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Protein NMR resonance assignment by isotropic mixing experiments on random fractionally deuterated samples

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The 108-residue protein *E. coli* thioredoxin has been uniformly enriched to 50% with deuterium at all carbon-bound hydrogen positions. Isotropic mixing (i.e. TOCSY) experiments have been conducted for both the deuterated and natural-abundance samples. Using a 54 ms mixing time correlation peaks can be seen for all four protons on the benzenoid ring of tryptophan in both samples. The deuteration results in an average decrease in cross-sectional area of a factor of 2–3 for the TOCSY cross-peaks. The cross-peak intensities for the deuterated sample systematically decrease as a function of the number of protons involved in the transfer process thus overcoming a common ambiguity in the TOCSY experiment.

Protein NMR; Random fractional deuteration; TOCSY

1. INTRODUCTION

The two basic 2D ^{1}H NMR pulse experiments, COSY which allows observation of directly spin-coupled proton resonances and NOESY which serves to monitor through-space dipolar coupled protons, have proven sufficient for the sequential assignment of several proteins of molecular mass below 10 kDa (e.g. [1–3]). However, due to the significant decrease in spectral resolution observed for larger proteins, researchers have found it necessary to use a number of different pulse sequences which extend the capabilities of the COSY experiment so as to monitor multi-spin coupling interactions. Sequences such as relayed COSY [4], double-relayed COSY [5], multiple quantum-filtered COSY [6] and TOCSY [7,8] as well as various multiple quantum experiments [9] enable the experimentalist to establish correlations between spins which do not have an observable spin coupling but are connected by a sequence of protons having observable couplings. This ability has been exceedingly useful in analyzing the complete spin-coupling patterns of the various protein side chains.

Another approach to dealing with the resolution problems of larger proteins is isotopic labeling, in particular random fractional deuteration [10]. By expression of the protein of interest in an organism grown on a medium containing a mixture of ^2H_2O and ^1H_2O supplemented with a carbon source similarly deuterated, a sample is prepared in which all carbon-bound hydrogen positions are substituted with deuterium to an approximately uniform level in a non-correlated fashion.

By diluting the local concentration of ^{1}H spins around any given proton the resultant decrease in dipolar interactions yields a narrower linewidth for that proton's resonance. Since this argument applies to all the resonances of the labeled protein, the overall resolution of a 1D or 2D spectrum can be significantly enhanced.

The obvious potential limitation of the random fractional deuteration technique is the sacrifice in sensitivity which results from the isotopic dilution. Earlier work has verified that for the NOESY and COSY experiments 75% deuteration offers enhanced resolution with only a modest loss in sen-

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sitivity [10]. This paper provides evidence for resolution enhancement with equally satisfactory sensitivity when an isotropic mixing experiment, commonly referred to as TOCSY, is applied to a 50% random fractionally deuterated sample of the 108-residue protein *E. coli* thioredoxin.

2. EXPERIMENTAL

The deuterated protein samples were prepared by expression of an overproduction vector for *E. coli* thioredoxin in a prototrophic *E. coli* strain grown on a $^1H_2O/^2H_2O$ mixture containing partially deuterated succinate and alanine as carbon sources [10].

8 mM samples of *E. coli* thioredoxin in 150 mM NaCl, 20 mM phosphate (pH 5.7) at 30°C were used in the data collection. The COSY spectra were collected on a Bruker WM500 while the TOCSY spectra were collected on a Bruker AM500 instrument. The data were processed using the software written by D. Hare.

3. RESULTS AND DISCUSSION

In order to study the dependence of resolution on the level of uniform deuteration a phase-sensitive COSY spectrum was collected on a 50%-^{2}H sample of *E. coli* thioredoxin and compared to the previously examined 75%-^{2}H and natural-abundance spectra [10] in fig.1.

Fig.1B shows the spectrum from a 75%-^{2}H random fractionally deuterated sample of *E. coli* thioredoxin. Each cross-peak is composed of four components arranged at the corners of a square. Projection of this pattern on either axis corresponds to the 1D doublet pattern expected from an isolated $^1H_\alpha$-$^1H_\beta$ spin-coupled pair. In fig.1A the corresponding spectral region from a natural-abundance sample collected and processed under identical conditions is shown. The first obvious observation is that the corresponding components appear significantly broader in the natural-abundance sample. This is because the proton resonance natural linewidth is determined predominantly by ^1H-^1H dipolar relaxation which has been strongly curtailed by isotopic dilution in the deuterated sample. This enhancement in resolution is manifest in the corresponding NOESY spectra as well. The other important contrast between the spectra in fig.1A and B is that there are more component peaks observed for the natural-abundance sample. In fact most cross-peaks in fig.1A should be composed of 16 components corresponding to a doublet of doublets in the 1D spectrum expected from the additional $^1H_\alpha$-$^1H_{\beta'}$ and $^1H_\beta$-$^1H_{\beta'}$ spin couplings. All of these couplings are not resolved in the natural-abundance spectrum of this 12 kDa protein, in part due to the fact that, perhaps due to aggregation effects, the linewidths observed for this protein are more characteristic of a somewhat larger protein. As is found throughout the spectrum, the appearance of the clean quartet pattern found for the 75%-^{2}H-labeled sample indicates that mutual spin interactions of more than two spins has been effectively suppressed.

In fig.1C is shown the corresponding spectrum for the 50% random fractionally deuterated sample. In contrast to the more highly deuterated sample illustrated in fig.1B the linewidths of the components of the cross-peaks are clearly increased as is the sensitivity. Nevertheless, the overall resolution is much closer to that of the 75%-^{2}H sample as compared to the natural-abundance spectrum. In particular, the simple quartet pattern of the isolated-pair approximation is still reasonably closely followed.

The use of random fractional deuteration combined with selective deuteration to obtain residue-type information were sufficient for establishing a main chain sequential assignment of *E. coli* thioredoxin using only NOESY and COSY experiments [10]. Nevertheless, for the purposes of assigning the side chain spin-coupling patterns of this protein as well as application to larger more difficult proteins, it is desirable to apply at least a subset of the experiments discussed previously which are capable of identifying multiple spin correlations. On the other hand, it would clearly be desirable to maintain the benefit of narrower linewidths obtained in the random fractionally deuterated sample. The efficient suppression of tertiary spin interactions apparent in the COSY spectra creates the illusion that these samples would not prove suitable for multi-spin correlation experiments.

This concern is in fact not justified. The benefits of the longer relaxation times largely compensate for the loss in sensitivity due to the isotopic dilution. In order to illustrate this result an isotropic mixing experiment was carried out on 50%-^{2}H *E. coli* thioredoxin. The TOCSY experiment is at present considered the most effective means of

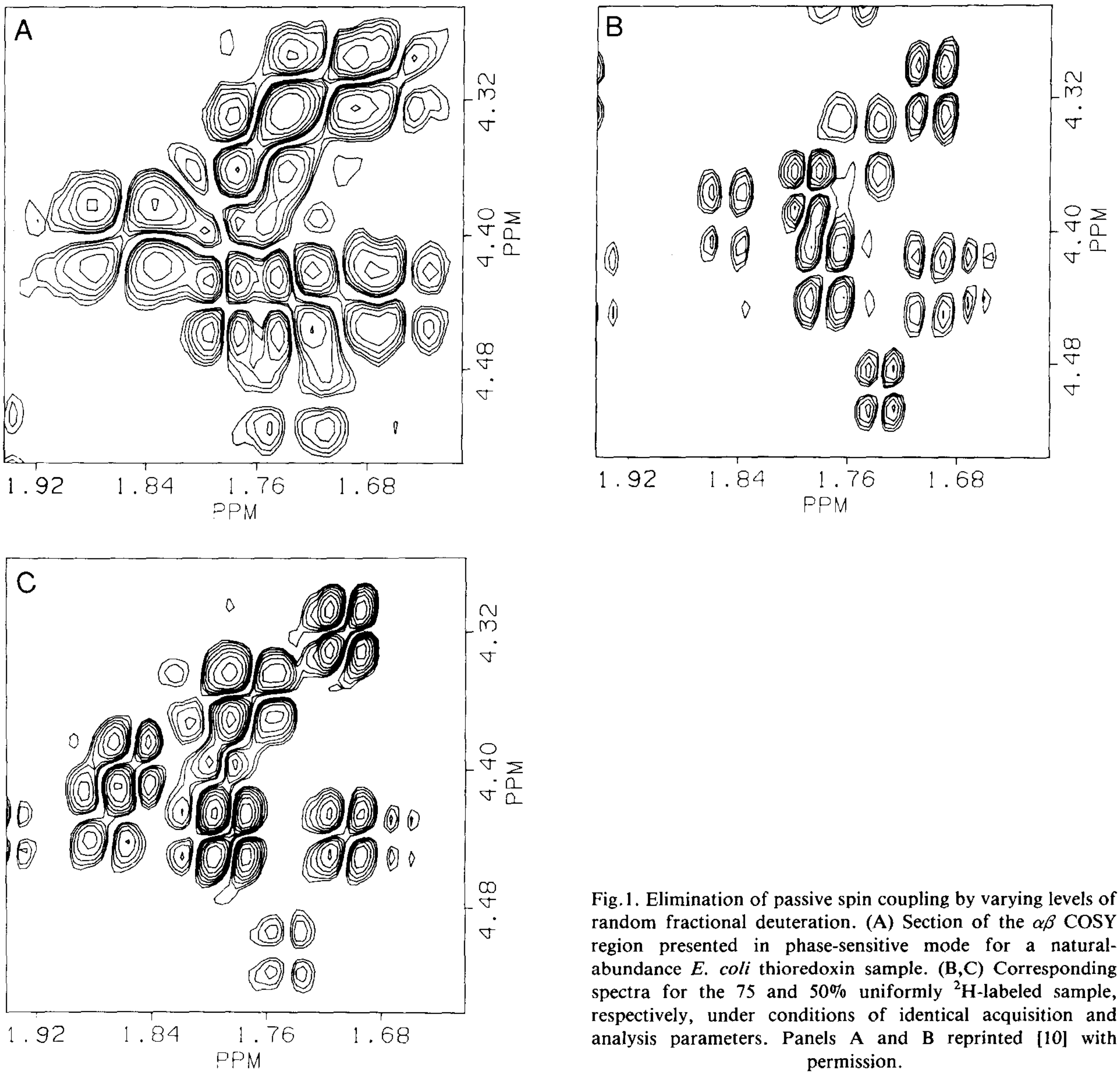

Fig.1. Elimination of passive spin coupling by varying levels of random fractional deuteration. (A) Section of the $\alpha\beta$ COSY region presented in phase-sensitive mode for a natural-abundance *E. coli* thioredoxin sample. (B,C) Corresponding spectra for the 75 and 50% uniformly ^{2}H-labeled sample, respectively, under conditions of identical acquisition and analysis parameters. Panels A and B reprinted [10] with permission.

establishing multi-spin correlations in moderately sized proteins. Fig.2 shows a section of the aromatic region of a TOCSY spectrum for the deuterated *E. coli* thioredoxin sample. The cross-peaks denoted W28 indicate that magnetization has been efficiently transferred from the H7 indole proton resonance at 7.78 ppm all the way to the H4 indole proton resonance at 7.10 ppm even though only 1/16 of all the protein molecules have all four positions of the benzenoid ring protonated and only 32 transients per t_1 point were collected. In fig.3 is shown a portion of the t_2 row at 7.78 ppm containing the cross-peaks between H7 and the H4, H5 and H6 protons of W28 for both the 50% deuterated sample in panel A and the natural-abundance sample in panel B. The potential limitation of the deuteration approach is indicated by the fact that although the COSY-like cross-peaks between H7 and H6 as well as the single relayed cross-peaks between H7 and H5 are

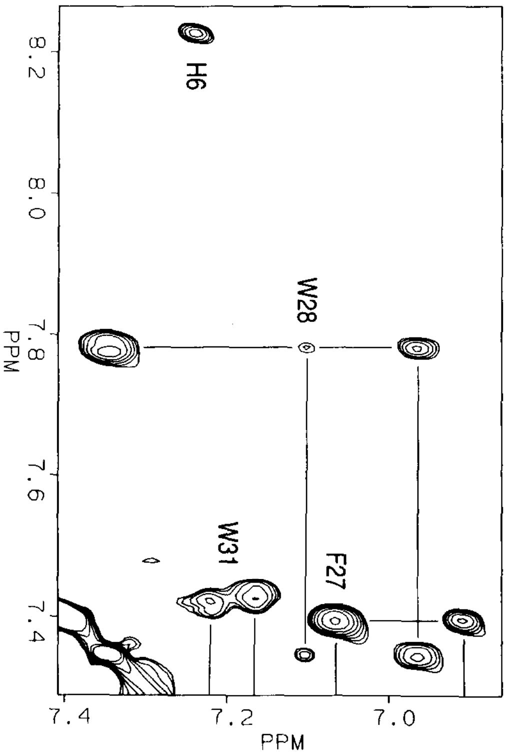

Fig.2. TOCSY spectrum of 50%-^{2}H random fractionally deuterated *E. coli* thioredoxin. A portion of the aromatic spectral is shown illustrating the cross-peaks of Trp 28 between H7 at 7.78 ppm and H6 (7.35 ppm), H5 (6.96 ppm) and H4 (7.10 ppm). The TOCSY experiment was carried out using the HOHAHA variation [8] with a 54 ms mixing time and a 2.0 s relaxation delay. 32 transients per t_1 point were collected.

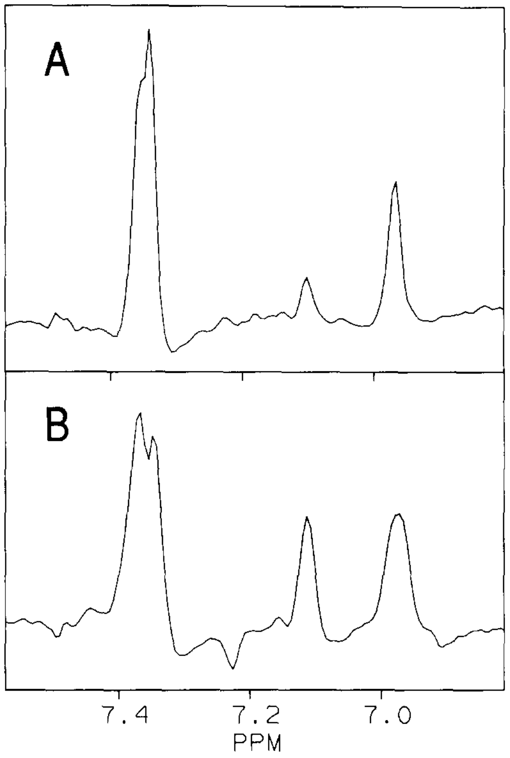

Fig.3. Slice of TOCSY spectra for Trp 28 cross-peaks involving the H7 resonance. (A) Cross-peaks to the H4, H5 and H6 resonances for the 50% random fractionally deuterated sample. (B) Corresponding spectrum for the natural-abundance sample processed under identical conditions except that a 1.0 s relaxation delay was used.

of comparable sensitivities in the two spectra, the double-relayed cross-peak to H4 is appreciably weaker for the deuterated spectrum. However, the comparison represents in many ways a worst-case situation. Most side chains have geminal hydrogens whose spin coupling it is often desirable to suppress as seen above for the COSY spectra. The sensitivity loss due to isotopic dilution is of course independent of molecular mass, while the longer relaxation times of the deuterated samples will result in an increase in sensitivity compared to that of the natural-abundance sample as larger proteins are studied. Even at this molecular mass the combined effects of relaxation times and small coupling constants can lead to considerable enhancement in sensitivity for the deuterated sample. In fig.2 can be seen a moderately strong cross-peak between the H2 and H4 protons of His 6 for which there is only a 1.5 Hz coupling. These histidine cross-peaks are often not observed in protein spectra including the case of the corresponding natural abundance TOCSY spectrum of *E. coli* thioredoxin.

The progressive 2-fold decrease in intensity at each step of the magnetization transfer represents a significant benefit of deuteration. One of the major limitations of TOCSY experiments on natural-abundance samples is that it is often difficult to determine the pathway of magnetization transfer so that, although a group of resonances can be identified as forming a spin system, the internal identification can be ambiguous. Attempting to determine the pathway of magnetization transfer by collecting spectra for different mixing times is not reliable. The single- and double-relayed COSY experiments mentioned above have been increasingly relegated to the task of resolving these ambiguities in TOCSY spectra [11]. However, several limitations of the relayed COSY experiments indicate that they will be of decreasing utility as assignment efforts move to larger proteins. In contrast, as seen in fig.3 and throughout the full TOCSY spectrum the pathway of magnetization development is obvious even at moderately long mixing times for the random fractionally deuterated sample as a result of the progressive isotopic dilution.

The primary motivation for carrying out the TOCSY experiment on the deuterated sample was, of course, the anticipated improvement in resolution. How much the resolution improves is dependent on not only the change in relaxation time but also the coupling pattern in which the particular coupling partners are involved. Nevertheless, a large portion of the cross-peaks of the deuterated spectrum have linewidths approx. 30–40% less than those of the corresponding natural-abundance cross-peaks. This represents a decrease in cross-sectional area at half-height of 2–3 as compared to the natural-abundance spectra.

In summary, random fractional deuteration has proven valuable in enhancing resolution with comparatively little loss in sensitivity in TOCSY experiments on moderately sized proteins while at the same time overcoming one of the basic limitations of the technique by clearly indicating the pathway of magnetization transfer. There appears to be no reason why such labeled samples should not prove equally suitable for all of the higher-order NMR experiments which are currently practical for moderately sized proteins. The quality of the COSY spectrum for the 50%-^{2}H sample in fig.1C indicates that for proteins of the size of *E. coli* thioredoxin or smaller for which the added resolution of the 75%-^{2}H sample is not necessary, only one random fractionally deuterated sample may be needed for complete spectral analysis.

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