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Optimising selective deuteration of proteins for 2D ^{1}H NMR detection and assignment studies

Application to the Phe residues of *Lactobacillus casei* dihydrofolate reductase

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A selectively deuterated dihydrofolate reductase from *L. casei* has been prepared containing partially deuterated aromatic amino acids. This provides simplified 2D NMR spectra and allows signals from all 8 Phe residues to be identified. The pattern of deuteration is such that (i) the only cross-peaks detected in the aromatic region of the 2D COSY spectrum are those between the Phe 2′,6′ and 3′,5′ protons and (ii) chemical shift degeneracy in the aromatic region is removed thus allowing unambiguous assignment of cross-peaks in 2D NOESY spectra required for specific assignment purposes.

NMR, 2D; Dihydrofolate reductase; Selective deuteration

1. INTRODUCTION

NMR spectroscopy can be used to obtain detailed information about interactions, conformations and dynamic processes in protein-ligand complexes in solution. However, in order to extract such information from NMR spectra it is necessary to assign the NMR signals to specific nuclei in the complex. The availability of high-field NMR spectrometers and the development of two-dimensional multipulse NMR experiments has considerably simplified this process, and the ^{1}H spectra of many small proteins can now be completely assigned by using the sequential assignment procedures pioneered by Wüthrich et al. (see [1] and references therein). However, for larger proteins (M_r > 15000), the increased complexity of the spectra, with inevitable degeneracy in chemical shifts, militates against the simple application of the direct sequential assignment method. Chemical shift degeneracies not only make it difficult to assign the signals to residue types (in 2D correlation experiments such as COSY and HOHAHA) but also complicate the analysis of 2D NOESY experiments. This is particularly difficult when one proton has NOE connectivities to several others; any ambiguity in the assignment of the nuclei contributing to the NOESY cross-peaks makes it very difficult to apply the sequential assignment method in a straightforward manner. Although 2D RELAYED NOESY [2] or 3D methods (for example, combining HOHAHA and NOESY [2]) will contribute to removing some of these ambiguities, the short T_2 values of larger proteins make these methods difficult to use. Isotopic labelling provides an obvious way of simplifying complex protein spectra [3-11] and it now seems likely that a combination of these methods with the available 2D NMR experiments will provide the basis for assigning ^{1}H spectra of larger proteins. For example, Le Masters and Richards [10] have examined

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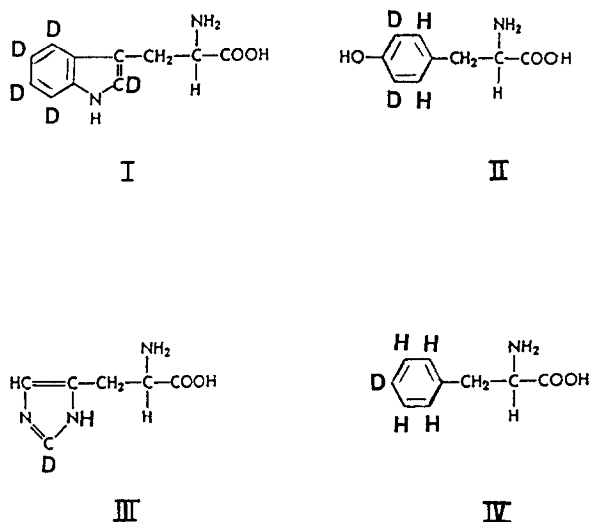

Scheme 1.

partially perdeuterated proteins and obtained improved 2D COSY spectra: in these experiments the level of non-selective deuteration was arranged such that only isolated pairs of protons interact in any one molecule thus simplifying the multiplicity on the detected cross-peaks in the 2D COSY spectra. Others have chosen to adopt more selective approaches to deuteration.

While the simplest method of examining an extensively deuterated protein containing only one non-deuterated amino acid residue type provides an unambiguous assignment of the signals to a residue type it does not allow one to make specific assignments to sequence positions. In order to do this it is necessary to make NOE connections between protons in several different residues. For this one needs to aim for a pattern of selective deuteration that is sufficient to remove the chemical shift

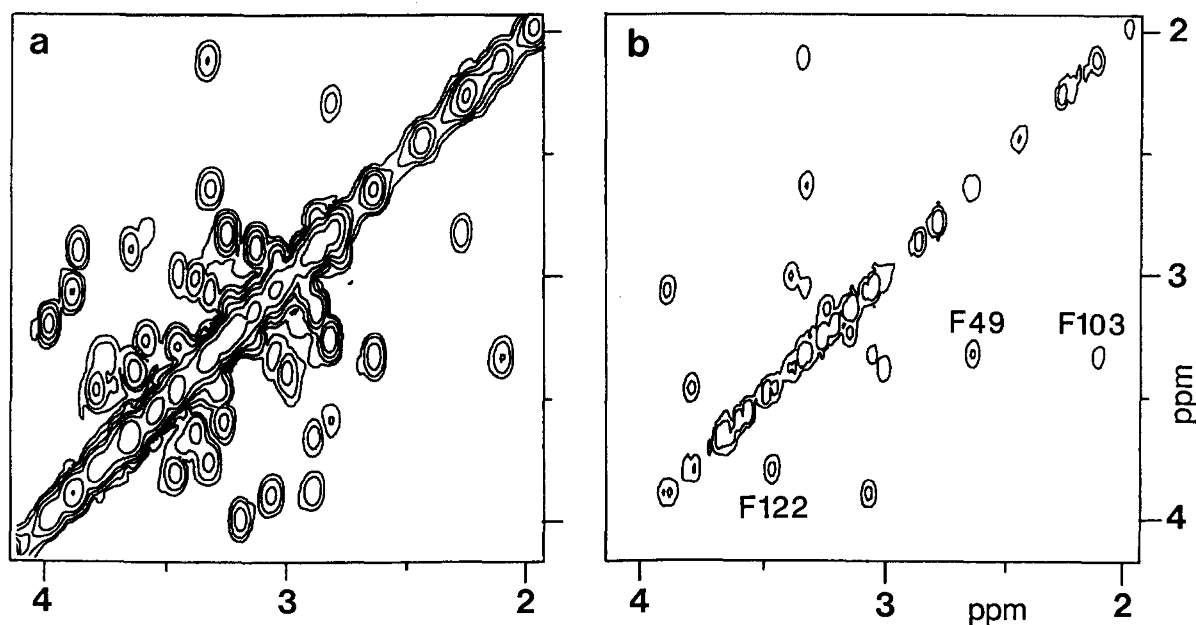

Fig.1. Aromatic region of the 2D COSY ^{1}H contour plot for the dihydrofolate reductase-methotrexate complex recorded at 308 K and 500 MHz with a Bruker AM500 spectrometer. (a) Non-deuterated enzyme. (b) Selectively deuterated enzyme incorporating the selectively deuterated aromatic acids (I–IV). The COSY spectra were obtained in the absolute value mode using a $(90\text{-}t_1\text{-}90\text{-}t_2\text{-}d)_n$ pulse sequence with a 16-step phase cycle to select N-type peaks and to suppress quadrature images and axial peaks [16]. 2D experiments were carried out with the carrier frequency placed at the centre of the spectrum using quadrature detection in both dimensions. The H_2O peak was decreased by irradiating it during the relaxation delay (0.8 s). The data was multiplied by a sine bell squared window function, unshifted, in each dimension. The ^{1}H NMR chemical shift reference, dioxane (1 mM) is 3.75 ppm downfield of 5,5-dimethyl-5-silapentane-2-sulfonate at 308 K. In spectrum (a) 308 scans were acquired and 1024 data points used in t_2 for each of the 256 t_1 values. The spectral width was 6410 Hz (12.8 ppm) giving a digital resolution of 6.3 Hz/point in F_2 and the time domain data matrix was zero-filled to 1024 points. The enzyme-methotrexate complex was 1.0 mM in D_2O containing 50 mM potassium phosphate/500 mM KCl. In spectrum (b) 144 scans were acquired and 2048 data points were used in t_2 for each of the 384 t_1 values. The spectral width was 6410 Hz (12.8 ppm) giving a digital resolution of 3.1 Hz/point. The time domain data matrix was again zero-filled to 1024 points. The deuterated enzyme-methotrexate complex was 1.0 mM in D_2O buffer.

ambiguities but which retains enough protons to allow the necessary NOE connections to be made for specific assignment purposes.

In the aromatic region of the ^{1}H spectrum of dihydrofolate reductase (M_r 18 300) there is a great deal of chemical shift degeneracy. In previous studies [13] we have been able to identify signals from 2 of the 4 Trp, the 7 His and 5 Tyr residues and from 5 of the 8 Phe residues. Here, we describe how selective deuteration experiments now allow us to identify signals from all 8 Phe residues and how the resulting simplification of the 2D NOESY spectra can assist in their specific assignment.

2. MATERIALS AND METHODS

[2′,4′,5′,6′,7′-^{2}H]Tryptophan (I), [3′,5′-^{2}H]tyrosine (II) and [2′-^{2}H]histidine (III) were prepared from the unlabelled amino acids by methods described previously [14]. [4′-^{2}H]Phenylalanine (IV) was synthesized starting from *p*-bromotoluene. The hydrolysis of the Grignard Mg-complex of *p*-bromotoluene with D_2O gave *p*-deuterotoluene, which was then brominated using Br_2 in CCl_4 under UV irradiation to give *p*-deuterobenzyl bromide. The *p*-deuterobenzyl bromide in ethanol was added to a solution of sodium acetamidodiethylmalonate in absolute ethanol and the resulting *p*-deuterobenzyl acetamidodiethylmalonate was hydrolyzed in HCl giving dl-*p*-deuterophenylalanine HCl. The various methods of deuteration used resulted in some racemisation of the deuterated amino acids (except for Trp): they were used in subsequent fermentation without resolution. *Lactobacillus casei* was grown in defined medium which contained the deuterated amino acids (I–IV) together with the other isotopically normal amino acids using the procedures described previously [5]. Dihydrofolate reductase was isolated from the *L. casei* cells and purified as described by Dann et al. [11].

The non-deuterated dihydrofolate reductase was isolated from *E. coli* containing a plasmid bearing the *L. casei* dhfr gene (under the control of the λ pL promoter [12]) and was purified in the same way as that used for the enzyme from *L. casei*.

Methotrexate was obtained commercially (Sigma) and was used without further purification.

^{1}H NMR spectra were obtained at 500 and 600 MHz using Bruker AM-500 and 600 spectrometers operating in the Fourier transform mode as described by Hammond et al. [13].

In some samples the exchangeable protons in the dihydrofolate reductase were replaced by deuterium by freeze-drying the enzyme from D_2O solution. Enzyme samples were redissolved to form 1–1.3 mM solutions in D_2O containing 500 mM KCl/50 mM potassium phosphate at pH* 6.5 (meter reading uncorrected for the isotope effect on the glass electrode) before adding one or more equivalents of methotrexate. The 3 mM enzyme-MTX sample was prepared by dialysing 1 mM enzyme in the presence of MTX and dilute buffer, which was then concentrated by freeze-drying and redissolving in a smaller volume of D_2O.

3. RESULTS AND DISCUSSION

Fig.1a shows the aromatic region of the ^{1}H

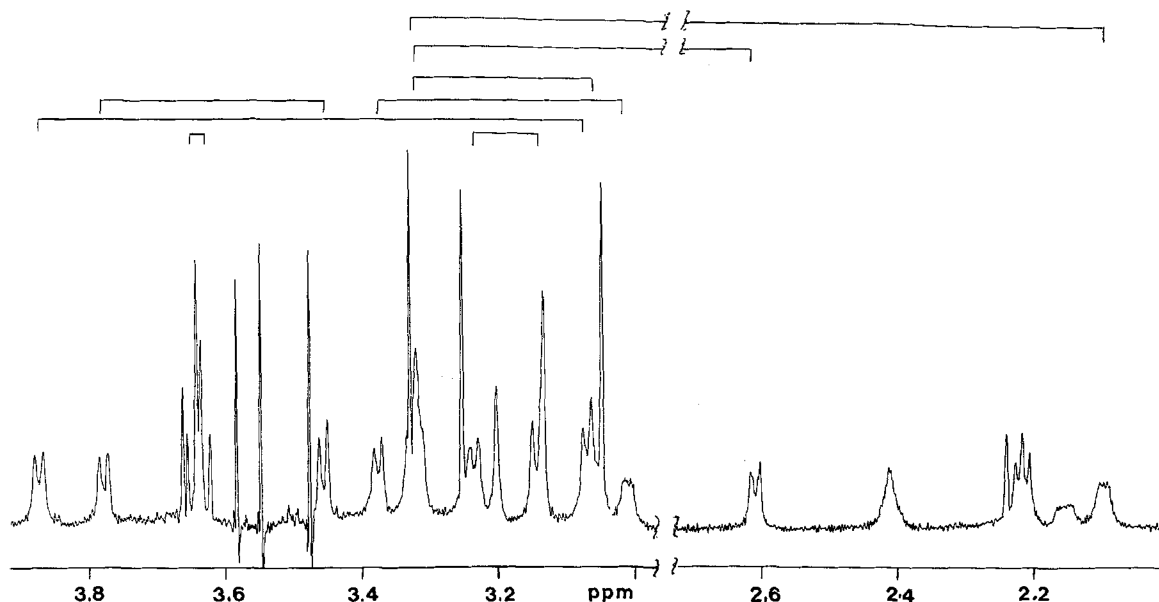

Fig.2. The aromatic region of the resolution enhanced ^{1}H spectrum of the selectively deuterated dihydrofolate reductase-methotrexate complex (1.3 mM solution in D_2O buffer) recorded at 600 MHz. The bars above the spectrum indicate the connected pairs of doublets from the 8 Phe residues. The chemical shift reference is dioxane (1 mM).

COSY spectrum of the complex of methotrexate with normal (non-deuterated) dihydrofolate reductase. Analysis of this spectrum is not straightforward and even when considered in conjunction with the HOHAHA and 2D double-quantum-coherence spectra it was only possible to detect cross-peaks for 5 of the 8 Phe residues. Fig.1b shows the COSY ^{1}H spectrum for the methotrexate complex with the selectively deuterated enzyme prepared by incorporating the aromatic amino acids I–IV. This pattern of deuteration was chosen so that only Phe residues will give cross-peaks in the aromatic region of the COSY spectrum (each expected to give a single cross-peak under conditions of rapid ring flipping). The dramatic simplification seen in fig.1b allows us to detect immediately signals from 7 of the 8 Phe residues. The absence of a cross-peak from one of the Phe residues could have resulted either from the coupled Phe protons having very similar chemical shifts or from line-broadening effects associated with some exchange process (such as ring flipping). Examination of the one-dimensional 600 MHz ^{1}H spectrum (see fig.2) clearly indicates that the former explanation is correct. In this spectrum it is possible to detect a strongly coupled AB multiplet at 3.64 ppm (from dioxan reference) in addition to the 7 pairs of doublets corresponding to the cross-peaks detected in the COSY spectrum. The sharp singlet signals seen in fig.2 can be assigned to the imidazole H4 protons of the 7 His and the H2′ and H6′ protons of the 5 Tyr residues.

The sequence-specific assignment of the signals from the Phe residues requires correlating NOE connectivities between Phe protons and protons from other residues with internuclear distance information obtained from the crystal structure [15]. Using this approach we have already assigned signals from Phe 49, 103 and 122 [13] and these are indicated in fig.1b. Selective deuteration simplifies not only the COSY spectra but also the 2D NOESY spectra as can be seen by comparing the NOESY spectra of normal and deuterated enzyme complexes (see fig.3). Such simplification will clearly assist in making assignments by removing ambiguities in the origins of the NOESY cross-peaks. For example, the cross-peaks in the row at F_1 -4.71 ppm in fig.3a correspond to several aromatic signals with NOE connections to the previously assigned CH_3 signal of Leu 113: fig.3b

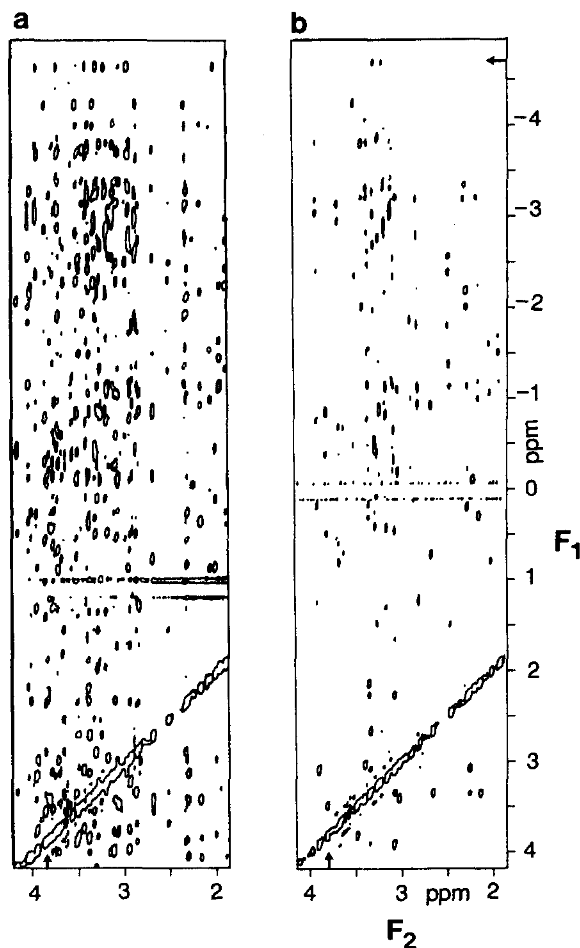

Fig.3. Selected regions of the ^{1}H NMR NOESY spectra of the dihydrofolate reductase-methotrexate complex. Spectrum (a) obtained with non-deuterated enzyme showing, (top) the NOESY peaks connecting aliphatic protons and aromatic protons and (bottom) the aromatic protons with other aromatic protons. Spectrum (b) shows the same regions obtained from the deuterated enzyme sample. NOESY spectra were obtained in the phase-sensitive mode using a $(90\text{-}t_i\text{-}90\text{-}\tau_m\text{-}90\text{-}t_2\text{-}d)_n$ sequence and the method of time-proportional phase incrementation [17,18]. Additional phase cycling was used to reduce quadrature images and axial peaks. 2048 data points were recorded in t_2 for each free induction decay. The time-domain data from a NOESY experiment was zero-filled to 1024 points in t_1 and processed with a sine bell function in each dimension, shifted $\pi/32$ in t_2 and $\pi/16$ in t_1. In spectrum (a) 320 scans were acquired for each of the 256 t_1 values. The spectral width was 7042 Hz (14.5 ppm) giving a digital resolution of 3.5 Hz/point in F_2. The mixing time was 0.15 s. The enzyme-methotrexate complex was 3.0 mM in D_2O in 50 mM potassium phosphate/ 500 mM KCl and the spectrum was recorded at 298 K. In spectrum (b) 272 scans were acquired for each of the 384 t_1 values. The spectral width was 6410 Hz (12.8 ppm) giving a digital resolution of 3.1 Hz/point in F_2. The mixing time was 0.1 s. The enzyme-methotrexate complex was 1.0 mM in D_2O buffer and the spectrum was recorded at 308 K.

indicates that for the deuterated enzyme there are now only two NOE cross-peaks in this region. Consideration of the crystal structure data reveals that Phe 136 is the only Phe residue which is close enough to Leu 113 to provide these NOE interactions thus allowing us to assign the corresponding signals for Phe 136. The additional cross-peaks seen at F_1 −4.71 ppm in fig.3a arise from NOE connections between Trp ring protons and Leu 113 CH_3 thus allowing further assignments to be made. The spectral simplification seen in the columns of cross-peaks at each F_2 value can also assist in the assignment. This is seen very clearly in the comparison of the columns of cross-peaks at F_2 3.78 ppm in fig.3a,b; the cross-peaks at F_1 0.47, −0.41 and −0.88 ppm in fig.3b can be assigned to the Phe 122 α, β, β' protons, respectively, from NOE interactions with H2′,6′ of Phe 122.

The ultimate success of these assignment methods based on combining selective deuteration with 2D NMR techniques will depend on carefully choosing the extent of deuteration to provide sufficient spectral simplification to remove chemical shift degeneracy while retaining enough protons to give an adequate number of NOE connectivities. The study of such deuterated proteins purified from organisms grown on carefully selected deuterated amino acids promises to become a routine approach for aiding assignments in the NMR spectra of large proteins.

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