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 $\textit{ner}$ 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, $\textit{ner}$; 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 $^{\alpha}$H and $^{\alpha}$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 $\alpha$-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...
well as difficult since it requires the use of aux-
ootrophic 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 fur-
ther 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 15N labelling.
In particular, the method involves the correlation
of proton-proton through-space and through-bond
connectivities with the chemical shift of the direct-
ly bonded 15N atom by means of relayed 15N-1H
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]. Com-
plete 15N labelling (+93%) was achieved by growing the
bacteria in a minimal medium using 15NH4Cl as the sole
nitrogen source. The sample for NMR comprised 2 mM protein
in 90% H2O/10% D2O containing 150 mM phosphate buffer,
pH 7.0.

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

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 enhance-
ment (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 dimen-
sions. In the F2 dimension this is accomplished by irradiation of the 15N nuclei with an energy effi-
cient WALTZ16 [24] or GARP [25] sequence. In the F1 dimension, 1H-15N zero and double quan-
tum coherence is present. The 180° 1H pulse at the
center of the evolution period (t1) interchanges
zero and double quantum coherence, with the final
result that observed resonances appear to be
modulated by the 15N chemical shift only [12].

Thus, for NH protons, no heteronuclear decoupl-
ing is needed during this interval. For NH2 groups,
on the other hand, the zero and double quantum
coherences are modulated by the passive J coupl-
ing to the second proton. Ideally, the effect of this
coupling is also removed by the 180° 1H pulse. In
practice, however, rf inhomogeneity makes perfect
inversion of the passive proton difficult. Conse-
quently, a low intensity doublet superimposed on
an intense decoupled singlet resonance is often
observed for NH2 correlations.

As has been demonstrated recently [26], the
relaxation rate of the heteronuclear multiple quan-

![Fig. 1. Pulse schemes for heteronuclear MQC correlation and
relayed heteronuclear MQC-COSY, -HOHAHA and -NOESY
experiments. Each of the four schemes utilizes the 15N pulses
shown at the bottom of the figure. The phases are cycled as
follows: $\phi_1 = 2(x), 2(-x); \phi_2 = 4(x), 4(y), 4(-x), 4(-y); \phi_3 =
3x, -x, \phi_2 = x$ (and may be inverted together with the receiv-
er 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 $\Delta$ was 4 ms, slightly shorter than 1/(2J_{HH}). To obtain pure
phase absorption spectra using the time proportional
incrementation method [29] the phase of $\phi_1$ is incremented by
90° for every successive $t_1$ value. 15N 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 $\phi_2$ and the receiver phase
may be inverted after completion of the above phase cycles [30].]
tum coherence is to first order not influenced by the strong $^1H$-$^{15}N$ dipolar coupling. As a result, the linewidths in the $F_1$ 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 nε 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

![Diagram](image-url)
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 NH2 groups. This low intensity doublet component facilitates identification of NH2 resonances, which for magnetically non-equivalent protons is confirmed by the presence of a second proton correlating with the same 15N chemical shift (fig.2A).

The 15N(F1)-NH(F2) region of the relayed 15N-1H HMQC-NOESY spectrum is shown in fig.2A. 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 15N chemical shift differences are in general not correlated with 1H 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 15N(F1 axis)-1H aliphatic(F2 axis) region of the relayed 15N-1H HMQC-NOESY spectrum is shown in fig.2C, and some examples of CαH(i)-NH(i+1) and CαH(i)-NH(i+1) NOE connectivities are indicated. The positions of the CαH resonances are easily determined from the relayed 15N-1H HMQC-COSY spectrum as well as by reference to a 1H-1H HOHAHA spectrum (not shown). This region is analogous to the NH-aliphatic region of a 1H-1H NOESY experiment. The NH-aliphatic NOEs, however, are spread according to the 15N chemical shift of the directly bonded nitrogen. Because it is very rare to find that both the 1H and 15N 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 15N, 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 1H-detected 15N-1H 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 15N chemical shift in one dimension and 1H-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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REFERENCES