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Chiral β and random fractional deuteration for the determination of protein sidechain conformation by NMR

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Stereospecific assignments of the aspartic acid and asparagine β-protons of the 108 residue protein *E. coli* thioredoxin have been obtained by the use of chiral deuteration. In addition protein samples have been prepared in which all carbon bound hydrogen positions are substituted to an extent of 75% with deuterium. These random fractionally deuterated samples significantly facilitate the measurement of coupling constants and intraresidue NOE intensities which combined with the stereospecific assignments have provided determination of the first sidechain dihedral angle χ_1 for all four asparagine residues and eight of the ten assigned aspartic acid residues.

Chiral deuteration; Sidechain conformation; Protein NMR

1. INTRODUCTION

Recent NMR studies have demonstrated the ability to determine protein mainchain conformations in solution (e.g. [1]). Difficulties in determining the sidechain conformations appear to be the main impediment to obtaining atomic resolution structures. Of particular note is the problem of determining the first sidechain dihedral angle χ_1 for residues possessing two β-protons. This requires the stereospecific assignment of these protons combined with the $\alpha\beta$ coupling constants and intraresidue NOE crosspeak intensities. Wagner and co-workers [2] recently published the first thorough attempt to extract χ_1 values from natural abundance samples using standard NOESY and COSY data. For the 70 residue protein Elgin c they were able to obtain χ_1 values for 9 of the 24 AMX spin systems (i.e. amide, α- and two β-protons) and 3 of the 18 longer (non-proline) sidechains containing β-methylene protons. The main limitation arises from problems in extracting coupling constant information. The two actively coupled protons, which give rise to the frequency labeling of the crosspeak, yield a fundamental four peak COSY pattern arranged as a square with the sides equal to the coupling constant. However, when these protons are also coupled to other protons this results in splitting of the basic quartet along each axis analogous to the multiplet patterns seen in 1D spectra. When further complicated by the effects of larger natural linewidths seen for proteins, deconvolution of the coupling constant information from a highly coupled crosspeak is generally challenging. In addition to pulse sequences which partially simplify the crosspeak multiplicity [3,4] a particularly promising approach involves the use of protein samples deuterated at all carbon bound positions to the extent of roughly 75%. At this level all crosspeaks appear as the basic quartet pattern since the secondary couplings have been largely removed by dilution. Besides providing ready determination of coupling constants the simplified crosspeak pattern should prove exceedingly valuable for automated pattern recognition analysis [5,6].

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This coupling constant information can be complemented by the use of chiral β-deuteration to obtain unambiguous stereospecific assignments as has previously been used in peptide conformational studies (e.g. [7]). Aspartic acid chirally deuterated in the β-position has been incorporated into the 108 residue protein *E. coli* thioredoxin. These data, combined with NOE intensity and COSY coupling constant measurements from random fractionally deuterated samples, have provided χ_1 angle determinations for 12 of the 14 assigned (LeMaster and Richards, submitted) aspartic acid and asparagine residues.

2. EXPERIMENTAL

[2,3-$^2H_2$]Fumaric acid was prepared by triphenylphosphine reduction of dimethyl acetylenedicarboxylate [8]. 4 ml of 0.75 M ammonium [2,3-$^2H_2$]fumarate and 5 mM $MgCl_2$, 1 mM EDTA at pH 8.5 were incubated at 37°C overnight with 25 units of l-aspartase (Sigma) [9,10]. After acidification and centrifugation the sample was loaded onto an AG50 cation-exchange resin and displaced with 50 mM NH_4OH. The yield of 3*S*-[2,3-$^2H_2$]aspartic acid was 70%. 3*R*-[3-$^2H_1$]Aspartic acid was similarly prepared from natural abundance fumarate by use of D_2O as solvent for the aspartase reaction. Procedures for the production of the selectively enriched and random fractionally deuterated *E. coli* thioredoxin samples have been described ([11] and LeMaster and Richards, submitted).

8 mM samples of *E. coli* thioredoxin in 150 mM NaCl, 20 mM phosphate, pH 5.7, at 30°C were used in data collection on a Bruker WM500. Data were collected to 4.2 Hz in the T2 dimension and 6.0 Hz in the T1 dimension. The data were multiplied by an unshifted skewed sine bell squared in the T2 dimension and unshifted skewed sine bell in the T1 dimension before transformation using the software written by D. Hare. In the NOESY experiments a mixing time of 100 ms was used for the aspartic acid labeled samples and 250 ms was used for the random fractionally deuterated sample.

3. RESULTS AND DISCUSSION

The 3*S*-[2,3-$^2H_2$]- and 3*R*-[3-$^2H_1$]aspartic acid samples used for labeling *E. coli* thioredoxin were obtained via the reaction between fumarate and ammonia catalyzed by aspartase. This enzyme is known to add ammonia with *trans* stereospecificity [12]. Since one β-proton of the resultant aspartic acid is derived from fumarate and the other from solvent, use of deuterated fumarate or deuterated solvent provides both stereospecific labelings. In fig.1 a portion of the amide-β NOESY spectral region for these two samples is displayed as well as the corresponding spectrum of the random fractionally deuterated sample. For all but one of the 14 sequentially assigned aspartic acid and asparagine residues the two β-proton resonances are resolved in the chirally β-deuterated spectra directly yielding chiral assignments.

The so-called 'Karplus equation' (i.e. $^3J = A\cos 2\theta + B\cos\theta + C$) is standardly used to relate the observed vicinal coupling constant to the dihedral angle. For a given pair of coupling constants between the two β-methylene protons and the α-methine proton the Karplus curve is generally 2-fold degenerate, and for certain ranges of dihedral angle 4-fold degeneracies are observed [13]. The situation is further complicated by the fact that the parameters used in the Karplus curve calculations are imprecisely determined by empirical correlations with model compounds. Thus even given the stereoselective assignments, dihedral angle determinations can still be difficult if no assumptions are made as to the χ_1 angle distribution. It has long been realized that the three staggered conformations of 180°, +60° and −60° are strongly preferred in most model compounds. Although various sterically strained model systems have been found which adopt eclipsed conformations, there is little reason to believe that the protein structure commonly forces sidechain conformations far from their preferred staggered positions. In an analysis of the sidechain conformations of 19 of the most highly refined protein structures roughly 50, 35 and 15% were found to adopt the −60°, 180° and 60° conformations, respectively. Less than 5% of all χ_1 values fell outside of the three staggered distributions having standard deviations of approx. 10° [14]. A more recent analysis indicates that the vast majority of the sidechains with non-staggered conformations lie in loop and turn regions which are generally less well determined thus suggesting that the propor-

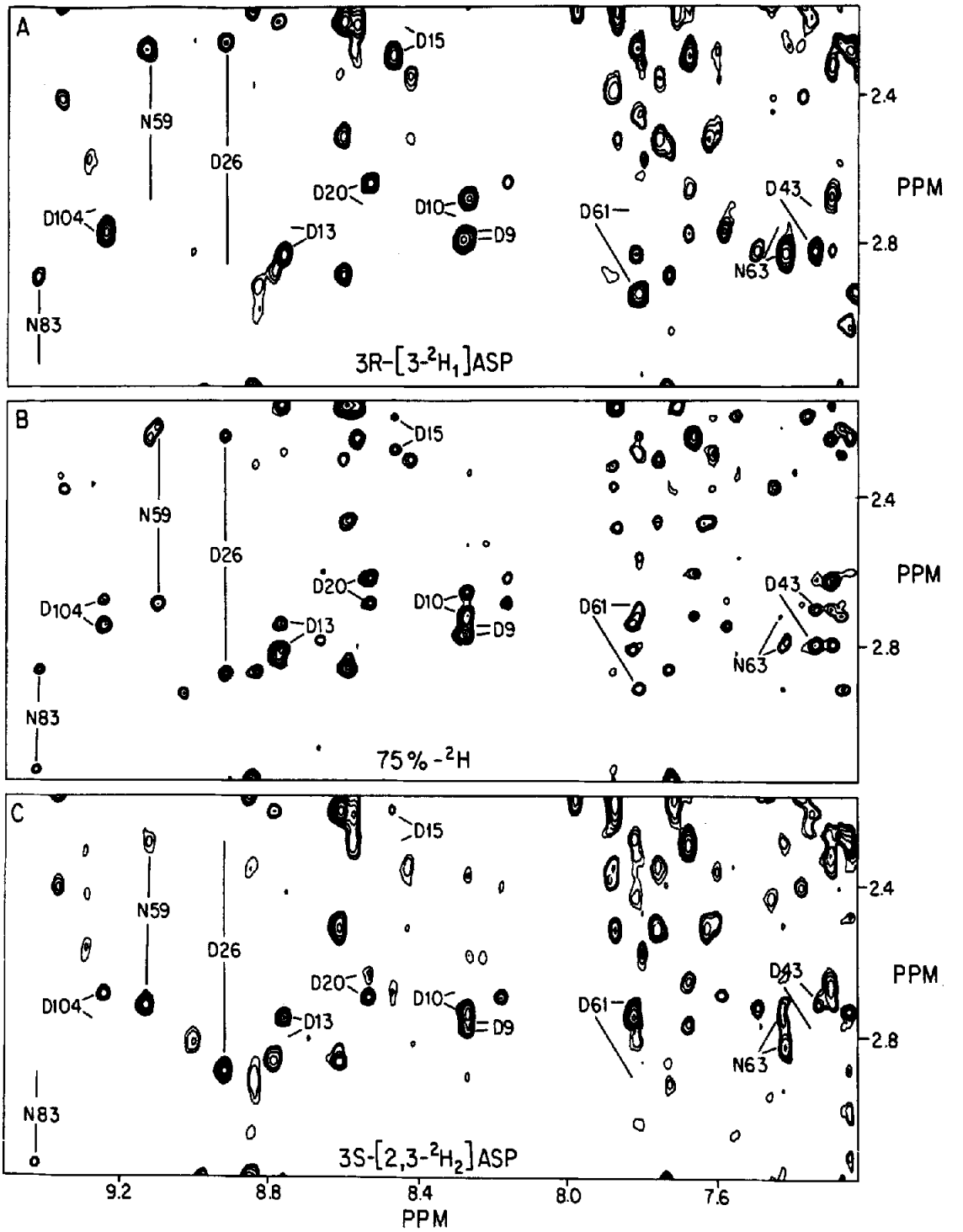

Fig. 1. Chiral β-deuteration editing of amide-β NOESY spectra. (A and C) Present a portion of the NOESY spectra of aspartic acid labeled *E. coli* thioredoxin in which each of the β-hydrogen positions are selectively deuterated. The arrows indicate the positions of the pro-*R* and pro-*S* intraresidue amide-β crosspeaks for the aspartic acid and asparagine residues as verified by reference to the random fractionally deuterated spectrum in B.

tion of sidechains deviating from staggered conformations in protein structures may be exceedingly small (Rose, G. and Presta, L., personal communication). The few deviant sidechains that remain would generally be recognized by their failure to match the NOE and coupling constant values for the staggered conformations. In practice we anticipate that conformational averaging will offer a considerably more significant impediment to sidechain angle determination by NMR than will non-staggered conformations.

The expected coupling constants and relative interproton distances for the three staggered conformations are given in table 1. The relative values for the amide-β distances are weakly dependent on the mainchain dihedral angle ϕ. In table 1 ϕ is set to $-90°$ which is roughly the mean position observed in proteins. This table also gives the corresponding coupling constants and relative NOE intensities for the aspartic acid and asparagine residues of *E. coli* thioredoxin. These values are derived from the fractionally deuterated spectra of the amide-β NOESY (fig.1B), $\alpha\beta$ COSY (fig.2A) and $\alpha\beta$ NOESY (fig.2B). In addition to a simplified crosspeak pattern in the COSY spectrum, the partial deuteration results in a significant reduction in spin diffusion, thus rendering quantitative use of NOE intensities more reliable. For short mixing times in the NOESY experiment the intensities should grow at a rate proportional to $1/r^6$. The observed $\alpha\beta$ NOE intensity ratios match the predicted values fairly well while amide-β values show attenuated variation presumably in part due to the more rapid relaxation of the amide protons.

For 8 of the 14 assigned aspartic acid and asparagine residues both the $^3J\alpha\beta_S$ and $^3J\alpha\beta_R$ couplings agree with one of the three staggered conformations and the corresponding intraresidue NOE intensities are consistent with those determinations. The $\alpha\beta_S$ crosspeaks for D20 and N106 residues are degenerate thus precluding the determination of the corresponding coupling constants.

Table 1

χ_1 dihedral angle determination in *E. coli* thioredoxin

	χ_1 angle	$^3J\alpha\beta_S$ (Hz)	$^3J\alpha\beta_R$ (Hz)	$d\alpha\beta_S/d\alpha\beta_R$[a]	$d_N\beta_S/d_N\beta_R$[a]	χ_1 angle
	180	3.5	10.0	1.24	0.89	180
	−60	10.0	3.5	0.80	1.51	−60
	60	3.5	3.5	1.0	0.74	60
Residue	(β_S,β_R)	$^3J\alpha\beta_S$ (Hz)[b]	$^3J\alpha\beta_R$ (Hz)[b]	$(N\alpha\beta_S/N\alpha\beta_R)^{1/6}$[a,c]	$(N_N\beta_S/N_N\beta_R)^{1/6}$[a,c]	χ_1 angle
D9	(2.78,2.75)	5.0	6.5	1.35	d	
D10	(2.65,2.71)	9.0	4.0	0.70	1.13	−60
D13	(2.82,2.74)	7.0	6.0	0.94	1.10	−60+180
D15	(2.27,2.18)	10.5	5.0	<0.86	1.10	−60
D20	(2.62,2.69)	d	5.0	0.70	1.10	−60
D26	(2.23,2.87)	3.5	11.0	1.16	0.94	180
D43	(2.80,2.69)	10.0	4.0	0.67	1.10	−60
D47	(2.85,2.85)	d	d	d	d	
N59	(2.25,2.69)	–	11.5	1.20	0.94	180
D61	(2.91,2.71)	10.5	4.0	0.65	1.13	−60
N63	(2.80,2.72)	11.0	4.0	<0.88	1.20	−60
N83	(2.86,3.13)	9.0	5.0	0.74	1.10	−60
D104	(2.74,2.68)	11.5	5.0	large $N\alpha\beta_R$	1.13	−60
N106	(2.63,2.23)	d	3.5	0.70	>1.03	−60

[a] In the linear region of NOE buildup the ratio of the interproton distances should be proportional to the 1/6 power of the ratio of the NOE intensities

[b] Observed couplings were corrected for antiphase cancellation effects [15]

[c] NOE intensities based on peak heights using a 1.2 multiplicative contour level

[d] Spectral overlap

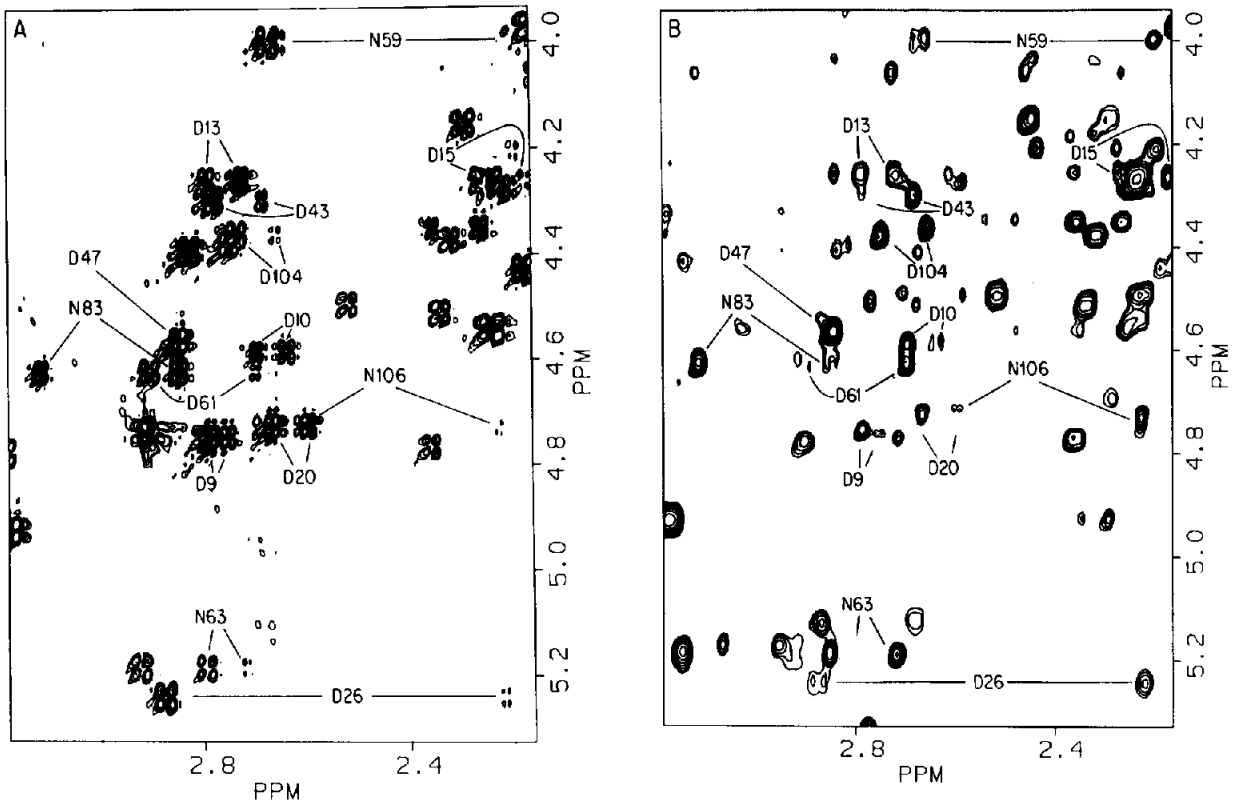

Fig.2. COSY and NOESY spectra of the $\alpha\beta$ region of *E. coli* thioredoxin. Random fractionally deuterated samples give rise to a COSY spectrum (A) with a simplified crosspeak pattern facilitating measurement of coupling constants as well as improved resolution and reduced spin diffusion in the NOESY spectrum (B).

However, in both cases small coupling constants are observed for $^3J\alpha\beta_R$ and the intraresidue NOE intensities are consistent with a χ_1 of $-60°$. Similarly the $^3J\alpha\beta_R$ of residue N59 is large and the intraresidue NOE values indicate a 180° χ_1 angle. The coupling constants and NOE intensities for residue D13 are not consistent with any single χ_1 angle. However, they are compatible with an approximately equal mix of 180° and $-60°$ conformers. Angle determinations were not feasible for residues D9 and D47. The β-protons of D9 were only poorly resolved and the derived parameters, although most consistent with a χ_1 value of 180°, could not be confidently interpreted. The β-protons of D47 were not resolved in any of the spectra collected. Under more favorable conditions, such as selective chiral β-deuteration in a random fractional deuterated background, it should be possible to obtain sufficiently accurate coupling constant and NOE intensity data necessary to determine the χ_1 angle in cases of β-proton degeneracy.

In summary, deuteration techniques have enabled us to obtain sidechain conformational information for almost all of the aspartic acid and asparagine residues of *E. coli* thioredoxin. We anticipate that refinement of the known X-ray structure [16] should provide an independent test of the validity of this approach.

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