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Observation of inter-subunit nuclear Overhauser effects in a dimeric protein

Application to the Arc repressor

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For the structure determination of symmetric protein dimers it is necessary to distinguish between intra- and inter-subunit NOEs. A method is presented to measure selectively the inter-subunit NOEs using uniform ^{15}N and ^{13}C isotope labelling. This is accomplished by doubly filtered 2D NOE experiments on mixtures of native protein with isotope-labeled protein. The method has been applied to the Arc repressor and allows the characterization of virtually all proton–proton NOEs in terms of their intra- or inter-subunit nature.

NMR; Arc repressor; Dimer; Inter-subunit NOE

1. INTRODUCTION

The quality of a three-dimensional NMR structure of a protein is primarily determined by the number of long-range NOEs. However, in the case of a symmetric dimer those NOEs are difficult to interpret, since it is a priori not possible to discriminate NOEs within monomers from NOEs between monomers. An essential step in the structure determination of dimeric proteins by NMR is therefore the characterization of inter- vs. intra-subunit NOEs, which by symmetry have the same spectral properties. In the case of a loosely coupled dimer this problem may not be so severe. However, for highly intertwined bio-molecules with many inter-subunit NOEs this ambiguity may prohibit the NMR structure determination.

We encountered this problem in our structural NMR studies of the Arc repressor, a small dimeric regulatory protein of 53 residues per monomer, involved in the lytic/lysogenic life cycle switch of *Salmonella* bacteriophage P22 [1]. Together with the MetJ and Mnt repressors it belongs to a family of sequence-specific DNA-binding proteins with an N-terminal β-sheet region as recognition motif [2,3]. Initially we solved the problem by exploiting the structural homology of the Arc and MetJ repressors [2]. The structure of the *E. coli* MetJ repressor had been determined by X-ray crystallography [4]. To obtain a crude structure we modelled the side-chains of the Arc repressor on the backbone of the MetJ repressor. This was used to establish the intra- or inter-monomer character for many NOEs. In a 'bootstrap' procedure these NOEs were used in distance-geometry and restrained MD calculations for a first round in the structure determination. The new structure was analysed again and an improved set of intra- and inter-monomer NOEs was obtained. After three iterations most of the NOEs were interpreted and a well defined structure of the Arc repressor was determined in accordance with all NOEs. In a similar vein, Nilges and Brünger [5] reported a method describing a simulated annealing procedure using floating intra- and inter-monomer NOE assignments. A limitation of these methods is that they both require a good starting structure, otherwise they do not converge for highly intertwined proteins such as the Arc repressor.

In principle, a more general approach would involve the use of isotope labelling. Using selective amino-acid deuteration a number of intra- and inter-monomer NOEs could be discriminated for the Trp repressor [6] and the λ repressor [7]. However, for dimers such as the Arc repressor, where most of the long-range NOEs are between subunits, uniform labelling with ^{13}C or ^{15}N would be more suitable than ^{2}H labelling since this allows a positive identification of inter-subunit NOEs. In this communication, we will describe an NMR method for the identification of inter-subunit NOEs, introducing the necessary asymmetry by uniform ^{15}N or ^{13}C

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Abbreviations: 2D, two-dimensional; MD, molecular dynamics; NMR, nuclear magnetic resonance; NOE, nuclear Overhauser effect; NOESY, 2D NOE spectroscopy; TPPI, time proportional phase increment.

labelling. The experiment involves mixing isotope-labelled and unlabelled Arc in a 1:1 ratio forming a hetero-dimer, and the use of heteronuclear X-half filters [8–11]. A very similar experiment was independently developed by Hilbers and coworkers [12].

2. MATERIALS AND METHODS

Arc repressor was isolated from *E coli* strain X90 containing plasmid pTA200 as described [13], with some modifications. The bacteria were grown on minimal media (S.V.) containing either $^{15}NH_4Cl$ or $^{13}C_6$ glucose (Isotec Inc) for uniform ^{15}N or ^{13}C labelling. NMR samples were prepared by making 1:1 mixtures of labelled and unlabelled Arc as judged by UV absorption. The hetero-dimer is formed together with the two types of homo-dimer in a 2:1:1 ratio after short equilibration [14]. The total amount of protein was 12 mg in either 450 μl H_2O (^{15}N Arc) or D_2O (^{13}C Arc) containing 200 mM NaCl and 50 mM KP_i at pH = 4 65.

All NMR experiments were performed on a Bruker AMX-500 spectrometer equipped with a three-channel interface. The pulse sequence used for recording of the $^{12}C/^{13}C$ doubly filtered NOESY spectrum is presented in Fig. 1 The phase cycling is as follows: $\phi1 = \phi9 = 2(x)2(-x)$, $\phi2 = \phi7 = (x,-x)$, $\phi3 = 16(x)16(y)$, $\phi4 = \phi5 = 16\ (x)16(-x)$, $\phi6 = 4(x)4(-x)4(y)4(-y)$, $\phi8 = 2(4(x)4(-x)4(-y)4(y))$ $2(4(y)4(-y)4(x)4(-x))$, Acquisition = (x,−x,−x,x,−x,x,x,−x,y,−y,−y,y, −y,y,y,−y) 2(−x,x,x,−x,x,−x,−x,x,−y,y,y,−y,y,−y,−y,y)(x,−x,−x,x,−x,x,x, −x,y,−y,−y,y,−y,y,y,−y).

During the recycle delay and the NOE mixing time low power H_2O irradiation was applied, and at the beginning of the NOE mixing time low power X purge pulses were given. These purge pulses (field strength of 5 kHz and a duration of four times 0.5 ms) were included in order to suppress the transfer of unwanted coherences through the NOE mixing time. The carrier of the ^{13}C pulses was placed in the middle of the ^{13}C spectrum in the C_α region (40 ppm). Prior to the acquisition with GARP heteronuclear decoupling a 90-degree X pulse-pair was included in the pulse sequence [15]. Quadrature detection in the t_1 dimension is achieved by the TPPI method, incrementing the phase of the second 90 degree proton pulse. The value of τ should be $(2^1J_{HC})^{-1}$, and therefore an average value of 3.94 ms for ^{13}C was used. A similar pulse sequence was used for acquisition of a $^{14}N/^{15}N$ doubly filtered NOESY, in which case a value of 5 55 ms for τ was used corresponding to the value of $(2^1J_{HN})^{-1}$.

All NMR spectra were processed on a Silicon Graphics workstation using the TRITON software package. The data set of the 2D $^{12}C/^{13}C$ doubly filtered NOESY (256 × 2K) was recorded with 320 scans and a NOE mixing time of 200 ms Separate baseline corrections were applied after the Fourier transformations and appropriate window function multiplications, to obtain a 1K × 1K spectral matrix. The spectrum was analysed directly on a workstation using the program REGINE written in our laboratory.

3. RESULTS AND DISCUSSION

Mixing of ^{13}C labelled Arc with an equimolar amount of unlabelled Arc leads to two types of homo-dimer (completely unlabelled and fully labelled) and a hetero-dimer in a ratio of 1:1:2. A doubly filtered NOESY experiment allows the selective observation of NOEs from protons of unlabelled Arc to protons of labelled Arc, which must be inter-subunit NOEs of the hetero-dimer. The NMR experiment as shown in Fig. 1 is designed around the NOE mixing time τ_m. Flanked at both sides is a spin-echo difference pulse sequence (conventional X half filter) to perform the specific ^{13}C-^1H (proton-**X**) or ^{12}C-^1H (proton-**H**) spin pair selection. The basic phase cycle of the pulse sequence of Fig. 1; ϕ_2 (x,−x) ϕ_7 (x,−x) and receiver phases (x,−x), simultaneously allows the selection of both the proton-**X** → proton-**H** and the proton-**H** → proton-**X** inter-subunit NOEs.

The complete 2D $^{12}C/^{13}C$ doubly filtered NOESY spectrum of the Arc repressor mixture is presented in Fig. 2. This spectrum shows many cross-peaks corresponding to NOE interactions from one monomer of the Arc dimer to the other. Many NOEs are present between the side-chain protons of aromatic and aliphatic residues and between aliphatic residues. Indeed, these residues make up most of the dimeric hydrophobic interface of the Arc protein. Part of this spectrum is shown in Fig. 3 where the inter-subunit NOEs from the aromatic to aliphatic protons are visible, and compared with a regular 2D ^1H-^1H NOESY spectrum.

A similar $X(\omega_1,\omega_2)$ filtered experiment has been performed by Otting and Wüthrich [10]. They recorded a set of four different experiments, which after a suitable linear combination resulted in four subspectra ($^{13}C(\omega_1,\omega_2)$ doubly filtered, $^{13}C(\omega_1)$ filtered/$^{13}C(\omega_2)$ selected, $^{13}C(\omega_1)$ selected/$^{13}C(\omega_2)$ filtered and $^{13}C(\omega_1,\omega_2)$ doubly selected). Since our interest is only in the combined filtered/selected spectra, we can reduce the experimental time by a factor two using the pulse sequence and phase cycling of Fig. 1.

In principle the 2D $^{12}C/^{13}C$ doubly filtered NOESY spectrum should not contain any diagonal intensity and

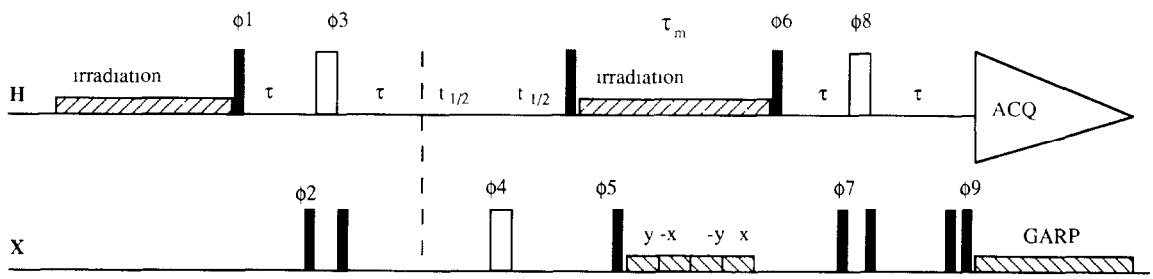


Fig. 1. Pulse sequence for recording the 2D $^{12}C/^{13}C$ doubly filtered NOESY spectrum.

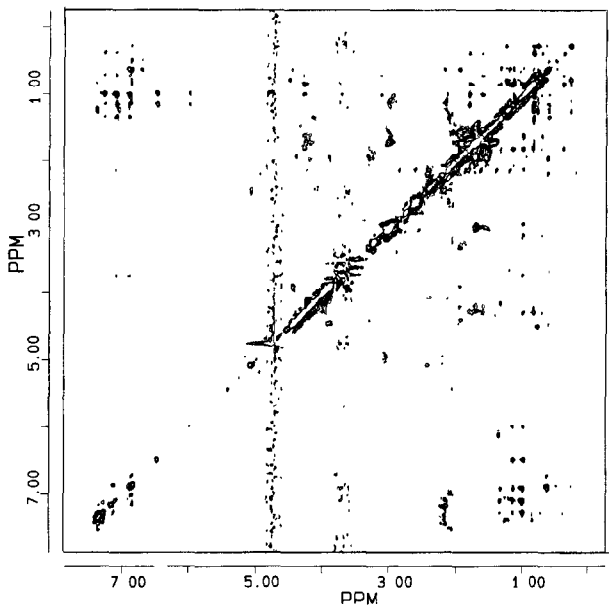


Fig. 2. Complete 2D $^{12}C/^{13}C$ doubly filtered NOESY spectrum of a 1:1 mixture of labelled and unlabelled Arc repressor in D_2O. In the 2D spectrum some small artifacts are present which stem from small molecular weight contaminants.

indeed the diagonal intensity of this spectrum was dramatically reduced. As pointed out earlier [16] the remaining diagonal intensity is probably due to the leakage of ^{13}C-^{1}H spins when selecting for protons bound to ^{12}C. This is related to the nonuniform $^{1}J_{H,C}$ coupling constants of proteins. Improved methods for the selection of protons attached to ^{12}C in the presence of ^{13}C-^{1}H spins are available [16] and consist of X-half filters without refocussing delays with an additional short spin-lock period. However, these type of filters cannot take advantage of the reduction in recording time by a factor of two.

The results of the doubly filtered NOESY experiments are illustrated for the important DNA binding site of Arc, which consist of an N-terminal β-sheet from residue 8 to 14 with strands from two Arc monomers, represented schematically in Fig. 4A. The specific inter-subunit backbone NOE between the amides of Phe[10] and Leu[12] (Fig. 4B) is shown in a part of the 2D $^{14}C/^{15}N$ doubly filtered NOESY spectrum. The inter-strand $C_\alpha H$-$C_\alpha H$ NOEs of the β-sheet in part of the 2D $^{12}C/^{13}C$ doubly filtered NOESY spectrum are indicated in Fig. 4C. The off-diagonal $C_\alpha H$ NOE from Gln[9] and Arg[13] is clearly visible. The $C_\alpha H$ NOE cross-peak of Asn[11] to its symmetric counterpart is present as a diagonal peak, which can be concluded from its much larger intensity compared to other diagonal peaks.

Interestingly, some new inter-monomer NOEs in 2D $^{12}C/^{13}C$ doubly filtered NOESY spectrum were identified which previously could not have been found. These are the NOEs present between the mutual methyl protons of Leu[12], and among the C_α-C_β protons of Asn[11]. Naturally these NOEs had previously been assigned as intra-residue since the intra-residue distances are within 3 Å [17], but this experiment shows that also inter-monomer NOE contributions are present. These inter-monomer NOEs are in complete agreement with the solution structure of the Arc repressor, since both residues Asn[11] and Leu[12] are close to the dyad axis. Furthermore, new inter-monomer NOEs were assigned, which initially could not be deduced from the comparison with the MetJ crystal structure because they are located in the C-terminal part of the protein where the structural homology between Arc and MetJ no longer holds.

Because the present experiment yields a considerably simplified spectrum also some completely new inter-

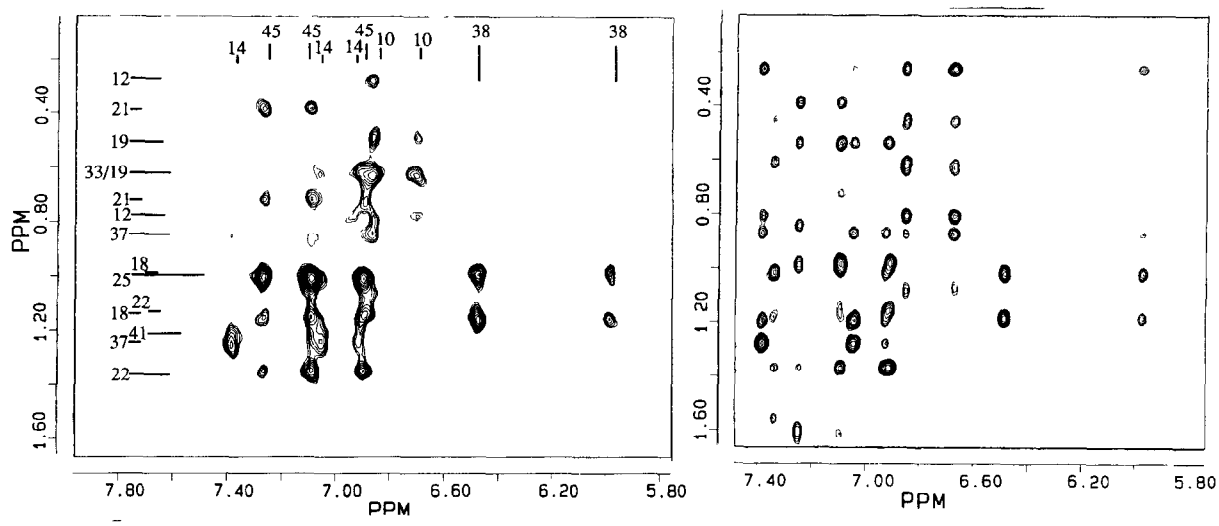
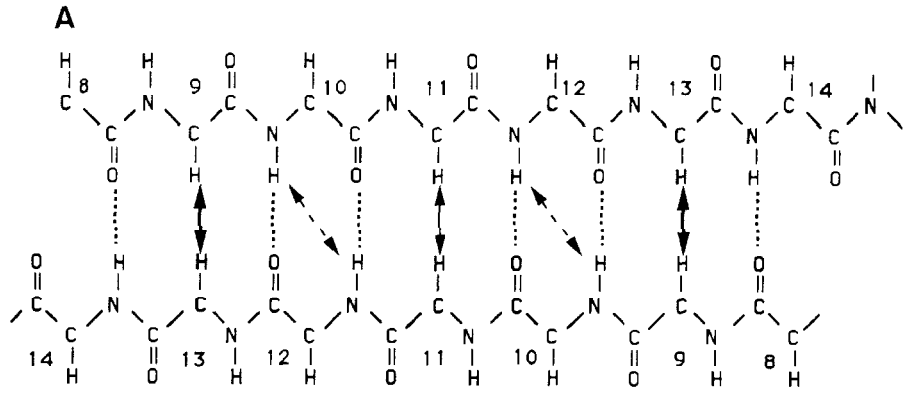
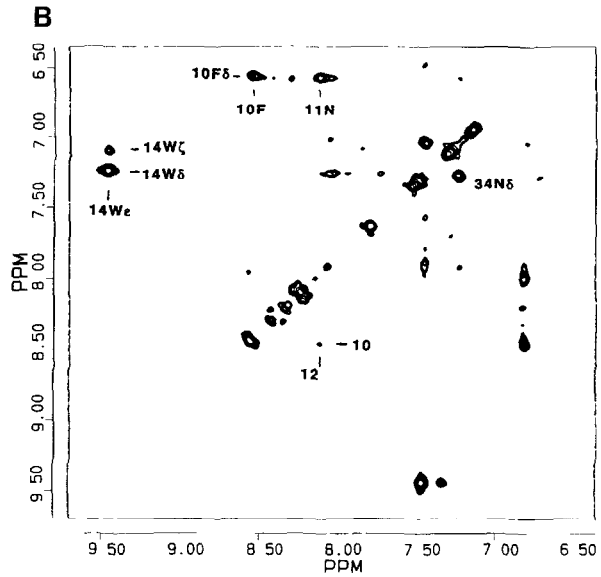


Fig. 3. In the left panel part of the 2D $^{12}C/^{13}C$ doubly filtered NOESY spectrum is shown. The methyl protons are indicated with their corresponding residue number along the vertical axis, the aromatic protons along the horizontal axis. In the right panel the identical part of a regular 2D ^{1}H-^{1}H NOESY is shown. Inter- and intra-subunit NOEs can be identified by comparison of the two spectra.





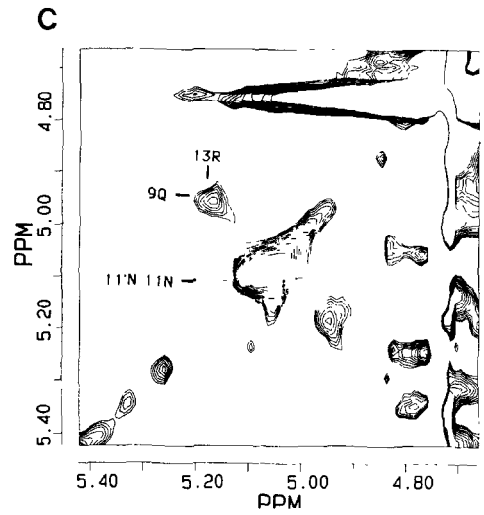


Fig 4. Schematic representation of the β-sheet DNA binding part of the Arc repressor (A). The arrows represent the observed inter-monomer backbone NOEs in the NOESY spectra below The dashed arrows represent the NOE observed in the spectrum of Fig. 4B, the straight arrows the NOES of Fig. 4C. (B) Part of a $^{14}N/^{15}N$ doubly filtered NOESY spectrum showing the inter-monomer amide–amide cross-peaks between residues 10 and 12 of the β-sheet. Some additional assignments are indicated between carbon-bound protons and amide protons which are also visible in this spectrum. (C) Part of the $^{12}C/^{13}C$ doubly filtered NOESY spectrum showing the $C_\alpha H$-$C_\alpha H$ cross-peaks of the β-sheet. The indicated cross peak of Asn^{11} is present as diagonal peak. The noise ridge at 4.75 ppm stems from the water frequency.

monomer NOEs could be assigned which were overlooked in the crowded regular 2D ^1H-^1H NOESY spectrum. For instance an inter-subunit NOE between the $C_\gamma H$ protons of Pro^8 and the methyl protons of Leu^{19} was found, and one between the $C_\delta H$ protons of Pro^{15} and the aromatic ring protons of Tyr^{38}.

In the experiments described in this communication all expected inter-monomer NOEs between carbon-bound protons were found which were initially assigned as such for the three-dimensional solution structure determination of the Arc repressor [2]. In addition some new inter-monomer NOEs could be assigned and a total of over 100 inter-subunit constraints could be deduced from these experiments. This provides sufficient structural information to obtain an independent three dimensional structure of the dimeric Arc repressor by NMR methods only.

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