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A powerful method of sequential proton resonance assignment in proteins using relayed ^{15}N-^{1}H multiple quantum coherence spectroscopy

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A powerful method of sequential resonance assignment of protein ^{1}H-NMR spectra is presented and illustrated with respect to the DNA-binding protein *ner* from phage Mu. It is based on correlating proton-proton through-space and through-bond connectivities with the chemical shift of the directly bonded ^{15}N atom. By this means, ambiguities arising from chemical shift degeneracy of amide proton resonances can be resolved. The experiments described involve combining the ^{1}H-detected heteronuclear multiple quantum coherence correlation experiment with homonuclear nuclear Overhauser enhancement, *J*-correlated or Hartmann-Hahn experiments.

Sequential resonance assignment; ^{15}N labeling; Relayed multiple quantum coherence spectroscopy; Protein, ner; Phage, Mu

1. INTRODUCTION

The assignment of the ^{1}H-NMR spectrum of a protein is an essential prerequisite for the determination of its three-dimensional structure in solution. The mainstay of sequential resonance assignment lies in the identification of through-space (< 5 Å) and through-bond connectivities between the NH protons, on the one hand, and the $C^{\alpha}H$ and $C^{\beta}H$ protons, on the other [1,2]. Further, because the chemical shift dispersion of the NH protons is, in general, larger than that of other proton types, the NH-aliphatic region of two-dimensional nuclear Overhauser enhancement (NOESY) spectra provides one of the main sources of long range NOEs between residues far apart in the sequence, which are essential for determining the polypeptide fold. For mainly α-helical proteins where the chemical shift dispersion of the NH resonances is small, as well as for proteins larger than 100 residues, spectral overlap and degeneracy within the NH region can present serious impediments towards successful assignment. To date two approaches have been used to tackle this problem. The simplest method exploits the differences in temperature and pH dependence of the NH backbone resonances. By recording a set of two-dimensional NMR spectra at a variety of temperatures and/or pH values, some degeneracies can be removed. In practice, however, this may not always be feasible owing to a limited range of conditions over which the protein under study is stable. A second approach involves the use of specific labelling. In general, this is expensive as

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Abbreviations: NMR, nuclear magnetic resonance; COSY, homonuclear correlated spectroscopy; NOESY, homonuclear nuclear Overhauser enhancement spectroscopy; HOHAHA, homonuclear Hartmann-Hahn spectroscopy; HMQC, ^{1}H-detected heteronuclear multiple quantum coherence spectroscopy; rf, radiofrequency

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well as difficult since it requires the use of auxotrophic strains. Spectral simplification may be achieved either by the incorporation of selected deuterated amino acids [3] or by the incorporation of heteronuclear spin labels at specific positions in the molecule. In this respect a number of heteronuclear filtered homonuclear experiments have been proposed in recent years [4–10]. A further serious drawback of all these methods is that it involves recording a great many spectra.

In this paper we demonstrate a much simpler strategy for facilitating the sequential assignment of proteins that have spectra that are too complex for analysis by the standard homonuclear methods alone. It involves the use of complete ^{15}N labelling. In particular, the method involves the correlation of proton-proton through-space and through-bond connectivities with the chemical shift of the directly bonded ^{15}N atom by means of relayed ^{15}N-^{1}H multiple quantum coherence spectroscopy.

2. EXPERIMENTAL

The protein ner from phage Mu was purified from *Escherichia coli* B containing the inducible plasmid pL-ner which directs high level production of the protein [11]. Complete ^{15}N labelling (~93%) was achieved by growing the bacteria in a minimal medium using $^{15}NH_4Cl$ as the sole nitrogen source. The sample for NMR comprised 2 mM protein in 90% H_2O/10% D_2O containing 150 mM phosphate buffer, pH 7.0.

All NMR spectra were recorded on a Bruker AM-600 spectrometer at 27°C.

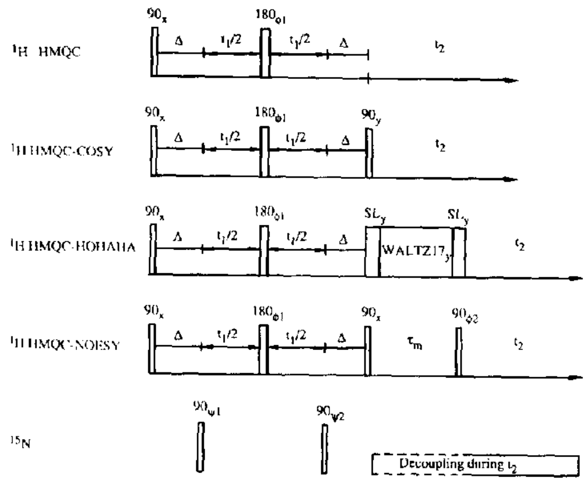

Fig.1. Pulse schemes for heteronuclear MQC correlation and relayed heteronuclear MQC-COSY, -HOHAHA and -NOESY experiments. Each of the four schemes utilizes the ^{15}N pulses shown at the bottom of the figure. The phases are cycled as follows: $\phi_1 = 2(x),2(-x)$; $\phi_2 = 4(x),4(y),4(-x),4(-y)$; $\psi_1 = x,-x$; $\psi_2 = x$ (and may be inverted together with the receiver phase after the basic phase cycle is complete); receiver (HMQC and relayed HMQC-COSY) $= x,-x$; receiver (relayed HMQC-NOESY) $= 2(x,-x),2(y,-y),2(-x,x),2(-y,y)$. The duration of Δ was 4 ms, slightly shorter than $1/(2J_{NH})$. To obtain pure phase absorption spectra using the time proportional incrementation method [29] the phase of ψ_1 is incremented by 90° for every successive t_1 value. ^{15}N decoupling during the acquisition time (t_2) is achieved using the WALTZ-16 decoupling sequence [24]. In addition, to avoid effects of an incomplete steady state, the phase ψ_2 and the receiver phase may be inverted after completion of the above phase cycles [30].

3. RESULTS AND DISCUSSION

The experiments we use rely on a combination of the heteronuclear multiple quantum coherence pulse scheme (HMQC) [12–19] with experiments such as homonuclear nuclear Overhauser enhancement (NOESY) [20], *J*-correlated (COSY) [21] and Hartmann-Hahn (HOHAHA) [22,23] spectroscopy. The pulse schemes with the minimum amount of phase cycling necessary for complete suppression of artifacts, are presented in fig.1.

To minimize spectral crowding and to maximize sensitivity we find it essential to remove the heteronuclear coupling in both frequency dimensions. In the F2 dimension this is accomplished by irradiation of the ^{15}N nuclei with an energy efficient WALTZ16 [24] or GARP [25] sequence. In the F1 dimension, ^{1}H-^{15}N zero and double quantum coherence is present. The 180° ^{1}H pulse at the center of the evolution period (t_1) interchanges zero and double quantum coherence, with the final result that observed resonances appear to be modulated by the ^{15}N chemical shift only [12]. Thus, for NH protons, no heteronuclear decoupling is needed during this interval. For NH_2 groups, on the other hand, the zero and double quantum coherences are modulated by the passive *J* coupling to the second proton. Ideally, the effect of this coupling is also removed by the 180° ^{1}H pulse. In practice, however, rf inhomogeneity makes perfect inversion of the passive proton difficult. Consequently, a low intensity doublet superimposed on an intense decoupled singlet resonance is often observed for NH_2 correlations.

As has been demonstrated recently [26], the relaxation rate of the heteronuclear multiple quantum coherence is to first order not influenced by the strong ^1H-^{15}N dipolar coupling. As a result, the linewidths in the F1 dimension of spectra recorded using the relayed HMQC-NOESY, HMQC-COSY or HMQC-HOHAHA method are narrower by about 25% than the corresponding NH linewidths in the homonuclear NOESY, COSY or HOHAHA spectra, respectively, thereby providing increased resolution.

Fig.2 presents the results obtained on the uniformly ^{15}N-labelled DNA-binding protein ner from phage Mu. This protein has been cloned and overexpressed in *Escherichia coli* [11] and the determination of its solution structure is currently under way in our laboratory. The simple ^{15}N-^1H correlation spectrum is shown in fig.2A. At pH 7, 61 of the potential 69 ^{15}N-^1H correlation peaks are present in the spectrum. (Note that the use of H_2O presaturation abolishes signals of NH resonances that exchange rapidly with water protons by saturation transfer.) In addition, correlation peaks involving the NH_2 groups of glutamine and

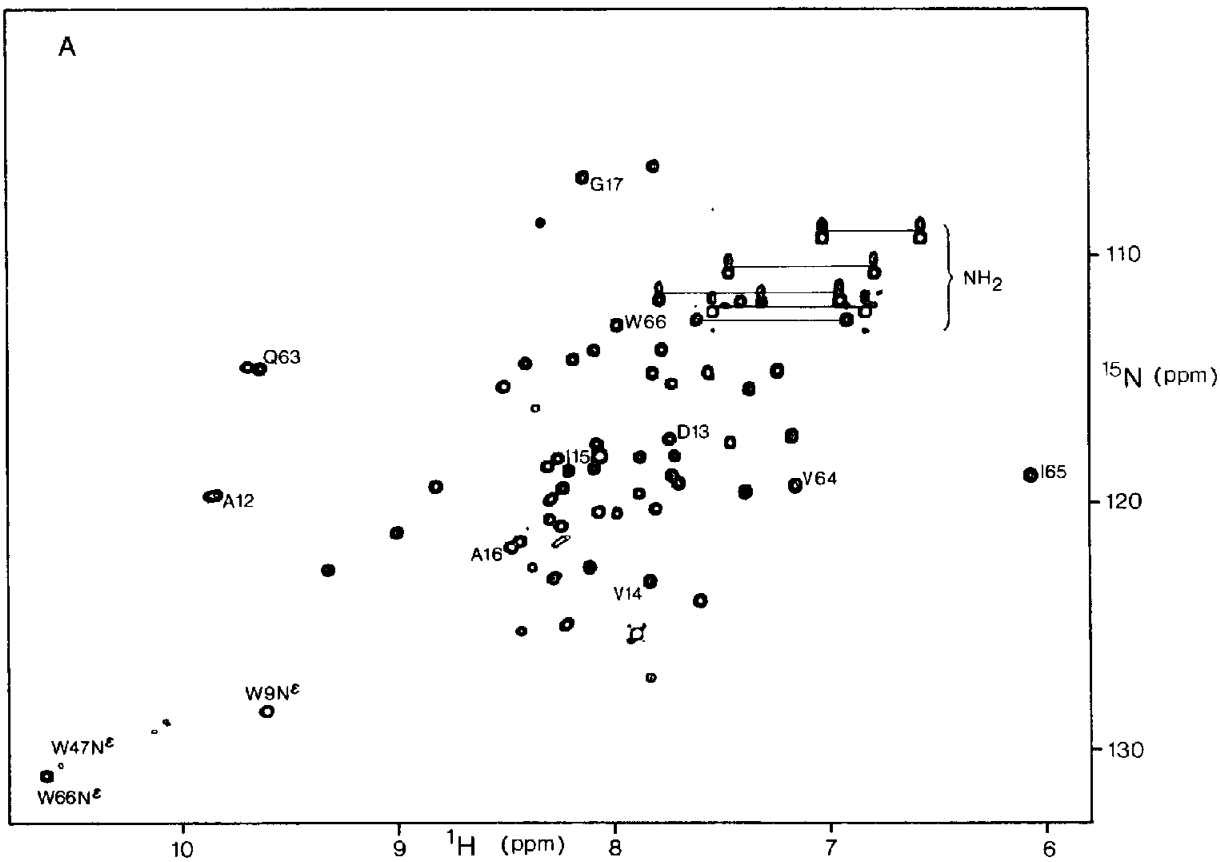

Fig.2. 600 MHz two-dimensional pure phase absorption ^{15}N-^1H HMQC correlation (A) and relayed ^{15}N-^1H HMQC-NOESY (B and C) spectra of Mu ner at 27°C. The ^{15}N(F1 axis)-NH(F2 axis) and ^{15}N(F1 axis)-aliphatic(F2 axis) regions of the relayed ^{15}N-^1H HMQC-NOESY spectrum are shown in (B) and (C), respectively. Selected NH(i)-NH($i+1$), $C^{\alpha}H(i)$-NH($i+1$) and $C^{\beta}H(i)$-NH($i+1$) NOE connectivities are shown in (B) and (C). The peaks in (A) and (B) are labelled at the position of $^{15}N(i)$-NH(i) connectivity; while those in (C) are labelled at the position of the $^{15}N(i)$-$C^{\alpha}H(i)$ or $^{15}N(i)$-$C^{\beta}H(i)$ connectivities, the latter being indicated by the letter β. The NOESY mixing time was 200 ms. Water suppression was achieved by pre-saturation during the relaxation delay and, in the case of the ^{15}N-^1H HMQC-NOESY experiment, during the mixing time τ_m as well. The sample contained 2.7 mM Mu ner in 90% H_2O/10% D_2O containing 150 mM sodium phosphate buffer, pH 7.0, with the protein ~93% ^{15}N labelled. 800 increments were recorded for each spectrum with 16 and 32 scans per t_1 value for the correlation and relayed-correlation experiments, respectively. The digital resolution is 9.8 Hz/pt in F2 and 4.1 Hz/pt in F1, the latter being obtained by appropriate zero filling. The spectra were recorded on a Bruker AM600 spectrometer. ^1H and ^{15}N chemical shifts are expressed relative to 4,4-dimethyl-4-silapentane-1-sulfonate and liquid NH_3, respectively.

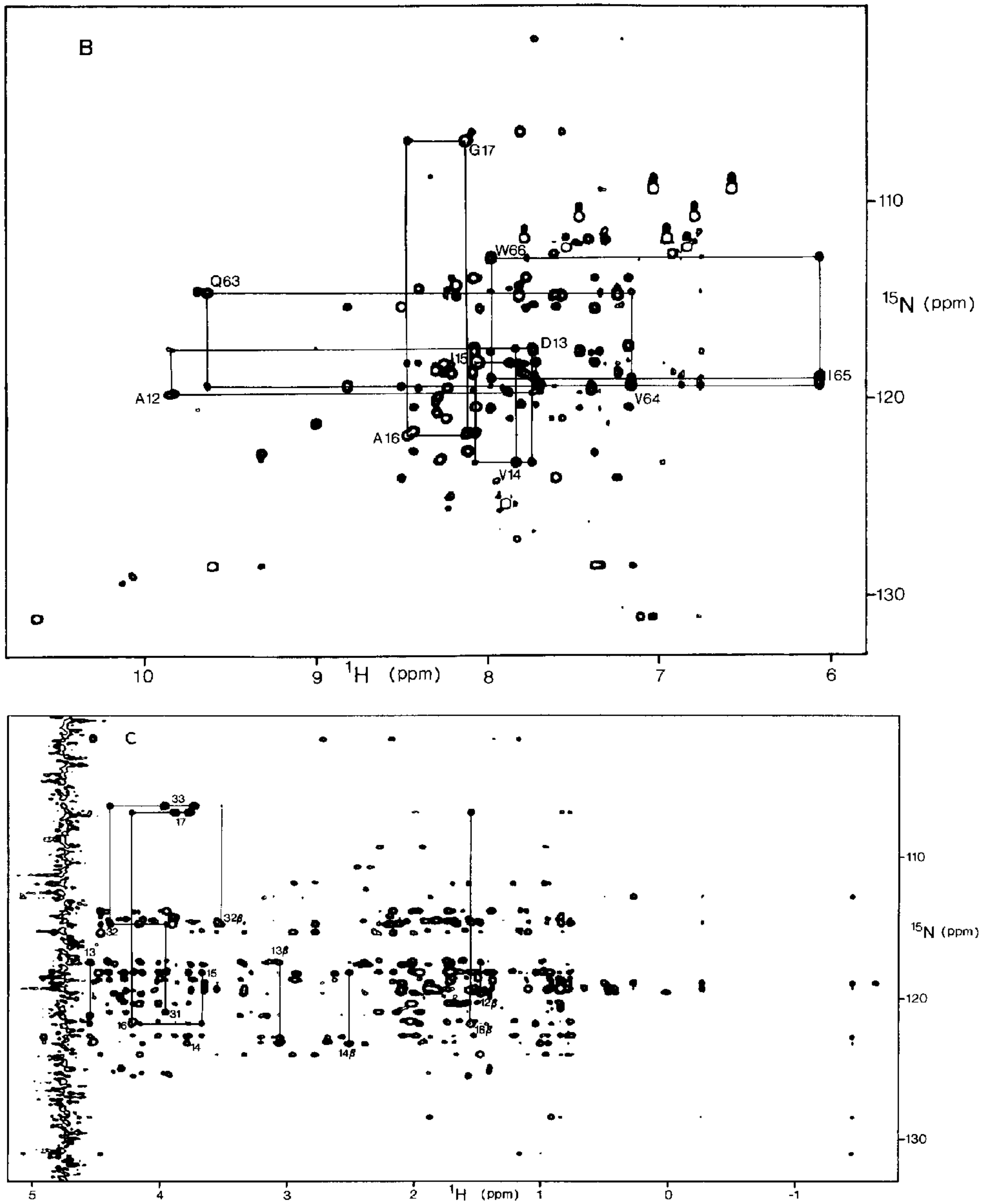
B
G17
W66
Q63
D13
I15
I65
A12
V64
A16
V14
110
120
130
^{15}N (ppm)
10
9
^{1}H (ppm)
8
7
6
C
33
17
32β
32
13
13β
15
12β
16
31
16β
14
14β
110
120
130
^{15}N (ppm)
5
4
3
2
1
0
-1
^{1}H (ppm)

Fig.2. (Contd.)

asparagine and three tryptophan indole protons are observed. Because of a small phase distortion, only one of the spurious doublet lines mentioned above is visible adjacent to each intense correlation for NH_2 groups. This low intensity doublet component facilitates identification of NH_2 resonances, which for magnetically non-equivalent protons is confirmed by the presence of a second proton correlating with the same ^{15}N chemical shift (fig.2A).

The ^{15}N(F1)-NH(F2) region of the relayed ^{15}N-^{1}H HMQC-NOESY spectrum is shown in fig.2B. It is readily appreciated that a large number of NH-NH NOEs are manifested in this spectrum, most of which arise from sequential connectivities between neighbouring NH protons along the polypeptide chain. These NH(i)-NH($i+1$) connectivities are the same as those observed in a conventional NOESY spectrum. The absence of a diagonal together with the fact that ^{15}N chemical shift differences are in general not correlated with ^{1}H ones, makes it easier to detect NOEs between NH protons with only slightly different proton chemical shifts. Two stretches of sequential NH(i)-NH($i+1$) connectivities are delineated, one from Ala-12 to Gly-17, the other from Gln-63 to Trp-66.

The ^{15}N(F1 axis)-^{1}H aliphatic(F2 axis) region of the relayed ^{15}N-^{1}H HMQC-NOESY spectrum is shown in fig.2C, and some examples of $C^{\alpha}H(i)$-$NH(i+1)$ and $C^{\beta}H(i)$-$NH(i+1)$ NOE connectivities are indicated. The positions of the $C^{\alpha}H$ resonances are easily determined from the relayed ^{15}N-^{1}H HMQC-COSY spectrum as well as by reference to a ^{1}H-^{1}H HOHAHA spectrum (not shown). This region is analogous to the NH-aliphatic region of a ^{1}H-^{1}H NOESY experiment. The NH-aliphatic NOEs, however, are spread according to the ^{15}N chemical shift of the directly bonded nitrogen. Because it is very rare to find that both the ^{1}H and ^{15}N chemical shifts of two NH groups are degenerate, NOEs involving NH protons with the same chemical shifts can be readily resolved in this manner.

The methodology described in this paper is of general applicability to any protein that can be uniformly labelled with ^{15}N, a relatively inexpensive and easy process for bacterially expressed proteins, and should significantly speed up the assignment process when combined with presently used homonuclear experiments. In the case of Mu ner, where the chemical shift dispersion of the NH protons is small due to a very high helical content, the experiments presented here were essential for successful sequential assignment. Further, because the ^{1}H-detected ^{15}N-^{1}H HMQC correlation experiment is itself very sensitive, the reduction in signal-to-noise over conventional NOESY, COSY or HOHAHA spectra is at most a factor of 2. It is also evident that the two-dimensional version of these experiments described here can be readily extended to three dimensions [27,28] with the ^{15}N chemical shift in one dimension and ^{1}H-chemical shifts in the other two, and it is hoped that this kind of 3D-NMR experiment will extend the size of proteins whose three-dimensional structures can be determined by NMR.

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