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Stereospecific assignments of the leucine methyl resonances in the ^{1}H NMR spectrum of *Lactobacillus casei* dihydrofolate reductase

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A general method is described for the stereospecific assignment of methyl resonances in protein NMR spectra based on selective deuteration procedures. A selectively deuterated dihydrofolate reductase from *L. casei* was prepared by incorporating stereoselectively deuterated L-leucine, (2*S*,4*R*)[5,5,5-$^2H_3$]leucine. By comparing the COSY spectra of the dihydrofolate reductase–methotrexate complexes formed using deuterated and non-deuterated enzyme the stereospecific assignments for resonances of all 13 leucine residues were obtained by noting the absence of cross-peaks in spectra from the deuterated proteins.

Dihydrofolate reductase; Stereospecific NMR assignment; Deuterated leucine

1. INTRODUCTION

NMR spectroscopy, in combination with computational procedures such as distance geometry calculations is now a well-established method for obtaining detailed three dimensional structures of proteins in solution. To apply such methods, it is first necessary to assign the NMR signals to specific protons in the protein. The precision of the structures obtained in this way is dependent not only upon the number of intraresidue constraints identified but also upon how well the constraints can be defined. In the absence of stereospecific assignments it is necessary to add relatively large correction factors to the intraresidue distance constraints to allow for either assignment being correct. For example, in the case of the diastereotopic methyl groups of valine and leucine residues, the correction used is 2.4 Å on distance constraints which typically fall in the range 2.0 to 6.0 Å. Thus for the most precise structure determinations it is essential to obtain stereospecific assignments for the various pairs of diastereotopic protons and methyl groups in the protein. Such assignments can also be important for defining specific protein–ligand interactions based on intramolecular NOEs.

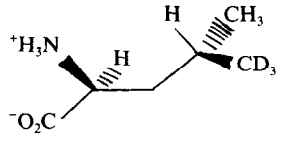
Clearly, it is important to have more direct methods of making stereospecific assignments for residues such as these. Here, we report a direct method for making such assignments, based on NMR studies of stereoselectively deuterated proteins prepared by biosynthetic incorporation of stereoselectively deuterated amino acids such as leucine. The usefulness of this approach is demonstrated by its application to *L. casei* dihydrofolate reductase (DHFR) where it has been used to obtain the stereospecific assignments for the methyl groups of 12 of the 13 leucine residues (the methyls of Leu-94 have degenerate chemical shifts).

Selective deuteration has frequently been used to identify the ^{1}H signals from a particular amino acid type [1–6] and has also been used to make stereospecific assignments for methylene protons in Gly [7] and Asp/Asn residues [8]. The present application is the first example of the use of stereoselective deuterium labelling for the assignment of diastereotopic pairs of methyl resonances in protein NMR spectra.

In earlier studies, stereospecific assignments for leucine and valine methyl resonances were obtained by applying 2D ^{1}H/^{13}C half-filter NMR experiments to proteins labelled stereospecifically in the leucine and valine residues with ^{13}C using the method of 'biosynthetic fractional ^{13}C labelling' [9–11].

2. MATERIALS AND METHODS

The synthesis of (2*S*,4*R*)[5,5,5-$^2H_3$]leucine (**1**) using pyroglutamic acid as a chiral template has already been described [12].



(1)

NMR studies of the stereospecifically labelled L-leucine used in this study showed the sample to contain about 25% of monoprotiated

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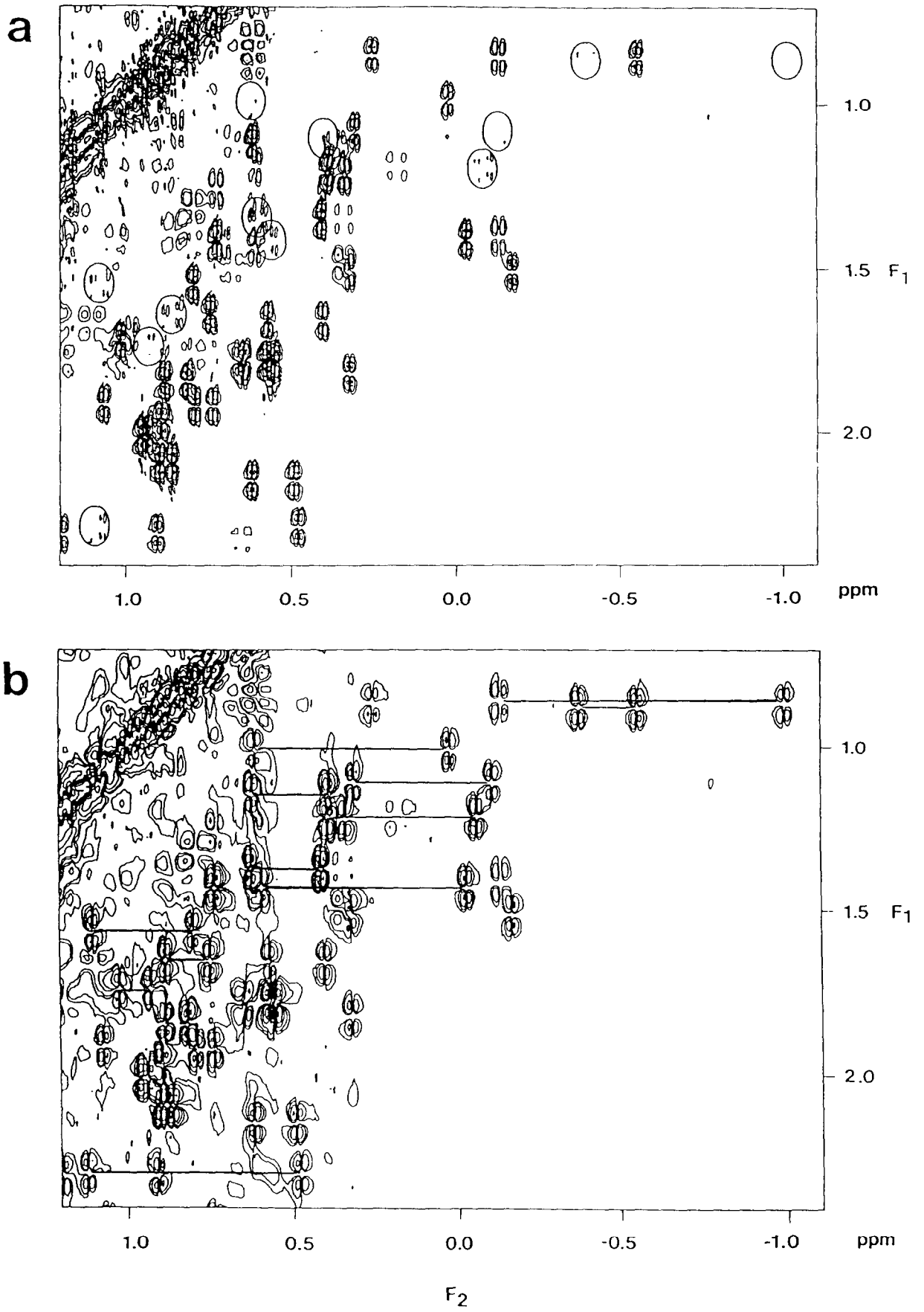
a
1.0
1.5 F1
2.0
1.0
0.5
0.0
-0.5
-1.0
ppm
b
1.0
1.5 F1
2.0
1.0
0.5
0.0
-0.5
-1.0
ppm
F2

Table I

Stereospecific assignments of leucine methyl protons for the DHFR·MTX complex

Residue	4-pro-R[a] chemical shift	NOEs[b] to 4-pro-R	4-pro-S[a] chemical shift	NOEs[b] to 4-pro-S
L4	1.17	MTX $2NH_2$, L4NH, L4α, Y29, $\varepsilon_1\varepsilon_2$	0.52	F30 $\delta_1\delta_2$
L12	0.93	T126NH	0.80	F122 $\delta_1\delta_2$
L19	0.66	MTX H7, MTX N10CH_3	0.46	
L23	0.65	H153 δ_2	0.06	
L27	0.64	H22 δ_2	0.01	L27α, MTX H7
L54	−0.07	F30 $\varepsilon_1\varepsilon_2$, M39$\varepsilon$	0.36	L54α
L62	0.43	F106 $\varepsilon_1\varepsilon_2$	0.66	
L94	0.78	L94α, V95NH	0.78	F83 $\varepsilon_1\varepsilon_2$
L113	−0.96	L131 δ_1, W133 ε_3	0.30	W5 ζ_3, L113α
L114	0.98	L4α	1.07	L114α, V157α
L118	−0.33	L118NH, Q7NH, W21 ε_1	−0.52	F122 $\varepsilon_1\varepsilon_2$, L151$\alpha$
L131	−0.02	W133 ε_1, W5η	0.44	F3 $\varepsilon_1\varepsilon_2$
L151	1.15	N148β_1, N148β_2	0.85	L151α

[a]Assignments obtained using the (2*S*,4*R*)[5,5,5-$^2H_3$]leucine DHFR

[b]These NOEs are those which best distinguish the ^1H resonances of leucine 4-pro-*R* and 4-pro-*S* methyl protons

species (CHD_2) in the pro-*R* methyl group but no deuterium in the pro-*S* methyl group.

To prepare the selectively deuterated protein an 18 litre fermentation of *Lactobacillus casei* was grown on a fully defined medium containing 1 g of the selectively deuterated leucine together with all the other amino acids. The labelled DHFR was isolated and purified from this fermentation using methods described previously [13]. The 1:1 binary complex of the labelled DHFR with methotrexate (MTX) (from Sigma Chemical Company) was prepared as described previously [14]. The final NMR sample contained 1.2 mM DHFR.MTX dissolved in D_2O, 500 mM in KCl, 50 mM in potassium phosphate at pH 6.5.

^1H NMR spectra were recorded at 308K using Varian 600 MHz and Bruker 500 MHz NMR spectrometers. The 2D DQF.COSY and NOESY spectra were recorded with the transmitter frequency on the residual HDO signal, the latter being set in the centre of the spectrum in both dimensions. Quadrature phase sensitive detection in F1 was obtained by incrementing the phase of the preparatory pulse [15,16]. 4K data points were recorded for each of 512 t_1 experiments.

The chemical shifts were measured from dioxan and then referenced to DSS (the dioxan resonance is 3.75 ppm downfield from DSS (2,2-dimethyl-2-silapentane-5-sulphonate) at 308K).

3. RESULTS AND DISCUSSION

L. casei dihydrofolate reductase has 13 leucine residues. In earlier studies, we were able to identify the leucine and valine methyl resonances by analysing 2D COSY and TOCSY spectra and also by comparing 2D COSY spectra of normal and [γ-$^2H_6$]valine DHFR complexes [6]. The availability of a sample of (2S,4R) [5,5,5-$^2H_3$]leucine DHFR now allows us to use a similar approach to obtain stereospecific assignments for each diastereotopic pair of leucine methyls. Fig. 1 shows the high field regions of DQF.COSY spectra obtained from the deuterated and non-deuterated DHFR.MTX complexes. A comparison of the two spectra immediately reveals the signal from each pair of Leu γ/δ cross-peaks which is missing in the spectrum of the deuterated enzyme. The missing peaks have been circled in Fig. 1a to indicate their location. In spectra plotted at a lower contour cut-off level small residual signals can still be seen in the circled regions: these signals arise from the 25% of the monoprotiated material which is present in the sample. At this level, the monoprotiated impurity clearly does not complicate the stereospecific assignment of the leucine methyl resonances.

In earlier studies, we assigned several of the leucine methyl resonances by using a combination of NOE and crystal structure data and assuming that the solution and crystal structures are similar [14]. More recently we have also used this approach to provide stereospecific assignments for some of the leucine residues and Table I summarises the relevant NOEs observed and used for making the assignments for the DHFR.MTX complex (spectra not shown). Included in Table I, are the stereospecific assignments obtained directly from the stereospecific deuteration experiments and these agree completely with those obtained by correlating NOE and X-ray structural data. This is further evidence that the solution and crystal structures of dihydrofolate reductase complexes are similar. More recently 3D $^{13}C/^1H$ and $^{15}N/^{13}C/^1H$ experiments on complexes formed with uniformly ^{13}C- and ^{15}N-labelled DHFR [17] have provided complete $^1H/^{15}N/^{13}C$ resonance assignments for all the leucine residues of DHFR without recourse to

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Fig. 1. The high field 'aliphatic' region of the 2D DQF.COSY ^1H contour plot for the dihydrofolate reductase.methotrexate comlex recorded at 308K (a) selectively deuterated enzyme incorporating (2*S*,4*R*)[5,5,5-$^2H_3$]leucine (**1**) (b) non-deuterated enzyme. The positions of the leucine cross-peaks involving the 4-pro-*R* methyl group are circled in (a) and the methyl pairs from each leucine are joined with a line in (b). Although the two samples were recorded at different frequencies (600 and 500 MHz, respectively) the spectra presented on the ppm scale are directly comparable for present purposes.

crystallographic data thus confirming the earlier assignments.

The ^{13}C-labelling method previously used to make stereospecific assignments for methyl groups [9–11] has the advantage of being general for both valine and leucine residues; however, it requires the use of relatively complex $^{1}H/^{13}C$ half filtered NMR experiments. In contrast, the incorporation of stereospecifically deuterated leucine into a protein allows direct access to the stereospecific assignment information using simple ^{1}H–^{1}H correlation experiments.

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