which is a dimer in solution (Knight and Sauer, 1988). The NMR sample contained a 1:1 mixture (12 mg) of uniformly ($^{15}N,^{13}C$) labeled protein with unlabeled protein. After a short equilibration time a heterodimer is formed with one monomer ($^{15}N,^{13}C$) labeled and the other monomer unlabeled, together with two types of homodimer in a 2:1:1 ratio.

Figure 2 shows the 600 MHz spectrum obtained with the new pulse sequence. The figure shows a large number of intermonomer NOEs within the Mnt (1–76) dimer. For example, shown in Fig. 3a, in the $C^{\alpha}H$–amide region intermonomer NOEs are present belonging to the DNA-binding β-sheet region of the Mnt dimer, which comprises two β-strands, one from each monomer. In fact, these NOEs suggest a secondary structure of the DNA-binding part which is similar to that of the

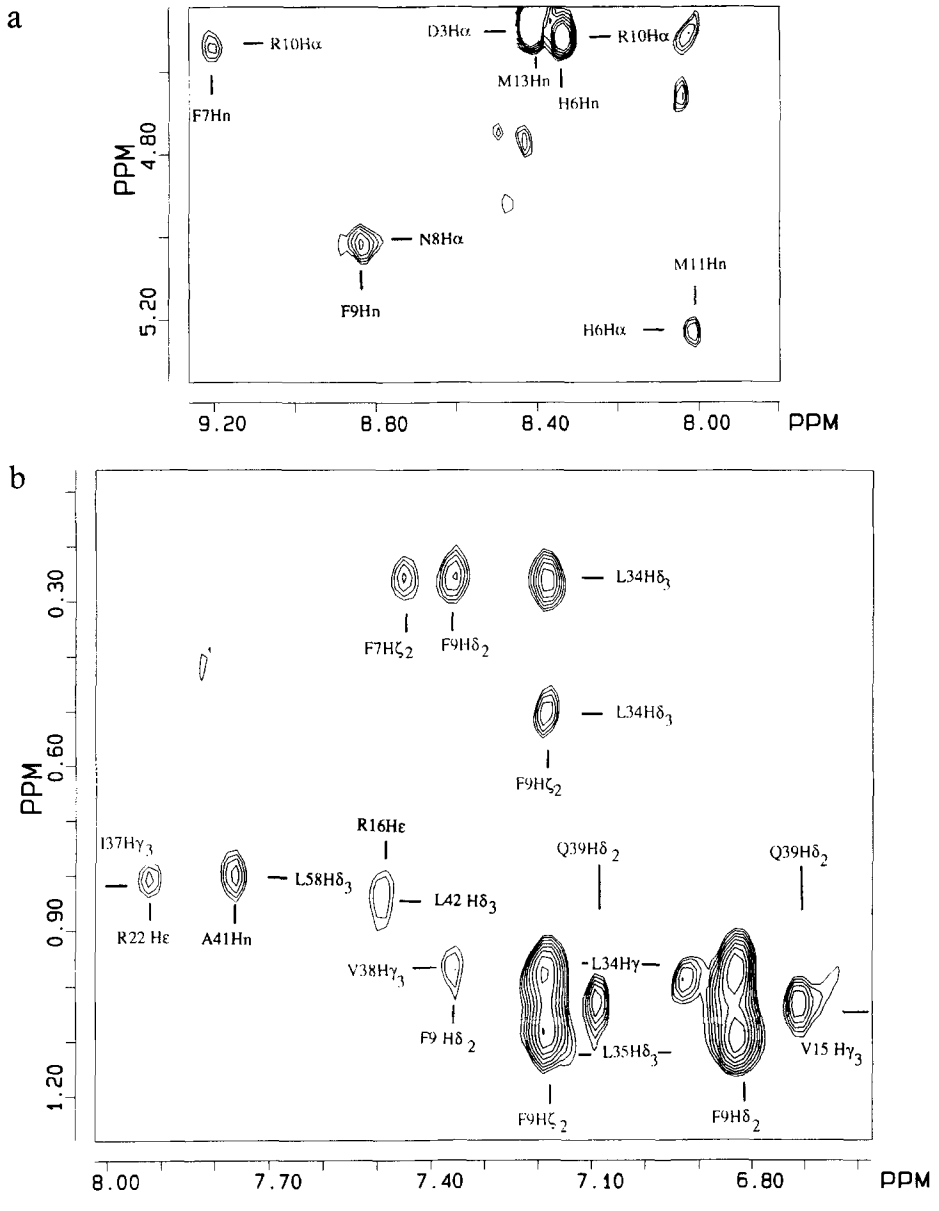

Fig. 3. (a) Part of the $C^{\alpha}H$–amide region of the 2D $^{12}C/^{13}C,^{14}N/^{15}N$ double filtered NOESY spectrum. The intermonomer NOEs of the DNA-binding β-sheet region of the Mnt repressor are indicated. (b) Part of the 2D $^{12}C/^{13}C,^{14}N/^{15}N$ double filtered NOESY spectrum displaying some specifically indicated intermonomer NOE cross peaks of the mutant Mnt (1–76) dimer. Examples are shown of intersubunit NOEs between carbon-bound protons as well as between carbon-attached and nitrogen-attached protons.

homologous proteins Arc and MetJ (Breg et al., 1990). Figure 3b shows another part of the spectrum with several intermonomeric NOEs. This demonstrates that in the NOESY spectrum interactions between carbon-bound protons as well as between carbon- and nitrogen-attached

protons are obtained (e.g., NOEs are present between the aromatic protons of Phe^{9} and the methyl protons of $Leu^{34'}$, and between the amino protons of Gln^{39} and the methyl protons of $Val^{15'}$).

Intermonomer NOEs observed between protons of residues in the C-terminal part of Mnt (1–76) included NOEs between Tyr^{51} and $Asp^{60'}$, between $Ala^{59'}$ and $Arg^{57'}$, between Asn^{53} and $Ala^{55'}$, and between Ala^{41} and $Leu^{58'}$. For this part of the protein (residues 45–76) no apparent homology exists with the related Arc and MetJ repressors and no structural data is available which would allow assignment of these NOEs as either intra- or intermonomeric on the basis of the assumption of structural relationship. Therefore, the observation of these intermonomeric NOEs is the only possibility to obtain a 3D solution structure of the dimeric Mnt mutant.

In conclusion, it was demonstrated that a complete set of intersubunit NOEs of symmetrical dimers can be identified unambiguously in a single NMR experiment. This is achieved by using a heterodimer consisting of both uniformly labeled (^{15}N,^{13}C) and unlabeled monomer. Applied to the Mnt repressor, the method has provided important structural information on the interactions between the monomers and it should be applicable to proteins of a relatively large size (20–30 kDa).

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