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**FOR THE RECORD**

# Nuclear magnetic resonance assignment and secondary structure of an ankyrin-like repeat-bearing protein: Myotrophin

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**Abstract:** Multidimensional heteronuclear NMR has been applied to the structural analysis of myotrophin, a novel protein identified from spontaneously hypertensive rat hearts and hypertrophic human hearts. Myotrophin has been shown to stimulate protein synthesis in myocytes and likely plays an important role in the initiation of cardiac hypertrophy, a major cause of mortality in humans. Recent cDNA cloning revealed that myotrophin has 118 amino acids containing 2.5 contiguous ANK repeats, a motif known to be involved in a wide range of macromolecular recognition. A series of two- and three-dimensional heteronuclear bond correlation NMR experiments have been performed on uniformly $^{15}N$-labeled or uniformly $^{15}N/^{13}C$-labeled protein to obtain the $^{1}H$, $^{15}N$, and $^{13}C$ chemical shift assignments. The secondary structure of myotrophin has been determined by a combination of NOEs, NH exchange data, $^{3}J_{HN\alpha}$ coupling constants, and chemical shifts of $^{1}H_{\alpha}$, $^{13}C_{\alpha}$, and $^{13}C_{\beta}$. The protein has been found to consist of seven helices, all connected by turns or loops. Six of the seven helices (all but the C-terminal helix) form three separate helix-turn-helix motifs. The two full ANK repeats in myotrophin are characteristic of multiple turns followed by a helix-turn-helix motif. A hairpin-like turn involving L32–R36 in ANK repeat #1 exhibits slow conformational averaging on the NMR time scale and appears dynamically different from the corresponding region (D65–I69) of ANK repeat #2.

**Keywords:** ANK repeats; myotrophin; NMR; secondary structure

Cardiac hypertrophy is an important adaptive response of the heart and is a central feature of many human cardiac diseases. Although hypertrophy-related heart failure continues to be a major cause of mortality in humans, the detailed molecular mechanism of hypertrophy is not known. A protein called myotrophin has been identified in spontaneously hypertensive rat hearts and hypertrophic human hearts (Sen & Petscher, 1987). Myotrophin has been shown to stimulate protein synthesis in neonatal rat ventricular cardiac myocytes (Sen et al., 1990) and increases transcript levels of immediate early genes, c-*myc*, c-*fos*, and c-*jun*, and hypertrophy markers such as $\beta$-MHC and $\beta$-MHC ANF (Mukherjee et al., 1993). Myotrophin levels have been reported to be elevated in cardiomyopathic human heart (Sil et al., 1993) and hypertensive rat heart (Sil et al., 1995). The evidence suggests that myotrophin plays an important signaling role in the initiation of myocardial cellular hypertrophy (Sen et al., 1990; Mukherjee et al., 1993; Sil et al., 1993, 1995; Sivasubramanian et al., 1996). The cDNA of myotrophin has been cloned recently, and the recombinant protein has been found to be as biologically active as native myotrophin (Sivasubramanian et al., 1996). cDNA sequence analysis revealed that myotrophin consists of 118 amino acids containing 2.5 contiguous ANK repeats (Taoka et al., 1994; Sivasubramanian et al., 1996) and hence belongs to the ANK repeat-bearing protein superfamily. The ANK repeat is a 33-amino acid motif that is found in tandem arrays of multiple copies in a wide range of proteins (Michaely & Bennett, 1992). Intensive studies of the ANK family of proteins have demonstrated that the ANK repeat motif plays critical roles in protein–protein and protein–nucleic acid interactions (Lux et al., 1990; Thompson et al., 1991; Michaely & Bennett, 1992; Hassel et al., 1993; Schneider et al., 1994; Jaffray et al., 1995; Sun et al., 1996). However, the detailed molecular mechanism of how ANK repeats mediate macromolecular interactions and confer specificity in diverse protein functions remains obscure. Of the 2.5 contiguous ANK repeats in myotrophin, one was found to be highly homologous to those of ANK repeat-bearing protein I$\kappa$B$\alpha$, an inhibitor of eukaryotic transcription factor NF$\kappa$B/*rel* (Sivasubramanian et al., 1996). Moreover, the two proteins share the same putative consensus phosphorylation sites for protein kinase C and casein kinase II (Sivasubramanian et al., 1996). $\kappa$B gel mobility shift assays show that myotrophin has the ability to interact with NF$\kappa$B as revealed by the formation of ternary protein–DNA complexes (Siva-

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*Abbreviations:* NOESY, NOE spectroscopy; HOHAHA, homonuclear Hartman–Hahn; ANK, ankyrin-like; MHC, myosin heavy chain; ANF, atrial natriuretic factor.

subramanian et al., 1996). Because NF$\kappa$B regulates a variety of cellular and viral gene expressions, these observations have led to the suggestion that myotrophin is involved in regulating the expression of cardiac hypertrophy-specific genes in the myocardium through ubiquitous NF$\kappa$B/*rel* factors and $\kappa$B DNA sites (Sivasubramanian et al., 1996).

To understand the molecular basis of myotrophin in the mechanism of hypertrophy as well as general features of ANK repeats in solution, we have initiated solution structural studies of myotrophin using multidimensional heteronuclear NMR spectroscopy. Herein, we report the secondary structure and folding topology of myotrophin. The results show that the protein is mainly composed of seven helices, all connected by turns or loops. The ANK repeats in myotrophin have been found to contain multiple turn-like structures and a helix-turn-helix motif.

**Results and discussion:** *Resonance assignment:* A $^1$H-$^{15}$N HSQC spectrum for uniformly $^{15}$N-labeled myotrophin (118 aa) is shown in Figure 1 with good chemical shift dispersion. Of 114 non-proline residues, only five overlapping pairs of $^1$H-$^{15}$N cross peaks were observed in the HSQC spectrum: (N29:E49), (Q47:H67), (I69:K114), (F53:L102), (N62:D100) (Fig. 1). Although there are some repeated residues in 2.5 ANK repeats, there was no severe resonance overlap in the NMR spectra that prevented data analysis. In fact, of the 18 leucines that are the most abundant amino acids in myotrophin, all are well resolved in the spectrum, except for L102, and have been assigned unambiguously (see Fig. 1).

Initial sequential assignments were performed through 3D $^{15}$N-separated NOESY and $^{15}$N-separated HOHAHA and HNHA experiments. We were able to obtain 85% of backbone and 60% of side-chain assignments based on sequential NOEs and side-chain spin systems (Wüthrich, 1986). After we made the $^{15}$N/$^{13}$C-labeled sample, backbone and side-chain assignments were completed readily through the combined use of through-bond correlation experiments HNCACB, C(CO)NH, HC(CO)NH, and HCCHTOCSY. A strip of HNCACB from residue Q47–I61 is shown in Figure 2. Virtually all the residues in myotrophin showed inter- as well as intraresidue $C^\alpha$ and $C^\beta$ correlations except prolines (only interresidue correlations), which made the backbone assignments straightforward. The assignments were further confirmed by C(CO)NH and HC(CO)NH spectra, which correlate all aliphatic side-chain carbons and protons with the amide of the next residue, respectively. Once completed, assignments were also checked on the basis of sequential NOE connectivities observed in the water-flip-back $^{15}$N-separated NOESY spectrum.

*Secondary structure:* Figure 3 summarizes the amide exchange data, the short- and medium-range NOE data involving amides obtained from the 3D $^{15}$N-separated NOESY spectrum, the $^3J_{HN\alpha}$ values calculated from HNHA spectrum, and the deviations from random coil values of the $H^\alpha$, $C^\alpha$, and $C^\beta$ chemical shifts, which correlate well with the secondary structure (Spera & Bax, 1991; Wishart et al., 1991). The secondary structural elements, as inferred from the NMR data, are indicated in Figure 3 as well. The protein is partly composed of seven $\alpha$-helices (50% of the sequence) as regular secondary structures. The other 50% of the myotrophin sequence is composed of turns and loops. The seven helices in myotrophin include residues D3–K11, L15–K24, P38–D44, L48–L56, P71–Y77, V81–S89, and N110–Q118. As shown in Figure 3, these helices have the following unique features: strong or medium $d_{NN(i,i+1)}$ connectivities, $d_{\alpha N(i,i+3)}$ connectivities, small $^3J_{HN\alpha}$ values, negative secondary $H_\alpha$ shifts, large positive second-

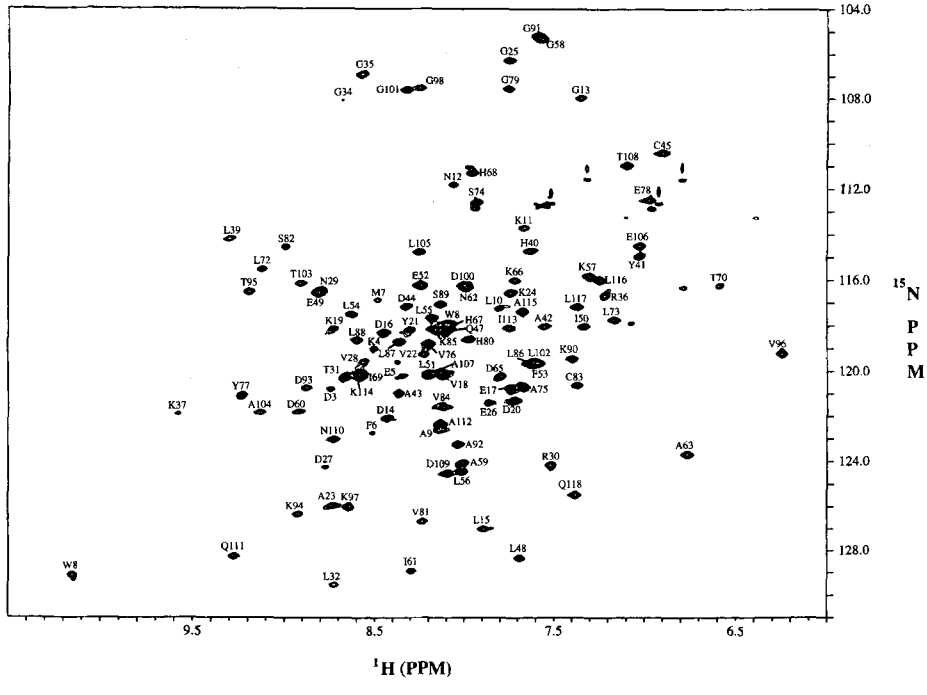


**Fig. 1.** Two-dimensional $^1$H-$^{15}$N HSQC spectrum of 1 mM uniformly $^{15}$N-labeled myotrophin at 25 °C, pH 6.2, in 93% $H_2O$/7% $D_2O$ with 50 mM phosphate buffer and 5 mM $\beta$-mercaptoethanol. The spectrum was acquired at a Varian Unity Plus 600 MHz spectrometer with 1,024 complex points in $t2$ and 256 complex points in $t1$ and processed by nmrPipe software. The assignments are labeled by the one-letter code of amino acids accompanied by a sequence number.

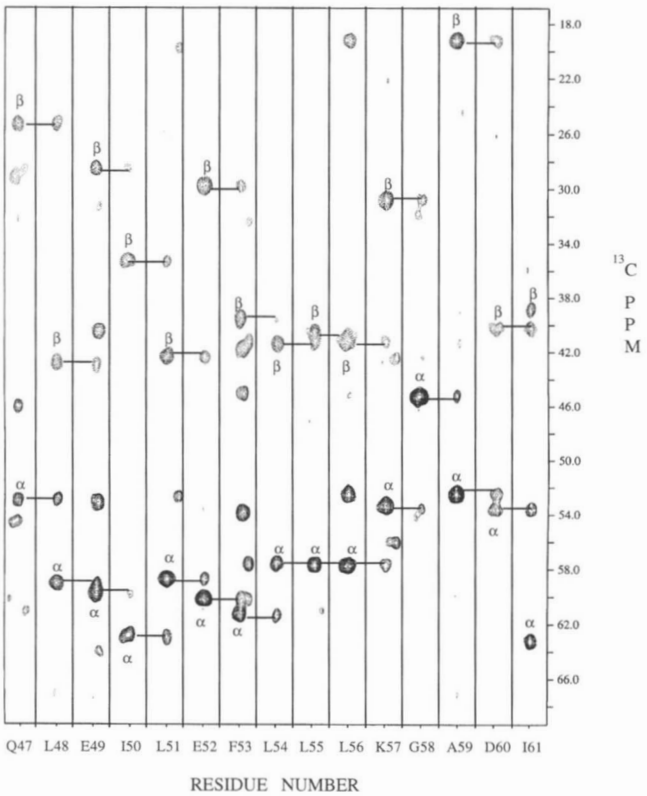


**Fig. 2.** Sequential strips from Q47–I61 extracted from the HN(CA)CB experiment. Intraresidue correlations are labeled with Greek symbols. Virtually all the interresidue correlations were observed in the HN(CA)CB spectrum.

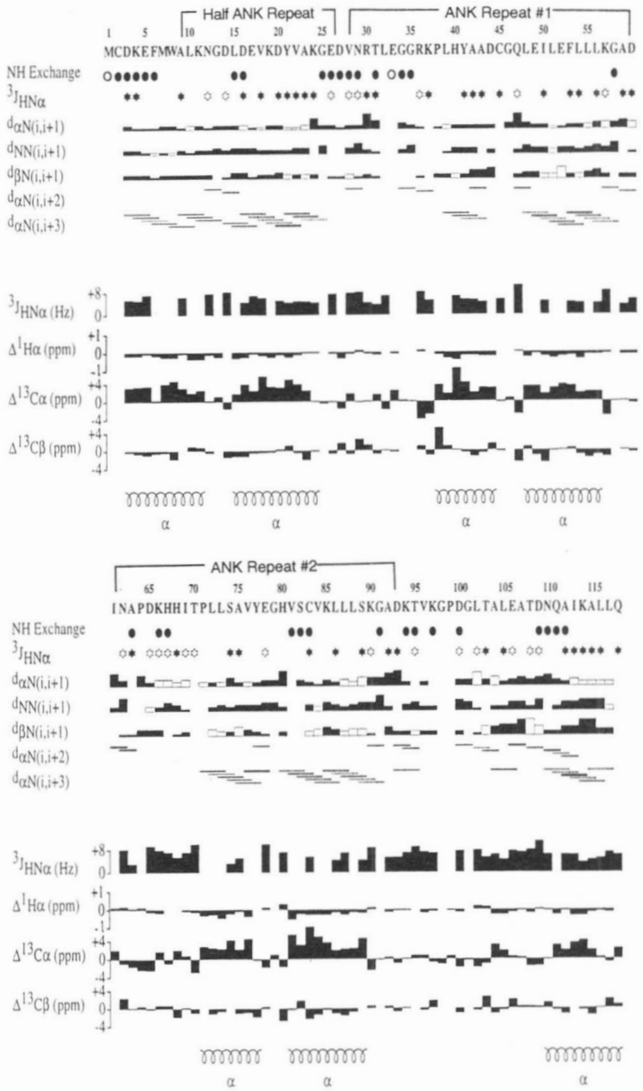


**Fig. 3.** Summary of the NMR data obtained for myotrophin on NH exchange, sequential NOEs involving NH, $^3J_{HN\alpha}$ coupling constants, $H^{\alpha}$, $C^{\alpha}$, and $C^{\beta}$ secondary shifts, the secondary structure deduced from these data. The 2.5 ANK repeats are indicated above the amino acid sequence. NH exchange: Filled circles indicate $^{15}N$-$^1H$ correlations subject to rapid hydrogen exchange; open circles indicate absent $^{15}N$-$^1H$ cross peaks. NOEs: The height of the bar indicates the strength of the NOE; open boxes and dotted lines denote ambiguity due to overlap. $^3J_{HN\alpha}$: Coupling constants obtained from the HNHA experiment (Vuister & Bax, 1993). Filled and open stars indicate small (<6 Hz) and large (>8 Hz) $^3J_{HN\alpha}$ coupling constants, respectively.

ary $C_{\alpha}$ shifts, and negative secondary $C_{\beta}$ shifts. Six of the seven helices, except for the C-terminal helix, form three separate helix-turn-helix motifs. The three-residue tight turns in these motifs (N12–D14, C45–Q47, and E78–H80, respectively) were determined by the following features: no consecutive secondary shifts typical for helices or sheets; discontinuous connectivities for both $d_{NN(i,i+1)}$ and $d_{\alpha N(i,i+3)}$, except for N12–D14; some strong and medium $d_{\alpha N(i,i+1)}$ NOEs; some $d_{\alpha N(i,i+2)}$ NOEs; some slow-exchanging NHs; and some large $^3J_{HN\alpha}$ coupling constants (>8 Hz). Turn-like structures were also observed in the stretches of G25–K37, G58–T70, and K90–D109 based on the above structural features for turns (see Fig. 3).

The second (P38–L56) and third (P71–S89) helix-turn-helix motifs belong to ANK repeats #1 and #2, respectively (Fig. 3). The beginnings of ANK repeat #1 (V28–K37) and #2 (I61–T70) are composed of multiple turn-like structures leading to the helix-turn-helix motif. It is noteworthy that the turns involving L32–R36 in ANK repeat #1 and D65–I69 in ANK repeat #2 show hairpin-like structures in addition to the above structural features for turns, as judged by the medium $d_{NN(i,i+4)}$ connectivities for L32:R36 and D65:I69, respectively, and slow NH exchanges for L32, R36, D65, and I69, which may be involved in hydrogen bonding. However, in contrast to the D65–I69 turn, the L32–R36 region appears to have slow conformational averaging because $^1H$-$^{15}N$ correlations for the latter are weak, particularly for L32, E33 (absent), G34, and K37 (Fig. 1). Unusually low intensities in the water-flip-back HSQC experiment can be attributed to slow conformational averaging instead of rapid exchange of amides (Grzesiek & Bax, 1993; Vuister et al., 1994). Indeed, L32, R36, and K37 exhibit slow NH exchanges despite the weak $^1H$-$^{15}N$ cross peak intensities.

In summary, myotrophin contains a high percentage of nonregular secondary structures, including turns and loops (50% of the protein sequence), indicating the flexibility of the protein conformation. The structural feature of the ANK repeats in myotrophin is unique, including the multiple turns followed by a helix-turn-helix motif. The helical feature of the ANK repeat motif has been suggested previously based on molecular modeling, CD, and NMR studies of several ANK repeat-bearing proteins (Lux et al., 1990; Michaely & Bennett, 1992, 1993; Gay & Ntwasa, 1993; Tevelev et al., 1996). After we completed our backbone assignment and

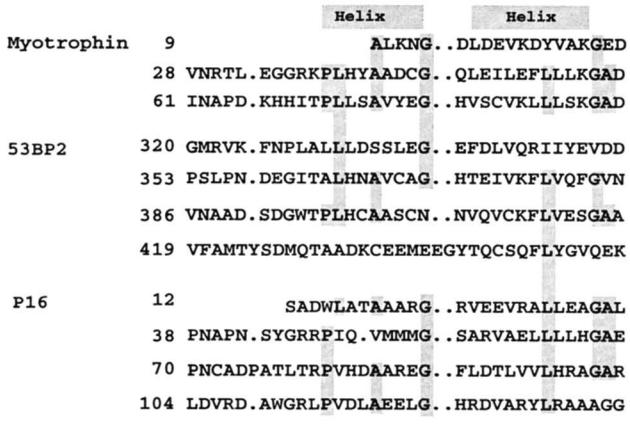


**Fig. 4.** Comparison of the ANK repeats among myotrophin, 53BP2, and p16. Most conserved regions are highlighted. Helical regions are indicated and the other regions are composed of turns and/or loops. The first repeats in myotrophin and p16 are incomplete.

secondary structure determination, the crystal structure of p53 binding protein 53BP2 containing four ANK repeats was published (Gorina & Pavletich, 1996). The secondary structure of the ANK repeats in the 53BP2 crystal structure is largely similar to the results of our solution studies on myotrophin. However, no clear evidence of interhairpin contacts as observed in 53BP2 were found in the myotrophin, indicating that the packing of the ANK repeats in myotrophin may be different from that of 53BP2; moreover, the dynamics of the various ANK repeats appear different in solution, such as hairpin-like turn regions in myotrophin. A comparison of the ANK repeats (Fig. 4) among functionally different myotrophin, 53BP2, and tumor suppressor p16 revealed that the sequence homology in the multiple turn regions of the ANK repeats are low. The amino acid variations as well as different packing and dynamics of the ANK repeats may be important for their diverse functions. The solution dynamics study and 3D structure determination of the myotrophin are under way to further elucidate the structure–function relationship of the protein and to understand the diverse functions of ANK repeats in general.

**Materials and methods:** *Sample preparation:* Two liters of *Escherichia coli* (BL21/DE3) LysS cells transformed with pET3a-51 encoding myotrophin were grown at 37 °C in minimal media containing 0.4% glucose/0.1% $^{15}NH_4Cl$ or 0.4% [$^{13}C_6$] glucose/0.1% $^{15}NH_4Cl$ in order to obtain $^{15}N$- and $^{15}N/^{13}C$-labeled proteins, respectively. Cells were grown in log phase to $OD_{600nm} = 0.7$, and protein expression was induced for 5 h with 1 mM isopropyl $\beta$-thiogalactopyranoside. The cells were harvested by centrifugation for 15 min at 6,000 × *g* at 4 °C and resuspended in 50 mM Tris-HCl, pH 8.0, 100 mM NaCl, and 5 mM $\beta$-mercaptoethanol. Cells were lysed by French Press and the pellet was removed by ultracentrifugation at 100,000 × *g* for 90 min at 4 °C. The supernatant (40 mL) was diluted with 20 mM Tris, pH 8.0, 1 mM $\beta$-mercaptoethanol (buffer A) to 120 mL and applied to a 20-mL Q-sepharose Fast Flow column by FPLC (Pharmacia). The column was washed with buffer A and eluted in 0.1–0.5 M NaCl gradient. Peak fractions were pooled and further purified using a Centriprep-30 (30-kDa cutoff). The purified protein was concentrated using a Centriprep-10 (10-kDa cutoff) cartridge. Sample purity (>95%) was determined by SDS gel electrophoresis and Coomassie Blue staining. A small amount of precipitation occurs after a period of time if the protein concentration is larger than 2 mM. Cys 45 appears to be on the surface because it becomes oxidized and possibly forms disulfide-linked dimer after several months (judged by the chemical shifts and $T_2$ measurements of the amides).

*NMR spectroscopy:* The protein sample was prepared in argon-purged $H_2O$ solution (7% $^2H_2O$), pH 6.2, 50 mM potassium phosphate, and 5 mM $\beta$-mercaptoethanol in a 250-$\mu$L microcell NMR tube (Shigemi Inc., Allison Park, Pennsylvania) with ca. 1.0–1.5 mM concentration. All the NMR experiments were conducted at 25 °C using a Varian Unity Plus 600 MHz spectrometer equipped with a triple-resonance probe head and a shielded *z*-gradient unit. The following experiments were recorded on a 1.0-mM uniformly $^{15}N$-labeled protein: sensitivity-enhanced 2D $^1H$-$^{15}N$ HSQC (Kay et al., 1992) using water-flip-back for water suppression (Grzesiek & Bax, 1993), 3D water-flip-back $^{15}N$-separated NOESY (mixing times 100 ms and 150 ms, respectively), 3D $^{15}N$-separated HOHAHA (mixing time 40 ms), and HNHA (Vuister & Bax, 1993). Amide hydrogen-exchange rates were determined by lyophilizing the protein from $^1H_2O$, dissolving the protein in $^2H_2O$, and acquiring a series of 2D $^1H$-$^{15}N$ HSQC spectra at 20 min, 45 min, 70 min, 100 min, and 100 h. The following experiments were recorded on a 1.5-mM uniformly labeled $^{15}N/^{13}C$-labeled protein: HNCACB (Wittekind & Muller, 1993; Muhandiran & Kay, 1994), C(CO)NH (Logan et al., 1992; Grzesiek et al., 1993), H(CCO)NH (Grzesiek et al., 1993), and HCCH-TOCSY (Bax et al., 1990; Kay et al., 1993).

All the data were processed on Sun UltraSPARC workstation using nmrPipe software (Delaglio et al., 1995). In the acquisition dimension, all data sets were processed identically. A solvent-suppression filter was applied to the time-domain data, followed by apodization with a 66° shifted squared sine-bell window, zero-filling to the next power of 2, Fourier transformation, and phasing. The data were apodized in *t*2 by a 72° shifted sine-bell window prior to zero-filling to 256 complex points, Fourier transformation, and phasing. For HN(CA)CB, HNHA, C(CO)NH, and H(CCO)NH, the lengths of the $^{15}N$ time-domain data were doubled by mirror-image linear prediction, apodized by a squared cosine-bell window, zero-filled to 128 complex points, and Fourier transformed. The processed spectra were analyzed by the PIPP program (Garrett et al., 1991).

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