2D and 3D NMR Study of Phenylalanine Residues in Proteins by Reverse Isotopic Labeling

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Abstract: A protein isotopic labeling strategy is presented which offers improved NMR sensitivity and resolution relative to the commonly used uniform 13C labeling approach. Incorporation of specific residues at natural abundance into an otherwise fully 13C-enriched protein yields 1H line widths for the unlabelled residues which are not adversely affected by 13C and makes it possible to selectively focus on interactions between the unlabeled residues and the remainder of the protein. Modifications of 13C editing and 12C filtering procedures are described which optimize their sensitivity and resolution. The experiments are used to obtain complete assignments for all 10 phenylalanine aromatic spin systems in the DNA-binding domain of Drosophila heat shock factor and to obtain a large number of structurally important long-range NOE constraints. A novel J correlation experiment is also described which makes it possible to measure H-1H J couplings in larger proteins and yields quantitative values for all 10 phenylalanines in the DNA-binding domain of Drosophila heat shock factor.

In recent years, 2D and 4D NMR combined with uniform 13C and 15N enrichment has become an established procedure for determining the solution structures of proteins with spectra too complex for conventional homonuclear 1H studies. 1-3 The resonance assignment part of this approach relies largely on determination of intraresidue J couplings via the large and well-resolved one-bond 1H-13C, 1H-15N, 13C-13C, and 13C-15N J couplings. 4-7 The 1H-1H NOE interactions, which are critical for structure determination, are typically severely overlapped in the conventional 2D NOESY spectrum but are dispersed in a 4D spectrum according to the frequencies of the 13C or 15N attached to each of the protons.

Although the approach outlined above has been applied successfully to a large range of proteins, there are inherent problems associated with the use of 13C isotopic enrichment. Most importantly, the strong one-bond 1H-13C dipolar interaction is even larger than the dipolar interaction between geminal protons, causing a dramatic decrease in the 1H T2. Despite these short T2 values, the high intrinsic sensitivity of many of the 3D J correlation experiments allows their application to proteins larger than 30 kDa. However, the rapid transverse relaxation severely affects measurement of the inherently much weaker NOE interactions, particularly for Cα methylene and other non-methyl-group side-chain protons in the rigid parts of the protein. Measurement of the structurally important Hα-1H J couplings is also adversely affected by the increase in 1H line width caused by 13C, and very few accurate values have been reported for larger proteins.

Although the assignment problem has largely been solved by the uniform 13C labeling approach, identification of the aromatic resonances of phenylalanine residues remains notoriously difficult.

In addition to the 13C-induced 1H line broadening, the aromatic Phe resonances also suffer from the non-first-order character of their 13C spectrum, caused by the very poor 13C chemical shift dispersion and large 13C-13C J couplings (~55 Hz). Consequently, the assignment methods commonly used for other residues tend to give poor results for the phenylalanine ring system. For proteins with more than a few Phe residues, the resonance assignments therefore frequently do not extend much beyond the Cα-Hα resonances. For example, in the study of the structure of a calmodulin/target peptide complex, 6 less than half of the aromatic protons and none of the Hβ resonances of the nine calmodulin Phe residues had been assigned. Hβ resonances are particularly critical to NMR structure determination, as they frequently are located in the hydrophobic core of the protein, where they exhibit disproportionately large numbers of long-range NOE constraints. Moreover, in contrast to the other Phe ring protons, the Hβ position is independent of ring flip motions, and no pseudo-atom constraint-loosening approximations need to be made.

In practice, the small 13C chemical shift dispersion of protonated aromatic ring carbons and the large width (110 Hz) of the 13C-13C triplet make the 13C dispersion less useful than for other residues. Thus, the inherent √2 loss in sensitivity associated with the additional 13C dimension is not offset by an increase in resolution. In fact, for a number of uniformly 13C-enriched proteins with long rotational correlation times (>10 ns), we have found that even the short Hβ-1H J NOE interactions may be difficult to observe in the 4D spectrum.

Here, we demonstrate the power of reverse labeling for alleviating the problems mentioned above. Incorporation of natural abundance residues into an otherwise uniformly 13C-enriched protein makes it possible to study the NOE interactions between the reverse labeled residues and the remainder of the protein with increased sensitivity. A novel experiment for quantitative measurement of H-1H J couplings in such residues is also demonstrated. The reverse labeling approach is demonstrated by incorporating natural abundance Phe into the otherwise uniformly 13C/15N-enriched DNA-binding domain of Drosophila heat shock factor, dHSF(33-155), a 123-residue domain containing 10 Phe residues.

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Study of Phenylalanine by Reverse Isotopic Labeling


Figure 1. Pulse schemes of (A) the semi-constant-time doubly 13C-filtered 2D NOESY experiment and (B) the 13C-edited, 13C-filtered 3D NOESY experiment. Narrow pulses correspond to 90° flip angles, wide pulses to 180°. All pulses are applied along x, unless otherwise specified. All gradients are sine-bell shaped, 25 G/cm at center. The 180° 13C pulses are of the composite type (90,220,90). Phase cycling for scheme A: \( \phi_1 = x,-x; \phi_2 = 2(x), 2(-x); \phi_3 = 8(x), 8(-x); \phi_4 = 2(x), 2(y), 2(-x), 2(-y); \) acq = x,-x,-y,-x,y,x,y,-y; quadrature in t1 using States-TPPPI on \( \phi_1 \). Delay durations for scheme A: \( \tau_1 = 1/(4J_{12C}) + a_{11}; \tau_2 = 1/(4J_{12C}) + a_{11} \) and b = 1–2\( a \); \( \tau_3 = 1/(4J_{12C}); \tau_{rel} = \) NOE mixing period; \( G_{1,2,3,4} = 0.2, 1, 3.5, 0.6 \) ms. For scheme A, weak presaturation of the residual HDO resonance is used between scans. Phase cycling for scheme B: \( \phi_1 = x,-x; \phi_2 = x; \phi_3 = 2(x), 2(-x); \phi_4 = 8(x), 8(-x); \phi_5 = 4(x), 4(-x); \phi_6 = 8(x), 8(-x); \) acq = x,-x,-y,-x,y,x,y,-y; quadrature in t1 using States-TPPPI on \( \phi_1 \) and \( \phi_4 \) simultaneously, quadrature in t2 using States-TPPPI on \( \phi_4 \). Delay durations for scheme B: \( \tau_1 = 1/(2J_{12C}); \tau_2 = 1/(4J_{12C}); G_{1,2,3,4} = 0.2, 1, 3.5, 0.6 \) ms.

Figure 2. Pulse scheme of the 13C-filtered H1–H2 quantitative J correlation experiment. Narrow pulses have 90° flip angles, wide pulses 180°. All pulses are applied along x, unless otherwise specified. Gradients: sine-bell shaped, 25 G/cm at center; \( G_{1,2} = 0.9, 0.5 \) ms. Phase cycling: \( \phi_1 = x,-x; \phi_2 = x; \phi_3 = 2(x), 2(-x); \phi_4 = 8(x), 8(-x); \phi_5 = 4(x), 4(-x); \phi_6 = 8(x), 8(-x); \phi_7 = 16(x), 16(-x); \) acq = x,-x; quadrature in t1 using States-TPPPI on \( \phi_1, \phi_4 \) and \( \phi_5 \) simultaneously. Delays: \( \tau_{1,2,3} = 3.6, 3.2, 20 \) ms; \( \tau = 10.7 \) ms. Weak presaturation of HDO is used during the delay between scans.

Experimental Section

Sample Preparation. Escherichia coli (BL21/DE3) cells transformed with dHSF(33–155) plasmid were grown at 37°C in M9 minimal media containing 0.3% [13C]glucose/0.1% [15N]NH4Cl and 20 mg/L natural abundance L-Phe. The protein was purified as described previously,7 and a 0.4 L culture yielded 500 mg of purified protein, sufficient for a 2 mM sample in a Shigemi microcell (Shigemi Inc., Allison Park, PA). The 13C labeling of the residue was at least 90%, and no measurable increase in 

13C was observed for any of the other amino acids.

NMR. All NMR experiments were recorded in D2O solution, pH 6.3, 10 mM potassium phosphate, 50 mM KCl, at 27°C, using a Bruker AMX600 spectrometer equipped with a Bruker triple-resonance probehead and a self-shielded z-gradient. Sine-bell shaped pulse field gradients (25 G/cm at the center of the sine bell) were generated with an in-house developed pulse shaping unit and amplifier.

13C-Filtered HOHAHA. The 13C-filtered HOHAHA spectrum of the aromatic region of dHSF(33–155) (Figure 3A) was recorded using a recently proposed 13C filtering scheme that optimizes sensitivity by 13C filtering during the 1H isotropic mixing period,8 avoiding the need for relaxation-sensitive filtering delays. Experimental parameters: 1H carrier, 4.75 ppm; 13C carrier, 63.9 ppm; high-power 1H radio frequency field strength, 26 kHz; high-power 13C radio frequency field strength, 16.6 kHz; 1H isotropic mixing and 13C DIPSI irradiation, 9.1 kHz radio frequency field strength; matrix size, 300*1024 data matrix, where \( n^* \) refers to a complex data points; 32 transients acquired per complex \( \tau_1 \) increment; total measuring time, 4 h; digital filtering, 45°-shifted squared sine-bell filters in both \( \tau_1 \) and \( \tau_2 \) final digital resolution, 5.7 (F2) and 3.4 Hz (F3). The 1H isotropic mixing period consisted of 11 DIPSI-2 cycles (3.1 ms each), of which the first and the last three were synchronized with DIPSI-2 13C irradiation.8

13C-Filtered NOESY. The 13C-filtered spectrum of Figure 3B, which shows only interactions between 13C-attached protons, has been recorded with the pulse scheme of Figure 1A, which is a modified version of the previously proposed [F1=C,F2=C]NOESY filtering scheme.9 Relaxation losses are minimized by using 2-fold shorter filtering delay durations \( (\tau_1 + \tau_2 = 3.6 \) ms; \( 2\tau_1 = 3.1 \) ms) compared to the original scheme.11 Moreover, the first evolution period is of the "mixed-constant-time" type,12 for longer \( \tau_1 \) durations an increasing fraction of the 1H evolution takes place during the \( \tau_1 \) + \( \tau_2 \) filtering delay. This has the effect of reducing the decay rate of the magnetization in the \( \tau_1 \) dimension. Experimental

(9) This 13C carrier position was accidental. A better choice for filtering the aromatic region of the spectrum would have been to place the 13C carrier in the aromatic region of the spectrum (–125 ppm) and to use shorter (two DIPSI-2 cycles, ~6.3 ms) for the same 13C irradiation.
The spectrum displays $J$ connectivity for $^{13}$C-labeled residues (Phe) only, and the cross peak to diagonal peak intensity ratio is directly related to the magnitude of the $\text{H}^+\text{H}^-$ cross peak (see text).

parameters: $^{13}$C carrier, 64 ppm; $^1$H carrier, 4.75 ppm; $^{13}$C 90° pulse width, 11 µs; $^1$H 90° pulse width, 10 µs; NOE mixing period ($\tau_{\text{M}}$), 80 ms; 96 scans per complex $\tau_1$ increment; acquired data matrix size, 300 $\times$ 1024; final digital resolution, 5.7 Hz ($F_2$); digital filtering, 50°-shifted squared sine-bell ($t_1$) filters; total measuring time, 12 h.

The quantitative $H^+H^-$ $J$ Correlation spectrum of Figure 4 has been recorded with the pulse scheme of Figure 2. $^{13}$C frequency labeling in the $I_2$ dimension is obtained in the standard way by heteronuclear multiple quantum correlation (HMOC), using $J_{\text{CH}}$ de- and rephasing delays, $\tau_1$, of 3.5 ms. The scheme was optimized for suppressing detection of $^{13}$C-attached aromatic proton signals by using a $\tau_2$ value of 1.55 ms ($2\tau_2 = 1/(2/\text{Chemical})$). Other experimental parameters: $^{13}$C carrier, 63.9 ppm; $^1$H carrier, 4.75 ppm; $^{13}$C 90° pulse width, 11 µs; $^1$H 90° pulse width, 9.6 µs; NOE mixing period ($\tau_{\text{M}}$), 80 ms; 16 scans per complex $\tau_1$ increment; acquired data matrix size, 96° $\times$ 32° $\times$ 512°; final digital resolution, 23 ($F_1$), 39 ($F_2$), and 7.1 Hz ($F_3$); digital filtering, 72°-shifted sine-bell ($t_1$), 72°-shifted squared-sine bell ($t_2$) 60°-shifted squared-sine bell ($t_3$); in the $t_1$ and $t_2$ dimensions, the end of the sine-bell window is truncated such that the last time domain data point is multiplied by 0.1; total measuring time, 38 h.

The subsequent refocusing during the final period, $t_2$, and a 90° pulse ($\phi_1 = \gamma$) purges yields the following terms for the in-phase $A$ spin magnetization:

$$
\begin{align*}
\cos(2\pi f_1 t_1) \cos(2\pi f_{13} t_2) \cos(2\pi f_{15} t_3) A_y + \\
2 \sin(2\pi f_1 t_1) \sin(2\pi f_{13} t_2) \cos(2\pi f_{15} t_3) A_y + \\
2 \cos(2\pi f_2 t_2) \sin(2\pi f_{13} t_3) A_x M_x + \\
4 \sin(2\pi f_2 t_2) \sin(2\pi f_{13} t_3) A_x M_x \gamma (2)
\end{align*}
$$

During $t_1$, the first three terms oscillate with the chemical shift frequencies, $\delta_y, \delta_x$, and $\delta_y$ of spins $A$, $M$, and $X$. The last term represents three-spin coherence and is $\tau_1$-modulated by four frequencies, $\delta_x \pm \delta_y \pm \delta_x$. This three-spin coherence term may be safely ignored because under the experimental conditions used ($2\tau_1 << 1/\text{J}_{AM,1}/1/\text{J}_{AX}$), it is very weak, and in practice, cross peaks resulting from it fall well below the signal-to-noise threshold. The effect of $J$ coupling during the relatively short $\tau_1$ evolution period ($\tau_{\text{max}} << 1/\text{J}_{AM,1}$) may also be ignored as it only results in a small amount of line-broadening in the $t_1$ dimension. It can be shown that only those terms that are converted into $A$ spin transverse magnetization by the 90° pulse, applied at the end of the $t_1$ evolution period, can give rise to observable in phase $A$ spin magnetization during $t_2$. Neglecting $J$ coupling evolution during the $t_1$ period, these terms are described by:

$$
\begin{align*}
\cos(2\pi f_2 t_1) \cos(2\pi f_{13} t_2) \cos(2\pi f_{15} t_3) A_y + \\
2 \sin(2\pi f_2 t_1) \sin(2\pi f_{13} t_2) \cos(2\pi f_{15} t_3) A_y + \\
2 \cos(2\pi f_3 t_2) \sin(2\pi f_{13} t_3) \cos(2\pi f_{15} t_3) A_x M_x + \\
4 \sin(2\pi f_3 t_2) \sin(2\pi f_{13} t_3) \cos(2\pi f_{15} t_3) A_x M_x \gamma (3)
\end{align*}
$$

The 90° pulse ($\phi_1 = \gamma$) pulse converts these terms into the following:

$$
\begin{align*}
\cos(2\pi f_2 t_1) \cos(2\pi f_{13} t_2) \cos(2\pi f_{15} t_3) A_y - \\
2 \sin(2\pi f_2 t_1) \sin(2\pi f_{13} t_2) \cos(2\pi f_{15} t_3) A_y - \\
2 \cos(2\pi f_3 t_2) \sin(2\pi f_{13} t_3) \cos(2\pi f_{15} t_3) A_x M_x - \\
4 \sin(2\pi f_3 t_2) \sin(2\pi f_{13} t_3) \cos(2\pi f_{15} t_3) A_x M_x \gamma (4)
\end{align*}
$$

The first term gives rise to a diagonal $A$ spin resonance, whereas the second and third terms yield in-phase COSY cross peaks to spins $M$ and $X$, respectively. As is seen from these terms, the cross peak to a diagonal peak ratios for spins $M$ and $X$ depend directly on $\tau_{\text{max}}$ and $\tan(\pi f_{13})$ and $\tan(2\pi f_{15})$, respectively. As discussed previously for the measurement of heteronuclear $^{13}$C-$^{1}H$ couplings, the strong geometric $M-X$ dipolar interaction can cause relatively rapid $M-X$ spin flips, which have the effect of increasing the weaker of the two $\text{H}^+\text{H}^-$ cross peaks and attenuating the more intense ones. Relaxation caused by dipolar interactions with other protons attenuates both cross peaks. The intensity ratios of the cross and diagonal peaks are used directly to calculate the values of the $H^+H^-$ $J$ couplings, without accounting for these relaxation effects.

Experimental parameters: $^{13}$C carrier, 63.9 ppm; $^1$H carrier, 4.75 ppm; $^{13}$C 90° pulse width, 11 µs; $^1$H 90° pulse width, 9.6 µs; $\tau_1$, 10.7 ms; in 128 scans per complex $\tau_1$ increment; acquired data matrix size, 128° $\times$ 2048°; final digital resolution, 23 ($F_1$) and 3.5 Hz ($F_2$); digital filtering, 72°-shifted sine-bell ($t_1$), 72°-shifted squared-sine bell ($t_2$) 60°-shifted sine-bell ($t_3$); in the $t_1$ and $t_2$ dimensions, the end of the sine-bell window is truncated such that the last time domain data point is multiplied by 0.1; total measuring time, 6 h.

4D $^{13}$C/2D Separated 4D NOESY. The 4D NOESY spectrum was recorded on a 2 mM sample of uniformly $^{13}$C-$^{15}$N-labeled protein, at the same pH, temperature and ionic strength as the experiments described above. The HMOC-NOE-NOESY scheme of reference 24 was used, using 16 scans for each set of complex ($t_1, t_2, t_3$) values. Other experimental parameters; matrix size, 16° $\times$ 64° $\times$ 16° $\times$ 384°; spectral widths, 20.7 ($F_1$), 9.8 ($F_2$), 20.7 ($F_3$), and 12.1 ppm ($F_4$); final digital resolution, 97 Hz ($F_1$ and $F_2$); digital filtering, 50°-shifted squared-sine bell ($t_1$) and 60°-shifted squared-sine bell ($t_2$); total measuring time, 6 h.

Results and Discussion

Resonance assignments in the 123-residue DNA-binding domain of Drosophila heat shock factor (dHSF(33-155)), used

in the present study, were found to be identical to those reported previously for a construct that included 11 additional unstructured carboxyterminal residues. At the concentration and temperature used, the 13N relaxation indicates a rotational correlation time of ~10 ns, which is somewhat longer than expected for a monomeric 14 kDa protein. Sedimentation experiments indicate, however, that the domain is monomeric at lower concentration.16 A detailed analysis of the NOE data also shows no evidence for intermolecular NOEs.

Due to the relatively large line widths in uniformly 13C-enriched dHSF(33-155), a detailed analysis of 3D HCCH17J8 and (HO)C@-C(γ)CH319 J correlation (supplementary material) and 4D NOESY data previously had yielded tentative assignments for only half of the aromatic 1H resonances of the 10 Phe residues. The difficulties in obtaining reliable assignments and NOE constraints for the Phe rings, most of which are located in the hydrophobic core, limited the precision at which the protein structure could be calculated. Therefore, incorporation of natural abundance Phe in dHSF(33-155) provided a good test case for the reverse labeling procedure.

Figure 3A shows the aromatic region of the 12C-filtered HOHABA spectrum, displaying all 10 phenylalanine 1H=H connectivity patterns, free of overlap from the Trp and Tyr residues. As can be seen, the unusually large number of aromatic residues in the hydrophobic core of the protein does not lead to an untractable overlap of resonances, presumably because of the high density of aromatics also causes significant ring current effects throughout this region. Sequence-specific assignment of the ring systems can be obtained by correlating the H* protons with the previously assigned Hα and Hβ resonances in a 12C-filtered 2D NOESY spectrum (Figure 3B). Interactions between Phe109 and Phe128, anticipated on the basis of a low-resolution structure, are also seen in this figure.

The absence of 13C results in relatively narrow Phe-Hα resonance (T2 ~ 20–25 ms). Although the line widths of the 12C-attached protons, particularly those of the geminal Hβ, remain too large for a detailed ECOSY20 JHNHa measurement, a good estimate for JHNHa can be obtained from the quantitative J correlation experiment of Figure 2. The spectrum of Figure 4 shows H=HJ cross peaks for 9 out of the 10 Phe residues; cross peaks to both Hα protons of Phe25 can be observed at contour levels below than shown in the figure. As described in the Experimental Section, neglecting relaxation effects,21 the cross peak to diagonal peak intensity ratio is tan(2rJ{), and Jcouplings derived from this relationship are reported in Table 1. For Phe129 and Phe134, correlations to only one of the Hα protons is observed, even when the spectrum is plotted at the noise level. The spectrum therefore provides an upper limit for tan(2rJ{) and thereby an upper limit for the corresponding JHNHa couplings. Together with results from qualitative JHNHa and JCOHa measurements,2223 the JHNHa data extracted from Figure 4 indicate x1 averaging for Phe129 (J αβ = 7.1/8.9 Hz), located in an extended flexible loop. All other Phe x1 angles are well defined by the J coupling data, permitting stereospecific assignments of the prochiral Hα protons (Table 1).

Figure 5 illustrates the improvement in sensitivity that can be obtained by reverse labeling over conventional 4D 13C/13C separated NOESY. Figure 5A shows a small region of a F1/F2 cross section through the 13C-edited, 12C-filtered 3D NOESY spectrum of the reverse labeled sample. This cross section, taken at the F1 frequency of Pheα3-Hα, reveals NOE interactions between Phe35-Hα and the methyl protons of seven different residues. Due to the lower 1H resolution in the corresponding region of the 1- and 2D NOE interactions to Phe35-Hα (assigned using 12C reverse labeling) cannot be distinguished from Phe75-Hα, and clearly the signal-to-noise ratio in the 4D suffers from the additional relaxation losses incurred during the final HSQC part of the 4D pulse scheme24 and from the additional 13C-induced line broadening of the detected Hα resonance.

In recent years, the uniform 13C labeling strategy has proven to be extremely powerful for structure determination of proteins up to about 20 kDa, and resonance assignments for proteins significantly larger than 20 kDa have also been reported. In principle, the uniform labeling approach is capable of yielding 3D structures for these larger proteins. However, due to the rapid drop in the signal-to-noise ratio of the NOESY spectrum with increasing correlation time, the average number of NOEs per residue, and thereby the resolution of the protein structure,28 rapidly decreases for larger proteins.

Reverse labeling of otherwise uniformly 13C/13N-enriched proteins presents an attractive approach for increasing the spectral information obtainable, particularly for spin systems such as

phenylalanine, where the aromatic $^{13}$C dispersion is poor. It has become well established that isotope filtering is ideally suited for studying the interaction between labeled and unlabeled residues.\(^{(29)}\) The modified filtering procedures described above optimize sensitivity by reducing the duration of filtering delays. The longer $^1H-(^{13}C)T_2$ values, relative to $^1H-(^{13}C)$, improve both resolution and sensitivity compared to the study of uniformly $^{13}$C labeled material. This permits experiments such as the above-described quantitative $^1H-^1H$ J correlation, free from overlap of the multitude of $^{13}$C labeled residues, and it allows for additional experiments to complete the assignment process. For dHSF- (33-155), reverse labeling yielded 109 additional long-range NOEs, an increase of 39%. This resulted in a very significant reduction in the spread of the NMR structures, from 1.4 to 0.87 Å for the backbone plus ordered side-chain atoms.\(^{(30)}\)

The labeling strategy described above is conceptually not new. In fact, before nonauxotrophic bacterial strains suitable for protein expression became widely available, essential amino acids at natural abundance were routinely added to the M9 growth medium. However, the lack of labeling of the essential amino acids was considered mere nuisance than a useful avenue for extracting additional information. The reverse labeling strategy therefore is more comparable with the protein deuteration strategy, where a number of different protonated amino acids are incorporated into an otherwise perdeuterated protein. This deuteration strategy has proven to be quite powerful, both for obtaining resonance assignments and for obtaining detailed structural information.\(^{(31-35)}\) In comparison to the $^{13}$C reverse labeling procedure described above, reverse deuteration requires a considerably larger number of selectively labeled samples to probe all possible interactions between protonated amino acids, and high levels of deuteration for metabolically active amino acids can be difficult to achieve.\(^{(33)}\)

The $^{12}$C reverse labeling approach described in this paper requires a substantial amount of additional work in protein preparation and NMR data collection over the uniform $^{13}$C labeling approach. However, it is becoming increasingly clear that, for larger proteins, without this type of additional work it may frequently be impossible to gather a sufficiently high number of structural constraints for defining the solution structure with high accuracy.

Acknowledgment. We thank John Marquardt and Frank Delaglio for assistance. This work was supported by the AIDS Targeted Anti-Viral Program of the Office of the Director of the National Institutes of Health.

Supplementary Material Available: Figure showing strips through the 3D ($^1H$,$^1H$,$^1C$) spectrum of reverse labeled dHSF- (33-155) (1 page). This material is contained in many libraries on microfiche, immediately follows this article in the microfilm version of the journal, and can be ordered from the ACS; see any current masthead page for ordering information.


