A novel method of preparing totally α-deuterated amino acids for selective incorporation into proteins

Application to assignment of ^1H resonances of valine residues in dihydrofolate reductase

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The pyridoxal/^2H_2O exchange reaction of the α-CH of amino acids is known to be accompanied by racemisation: Thus by using a D-amino acid as the starting material any L-amino acid formed in the reaction will be essentially fully deuterated at its α-position. We have used this method to prepare α-deuterated L-valine and incorporated this biosynthetically into *L. casei* dihydrofolate reductase. A comparison of the αCH-NH fingerprint regions of COSY spectra of deuterated and normal DHFR complexes allows one to identify cross-peaks from 15 of the 16 valine residues.

NMR; 2D; Dihydrofolate reductase; Selective deuteration

1. INTRODUCTION

Nuclear magnetic resonance studies are being increasingly used to obtain information about conformations and interactions in protein-ligand complexes. In order to undertake such studies it is necessary to obtain specific assignments for the ligand and protein resonances. For the protein assignments the first stage in this procedure is to identify the ^1H signals of spin systems characteristic of particular amino acid types. Selective isotopic labelling has proved to be very effective in making spin system assignments and numerous applications have been reported. For ^1H NMR resonances the assignments can be made either by using selective deuterium labelling, to remove ^1H signals from the spectra [1-5], or by using specific ^{15}N or ^{13}C labelling to identify the signals from scalar coupled protons [6-9]. Spin system assignments in the NH to αCH 'fingerprint' region of COSY or HOHAHA spectra are essential for subsequent sequential assignment of protein spectra. However, the spectra of medium sized proteins (M_r 15-25 kDa) suffer from extensive chemical shift degeneracy even in 2D spectra and this complicates the analysis of COSY, HOHAHA and NOESY spectra. One approach to removing these ambiguities in the fingerprint region has been to examine uniformly ^{13}C- or ^{15}N-labelled proteins using 3D NOESY-HMQC and HOHAHA-HMQC experiments where indirect detection of the ^{13}C or ^{15}N nuclei via the coupled ^1H nuclei allows the heteronuclear chemical shifts to become effective in removing degeneracy in the ^1H chemical shifts [6-9]. A direct method of removing chemical shift degeneracy in this region of the spectrum is to use selective α-deuteration to simplify the ^1H spectrum, and it is surprising that there have been relatively few reports of such studies [5,10]. The most extensive work in this area has been that of Le Master and Richards [5] who demonstrated that selectively α-deuterated amino acids can be incorporated biosynthetically into proteins using a transaminase deficient *E. coli* strain, thereby producing selectively deuterated proteins with simplified NMR spectra [5,10].

The conventional method of preparing α-deuterated amino acids is to subject the L-amino acid to a base catalysed exchange reaction in the presence of pyridoxal in ^2H_2O [11,12] with the final extent of deuteration depending upon the reaction conditions, in particular on the reaction time. In the presence of Al^{3+}, pyridoxal catalyses exchange at both the α- and β-positions and by using this approach Le Master and Richards [11] have achieved high levels of α- and β-deuteration (>95%) for several amino acids. In many instances it is necessary to label only the α-position and this is achieved by carrying out the reaction in the absence of the polyvalent cations. However, under these conditions, some amino acids such as valine and isoleucine are only partially α-deuterated even after extended reaction times. Since it is desirable to have high deuteration

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levels we have devised an alternative method for preparing deuterated L-amino acids which provides almost total α-deuteration. This is based on the fact that in the pyridoxal/^2H_2O exchange reaction the α-deuteration is accompanied by racemisation of the amino acids [11,12]. Thus, if a D-amino acid is used as the starting material then the L-amino acid found in the racemate mixture will be essentially fully deuterated at its α-position. It should be noted that the D/L-mixture can be used without resolution in the bacterial growth medium since only the L-isomer is incorporated. In this present study we demonstrate the method by examining the NMR spectrum of a selectively deuterated dihydrofolate reductase isolated from *Lactobacillus casei* (MTX/R) grown on a fully defined medium containing totally α-deuterated L-valine together with the other isotopically normal amino acids. The *L. casei* (MTX/R) strain has the advantage of being highly auxotrophic and capable of incorporating any non-essential amino acids if these are provided in the medium [13], thus, removing the possibility of transferring the label into other amino acids. However, there is no evidence that it is transaminase deficient and one must face the possible complication of losing some of the label as a consequence of transaminase activity.

2. EXPERIMENTAL

2.1. *Preparation of α-deuterated valine*

D-valine was subjected to a base catalysed exchange reaction in the presence of 99.8% ^2H_2O, NaOD and pyridoxal hydrochloride. Initially exchangeable protons in the reactants were pre-exchanged with deuterium by repeated freeze drying from ^2H_2O. The lyophilised material (8.4 g) was then taken up in 72 ml ^2H_2O (99.8% ^2H) in the presence of 18 ml of 40% NaOD (99% ^2H) and 1.46 g of pyridoxal hydrochloride (Sigma) and refluxed for 20 h, with two further additions of pyridoxal hydrochloride (1.46 g) being made at intervals. After this reaction time the extent of deuteration at the α-position was determined using ^1H NMR and found to be 85% for the D/L-mixture. The final solution was adjusted to pH 6 (with HCl) and then lyophilised.

2.2. L. casei *dihydrofolate reductase (MTX/R) growth conditions*

The *L. casei* MTX/R cells were grown at 37°C for 26 h in 40 litres of fully defined medium [14] containing the α-deuterated valine mixture (containing >99% α-deuterated L-valine) together with the other isotopically normal amino acids. The isolation and purification of *L. casei* dihydrofolate reductase (DHFR) was carried out using methods described previously [14].

Growth curve experiments using fully defined medium were carried out and the results confirm that *L. casei* MTX/R is fully auxotrophic for L-valine and does not grow when L-valine is replaced by D-valine.

2.3. *NMR experiments*

The ^1H NMR spectra were acquired from 0.5 ml, 5 mM samples of 1:1 DHFR/methotrexate complexes in an $H_2O/^2H_2O$ (90:10) solution containing 500 mM KCl and 50 mM potassium phosphate at pH 6.5.

In the case of the α-deuterated valine DHFR sample the phase sensitive DQF COSY spectrum was obtained on a Bruker AM 400 spectrometer at 308K with the carrier frequency placed at the centre of the spectrum and a spectral width of 6024 Hz. Quadrature detection in F_1 was achieved using the TPPI method [15]. Data were recorded in 4 K points in t_2 for each of 512 t_1 values. The water signal was suppressed by selective presaturation during the relaxation delay followed by a SCUBA pulse sequence [16]. The DQF-COSY spectrum of the isotopically normal DHFR complex was recorded on a Bruker AM500 spectrometer under similar conditions except that the spectral width was 7042 Hz and the water was suppressed by using a simple presaturation pulse.

3. RESULTS AND DISCUSSION

A comparison of the fingerprint regions of a series of DQF-COSY spectra for the deuterated and normal DHFR complexes allows one to identify cross-peaks for 15 of the 16 valine residues: these are all present at normal intensity for the isotopically normal sample and at a much reduced level for the α-deuterated sample. In the spectra shown in Fig. 1, 13 of the 16 valine cross-peaks can be immediately detected in this way: two

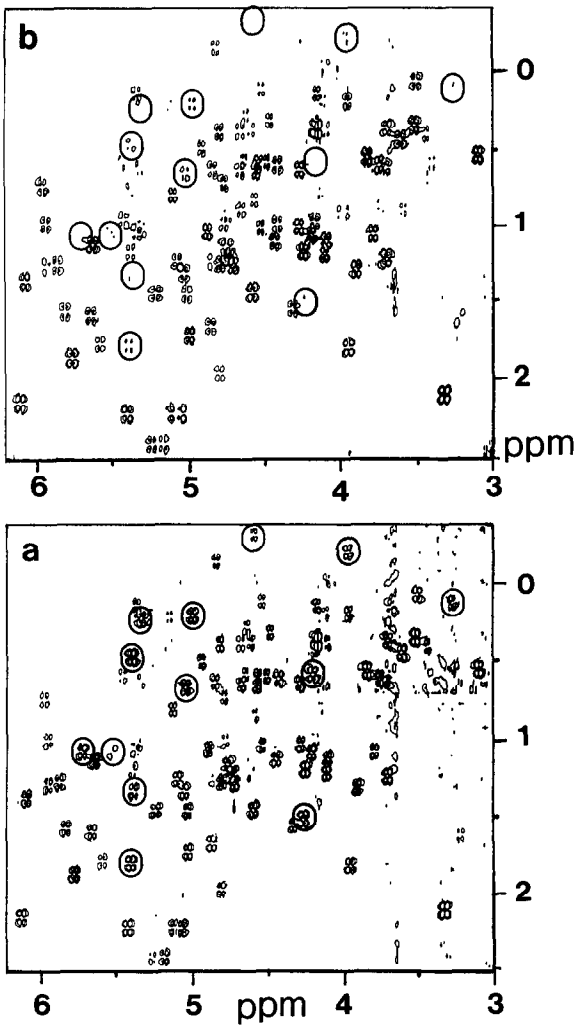

Fig. 1. The 'fingerprint' region of the 2D DQF COSY ^1H contour plot for the dihydrofolate reductase-methotrexate complex recorded at 308 K. (a) Non-deuterated enzyme. (b) Selectively deuterated enzyme incorporating α-deuterated valine. The positions of the valine cross-peaks are circled in the figure. Although the samples were recorded at different frequencies (500 and 400 MHz, respectively) the spectra presented on the ppm chemical shift scale are directly comparable for present purposes

cross-peaks are not seen in Fig. 1a because of the H_2O irradiation but these have been observed in other spectra recorded at different temperatures. One valine cross-peak has never been observed, probably because of an unusually small $J_{N\alpha}$ value. The 15 assigned valine signals provide ideal starting points for using sequential assingment methods to give specific residue assignments. In other experiments we have independently assigned several of the cross-peaks by identifying the unique spin-system patterns for valines in the COSY and HOHAHA spectra; however, only 11 of the 16 valines could be identified unequivocally by this method.

It should be noted that while the presence of weak valine cross-peaks in the spectrum of the deuterated sample indicates that there has been some loss of the deuterium label (probably resulting from transaminase action) this is not large enough to prevent clear identification of the valine cross-peaks. Careful intensity measurements on 9 of the well-resolved cross-peaks indicates that the level of deuteration retained is 70 ± 10%; clearly at this level there is no problem in identifying the cross-peaks influenced by the selective deuteration. Had a D/L-mixture with 85% α-deuteration been prepared from L-valine this would have had a L-valine deuteration of only 74% and the final incorporation of deuterated valine into DHFR would have been only 52% making it more difficult to identify the cross-peaks affected. This illustrates the advantages of starting with ~100% α-deuterated L-amino acids.

For much larger proteins (130 kDa) it seems likely that extensive selective deuterium labelling in combination with ^{15}N-labelling and 3D editing techniques will provide the spectral simplification necessary for assigning backbone protons. A particularly useful approach would be to incorporate a defined mixture of several amino acids with total α-deuteration. Clearly the method of α-deuteration proposed here could be used to advantage in such procedures.

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