The combined use of selective deuteration and double resonance experiments in assigning the ^1H resonances of valine and tyrosine residues of dihydrofolate reductase

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Selective deuteration is a general solution to the resolution problem which limits the application of double resonance experiments to the assignment of the ^1H NMR spectra of proteins. Spin-decoupling and NOE experiments have been carried out on *Lactobacillus casei* dihydrofolate reductase and on selectively deuterated derivatives of the enzyme containing either [γ-$^2H_6$]Val or [$\alpha,\delta_2,\varepsilon_1$-$^2H_3$]His, [$\alpha,\delta_1,\delta_2,\varepsilon_1,\varepsilon_2,\zeta$-$^2H_6$]Phe, [$\alpha,\delta_1,\varepsilon_3,\zeta_2,\zeta_3,\eta_2$-$^2H_6$]Trp and [$\alpha,\varepsilon_1,\varepsilon_2$-$^2H_3$]Tyr. When combined with ring-current shift calculations based on the crystal structure of the enzyme, these experiments allow us to assign ^1H resonances of Val 61, Val 115, Tyr 46 and Tyr 68.

NMR *Dihydrofolate reductase* *Deuterated protein* *NMR assignment*

1. INTRODUCTION

High-resolution NMR spectroscopy is capable of providing detailed information on protein structure in solution [1], but a prerequisite for this is the assignment of individual resonances in the spectrum to individual amino-acid residues in the sequence. Authors in [2–5] have described an elegant strategy for the assignment of the ^1H NMR spectra of proteins, using two-dimensional NMR experiments. However, as yet this strategy has only been successfully applied to very small proteins ($\lesssim$80 residues) and for larger proteins alternative procedures must be employed (review, [1,6]). One such procedure is to make assignments by reference to the crystal structure of the protein, using nuclear Overhauser enhancement (NOE) experiments to identify resonances of residues which are close together in space. In proteins of $M_r \gtrsim 15000$, the major limitation in the use of NOE experiments for assignment purposes is the limited resolution of the protein NMR spectrum even at the highest field strengths available. The most general solution to this resolution problem is isotopic substitution [1]; the application of this to the elucidation of NOE experiments on tRNA is described in [7]. We report the combined use of selective deuteration and NOE experiments in the assignment of valine and tyrosine resonances of *Lactobacillus casei* dihydrofolate reductase.

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2. MATERIALS AND METHODS

DL-[γ-$^2H_6$]Valine was synthesized as described for valine [8], by the reaction of [$^2H_6$]acetone with 2-mercaptothiazol-5-one (prepared by method B of [9]), to give 2-thio-4-[$^2H_6$]isopropylidene-thiazolid-5-one, which was then reduced and hydrolysed

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by HI and red phosphorus in acetic acid to give DL-[γ-$^2H_6$]valine (characterised by amino acid analysis and NMR spectroscopy). The growth of *L. casei* MTX/R on a defined medium containing a mixture of normal and deuterated amino acids [10], and the isolation and purification of dihydrofolate reductase [11] were carried out as described previously. The sample of enzyme in which all the aromatic protons except the *ortho* (2′,6′) protons of tyrosine had been replaced by deuterium was that studied earlier [10].

500 MHz ^1H NMR spectra were obtained on the Bruker AM500 Spectrometer of the MRC Biomedical NMR Centre at the National Institute for Medical Research. The samples consisted of 0.2–1.0 mM dihydrofolate reductase in 0.35 ml ^2H_2O containing 500 mM KCl, 50 mM potassium phosphate, pH* 6.5. (The notation pH* denotes a meter reading uncorrected for the isotope effect on the glass electrode.) The tightly binding ligands methotrexate or NADPH were added in a 1:1 molar ratio to the enzyme, and the more weakly binding 2,4-diamino-pyrimidine was added in a 10-fold molar excess. Dioxan (1 mM final concentration) was added as a chemical shift reference.

NOE experiments were carried out by applying selective ^1H irradiation for 0.2–1.5 s immediately prior to the 90° observation pulse. To minimise the effects of any long-term drift in the spectrometer, the irradiation field was cycled through a series of experimental and control frequencies, collecting 64 transients at each, until 1000–1500 transients had been accumulated. Difference spectra were calculated by subtraction of the free induction decays. Spin-echo decoupling experiments [12] were carried out using the 90°-τ-180°-τ-Acq pulse sequence, with $\tau = 60$ ms, so that doublets with $J = 7$–8 Hz are inverted. The selective decoupling field was applied continuously except during acquisition so as to avoid direct (Bloch-Siegert) effects; the results were examined as the difference between spectra obtained with on- and off-resonance irradiation.

The effects of the magnetic anisotropy of the aromatic rings of the Phe, Tyr, Trp and His residues on the shielding of nearby protons were calculated from the refined crystal structure [13], using the Johnson–Bovey equation [14] with the parameters of Giessner–Prettre and Pullman [15]. The only crystal structure available for *L. casei* dihydrofolate reductase is that of the enzyme–methotrexate–NADPH ternary complex [13], and thus calculations of ring-current shifts and interatomic distances refer to this complex. Most of our experiments were carried out on either the enzyme–methotrexate or enzyme–2,4-diaminopyrimidine complexes rather than on the ternary complex in order to obtain optimum resolution in the spectral regions of interest. Comparisons of chemical shifts and NOE effects in the spectra of these binary and ternary complexes indicate that the arguments used here to assign valine and tyrosine residues are applicable in all three cases. Detailed comparisons of the ^1H spectra of these complexes will be presented elsewhere [16].

3. RESULTS AND DISCUSSION

The first stage in assigning the ^1H resonances of a protein is identification by residue type, and here isotopic substitution provides a direct and unambiguous approach. With few exceptions [17,18], selective deuteration has hitherto been used in this way only for aromatic amino acid residues (e.g., [10,17,19,20]). We have now incorporated [γ-$^2H_6$]valine into *L. casei* dihydrofolate reductase and used this selectively deuterated enzyme to identify the valine methyl resonances in the spectrum of the isotopically normal enzyme.

Fig.1 compares the methyl region of the 500 MHz ^1H NMR spectrum of [γ-$^2H_6$]valine dihydrofolate reductase (in its complex with the inhibitor methotrexate) with the corresponding spectrum of the normal enzyme. It is apparent that a number of resonances are missing from the spectrum of the deuterated enzyme (fig.1b), and these must arise from the methyl groups of 16 valine residues in the enzyme. These valine resonances can be seen in isolation by taking the difference (fig.1c) between the spectra of the normal and the deuterated enzyme.

This difference spectrum shows two individual valine methyl resonances (labelled V_H and V_E) towards the high-field end of the spectrum, at -3.70 and -3.81 ppm from dioxan. Resonance V_E can be observed as a separate signal in the spectrum of the normal enzyme, but resonance V_H overlaps another methyl resonance. The two methyl resonances of valine in simple peptides ap-

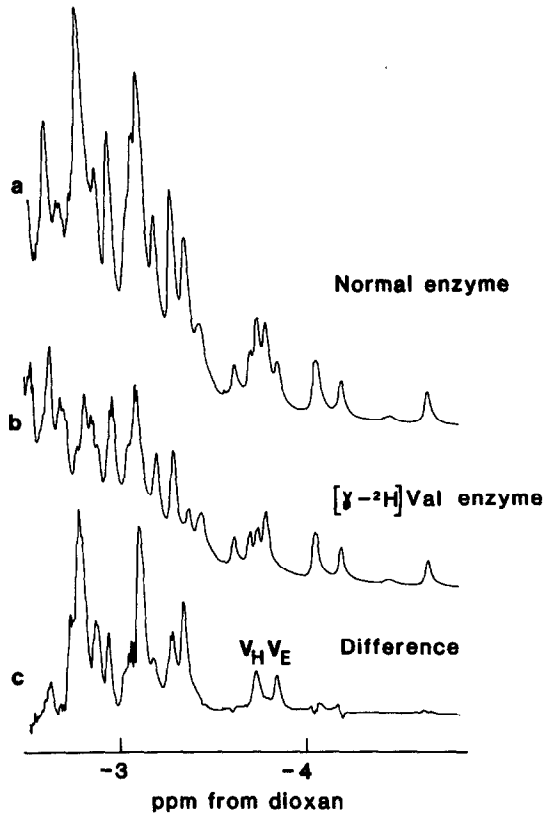

Fig.1. High-field region of the 500 MHz ^1H NMR spectra of (a) the dihydrofolate reductase–methotrexate complex and (b) the corresponding complex of the [γ-$^2H_6$]valine-containing enzyme. (c) The difference spectrum, (a) – (b).

pear at −2.74 and −2.77 ppm [21], so resonances V_H and V_E in the protein spectrum have been shifted approximately 1 ppm upfield, presumably by the magnetic anisotropy of nearby aromatic rings ('ring-current' shifts). The expected ring-current shifts can be calculated from the atomic coordinates derived from the refined crystal structure [13] as outlined in section 2. The limited accuracy of these calculations, together with the possible contributions from other shielding mechanisms, prevents us from using the calculated ring-current shifts alone to assign resonances such as V_H and V_E to individual residues. However, we can use these calculations to limit the number of valine residues we need to consider. With a difference of ~1 ppm between the observed chemical shifts and the random coil values, we have considered only valine methyl groups with a calculated upfield ring-current shift of >0.5 ppm as possible candidates for assignment to V_H and V_E. Four valine residues meet this criterion, residues 61, 79, 110 and 115; the calculated shifts range from 0.54 to 1.03 ppm upfield.

The next step in the assignment process is to establish whether resonances V_H and V_E arise from the same or different valine residues. This is most easily done by spin-decoupling experiments; in proteins where the multiplicity of the methyl resonances is incompletely resolved the spin-echo decoupling experiment [12] is the most useful, and the results of the relevant experiments are shown in fig.2. The two methyl resonances of a single valine will obviously both be decoupled by irradiation at the same (Hβ) frequency. However, irradiation at −2.15 ppm (fig.2A) decouples V_E but not V_H, demonstrating that these two signals do not arise

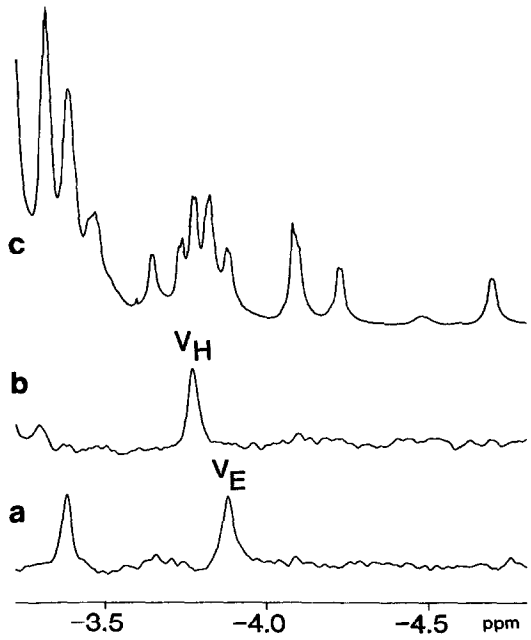

Fig.2. High-field region of the ^1H spin-echo decoupling difference spectra of the dihydrofolate reductase–methotrexate complex, with irradiation at (a) −2.15 ppm and (b) −3.03 ppm. (c) Corresponding region of a control spectrum. Spectra (a) and (b) are each the difference between spin–echo spectra (τ = 60 ms) obtained with irradiation at the indicated frequencies and with off-resonance irradiation.

from the same valine residue. Irradiation at this frequency also has a decoupling effect at −3.36 ppm, and comparison with the results of the same experiment with the [γ-$^2H_6$]valine enzyme shows that this arises from a valine methyl resonance, suggesting that it represents the second methyl signal from the residue giving rise to V_E. This is confirmed by the observation of a substantial NOE effect at −3.36 ppm on irradiation of V_E.

Assignment of V_E depends upon the use of NOE experiments to identify residues close in space to the valine from which it arises. Irradiation at −3.81 ppm gives rise to a number of NOE effects in the aromatic region of the spectrum (as would be expected if the high-field position of these signals is due to the effects of nearby aromatic rings). However, even at 500 MHz, the complexity of this region of the spectrum is such that we cannot immediately identify the type of residue (His, Phe, Trp or Tyr) from which these NOE effects originate, and thus we cannot use them to assign resonance V_E. This problem can be overcome by use of a selectively deuterated enzyme. Fig.3 shows the aromatic region of the spectrum of an enzyme sample in which all the aromatic protons except the *ortho* protons of tyrosine had been replaced by deuterium [10]; 5 resonances from the *ortho* protons of the 5 tyrosine residues can be clearly seen. (The rate of 'flipping' about the C_α–C_β bond of all the tyrosine residues in this protein is fast enough to average the resonances of the 2′- and 6′-protons [10]; each tyrosine residue thus gives rise to one *ortho* proton resonance.) As shown in fig.3, irradiation at −3.81 ppm (valine methyl signal V_E) produced clear NOE effects at 3.33 and 3.18 ppm (tyrosine *ortho* proton signals Y_A and Y_C). Thus the valine methyl corresponding to resonance V_E must be close in space (<4.5 Å) to the aromatic protons of two tyrosine residues. Examination of the crystal structure shows that, of the group of valine residues selected on the basis of the calculated ring-current shifts, only Val 61 has a methyl group within 4.5 Å of the aromatic protons of a tyrosine residue. This valine is in fact close to both Tyr 46 and Tyr 68. We can thus assign V_E (and its associated methyl and Hβ resonances located in fig.2A) to Val 61, and Y_A and Y_C to Tyr 46 and Tyr 68. (Additional NOE experiments involving irradiation of the *ortho* proton resonance of Phe 49 [16] allow us to assign Y_A to Tyr 46 and Y_C to Tyr 68.)

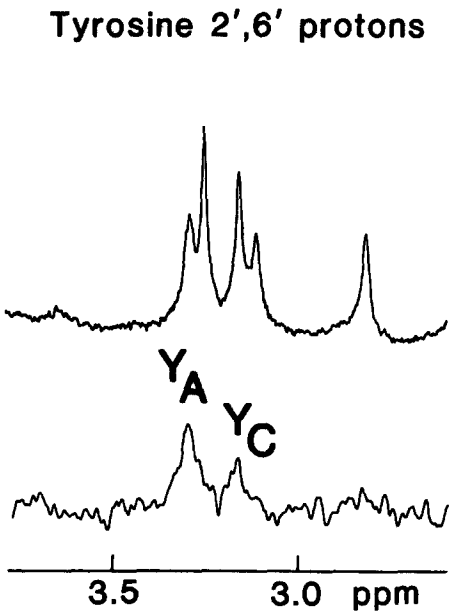

Fig.3. Top: Aromatic region of the 500 MHz ^1H NMR spectrum of the complex between 2,4-diaminopyrimidine and selectively deuterated dihydrofolate reductase, in which the only aromatic protons remaining were the 2′6′-protons of the 5 tyrosine residues. Bottom: Difference spectrum showing the NOE effects observed on irradiating resonance V_E in this sample.

Turning to the assignment of V_H, the starting point is again the spin-decoupling experiment. Since V_H overlaps another methyl signal (fig.1), this experiment would normally be ambiguous, it being impossible to establish which doublet has been decoupled. However, this ambiguity can be overcome by comparing the effects of irradiation at the same frequency in the normal and [γ-$^2H_6$]valine enzyme. In this way we can show that a decoupling effect on V_H is observed on irradiation at −3.03 ppm (fig.2B), much further upfield than expected for a valine Hβ resonance (−1.58 ppm in simple peptides [21]). Similarly, irradiation at −3.70 ppm produces a large NOE at −3.11 ppm, and comparison with the same experiment on the [γ-$^2H_6$]valine enzyme shows that this results from irradiation of V_H, and most probably represents the second methyl resonance of the same valine residue.

Thus, in the residue giving rise to V_H, both the β-proton and the protons of one methyl group experience substantial ($\geqslant 1$ ppm) upfield ring-current shifts, while the second methyl group is much less affected (a difference of 0.36 ppm from the random coil value). This pattern of shifts can be used, semi-quantitatively, to limit further the possible assignments of V_H. Of the group of 4 valine residues discussed above, only two, Val 110 and Val 115, are expected to show upfield shifts of the Hβ resonance of the required magnitude; for the other two residues the calculated shifts are $\leqslant 0.3$ ppm. For these two candidate residues the calculated ring-current shifts of the methyl protons are 0.54 and 0.56 ppm for Val 110, and 0.77 and 0.02 ppm for Val 115. The latter pattern, of one large and one small shift, clearly corresponds more closely to what is observed for the residue giving rise to V_H. We thus assign V_H to Val 115; this assignment is confirmed by NOE experiments to be described elsewhere [16].

4. CONCLUSIONS

The available methods of assigning resonances in proteins by using one- and two-dimensional NMR experiments to correlate nuclei through either NOE or scalar coupling interactions are of limited usefulness for proteins of $M_r \geqslant 15000$ because of the complexity of their ^{1}H NMR spectra. The experiments described here show that selective deuteration can overcome this problem and allow the extension to larger proteins of the assignment methods which have successfully been applied to small proteins.

Deuteration gives simplified spectra so that even in a large protein one can selectively irradiate an individual resonance and observe the effect on individual resonances. The improvement obtainable in this way is such that detailed assignments should be possible for proteins of M_r up to about 50000.

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