Identification of the ^1H resonances of valine and leucine residues in dihydrofolate reductase by using a combination of selective deuteration and two-dimensional correlation spectroscopy

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Lactobacillus casei dihydrofolate reductase (M_r 18500) contains 16 valine and 14 leucine residues. By comparing the 2D COSY NMR spectra of normal and [γ-$^2H_6$]valine enzyme we have been able to identify all 60 methyl resonances from these residues, and to connect the pairs arising from the same residue. This pairing of the methyl resonances was aided by the examination of the 2D RELAY spectrum which also allowed the $C_\alpha H$ resonances (and hence the complete spin systems) of 14 of the valine residues to be identified. The combination of selective deuteration with 2D NMR techniques is shown to be a powerful general method for resolving ^1H resonances in the complex spectra of proteins and for assigning them to amino-acid type.

Dihydrofolate reductase *Resonance assignment* *2D COSY NMR* *Deuteration*

1. INTRODUCTION

The resolution and assignment of resonances from individual amino-acid residues is a necessary first step in the study of proteins by high-resolution NMR spectroscopy, and it remains the most time-consuming part of such studies, particularly for proteins of $M_r \geqslant 15000$. Resonance assignments proceed in two stages: first, the resonance is resolved and assigned to an amino-acid type, and, second, it is assigned to an individual residue in the sequence.

Two approaches to the first stage of assignment have been used. If the spin system of an amino-acid side chain can be identified, for example, in a two-dimensional COSY experiment [1], the residue type can be determined in many cases. Alternatively, removal of signals from the spectrum by selective deuteration provides a direct method of identifying the type of residue from which they arise. We have earlier described the use of selective deuteration as an aid to ^1H NMR studies of dihydrofolate reductase [2–4]. For a protein of this size (M_r 18500), each of these approaches, when used in isolation, can only provide sufficient spectral resolution to permit a limited number of first-stage assignments.

We now report that a combination of selective deuteration and 2D shift correlation experiments (COSY and RELAY) allows resolution and first-stage assignment of an increased number of resonances. Using this approach, we have identified all 16 pairs of valine methyl resonances and all 14 pairs of leucine methyl resonances in the ^1H spectra of inhibitor complexes of dihydrofolate

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reductase. In addition the RELAY experiment has enabled us to extend these assignments to a large proportion of the $C_\alpha H$ resonances of valine residues in this protein.

2. MATERIALS AND METHODS

DL-[γ-$^2H_6$]Valine was synthesized using the method described for unlabelled valine [5] incorporating the method of Cook et al. [6] for the preparation of the starting material, 2-mercaptothiazol-5-one. *Lactobacillus casei* MTX/R was grown on a defined medium containing DL-[γ-$^2H_6$]valine and a mixture of the other 19 amino acids. Isolation and purification of dihydrofolate reductase were carried out as described in [7].

500 MHz ^1H NMR spectra were obtained on a Bruker AM500 spectrometer using samples of [γ-$^2H_6$]valine dihydrofolate reductase (0.5 mM) and of isotopically normal enzyme (4 mM) in ^2H_2O containing 500 mM KCl and 50 mM potassium phosphate at pH 6.5. The ligands trimethoprim and NADPH were added as 1.1–1.3 molar equivalents to ensure complete complexation of the enzyme. Dioxan (1 mM final concentration) was added as a chemical shift reference.

2D NMR experiments, including absolute value mode COSY and RELAY experiments, were obtained with a 16-step phase cycle to select N-type peaks and to suppress quadrature images and axial peaks [8]. The RELAY experiment [9] using a $(90–t–90–\tau_m/2–180–\tau_m/2–90–d)_n$ pulse sequence was used with a mixing time τ_m = 30 ms, optimized according to Bax and Drobny [10] for an AMQ_3X_3 spin system (JMQ = JMX ≃ 7 Hz, JAQ = JAX ≃ 0).

Both experiments were carried out with the carrier frequency placed at the centre of the spectrum using a spectral width of 6410 Hz and quadrature detection in both dimensions. In the lower resolution experiment (12.8 Hz/point) data were recorded as 1024 points in t_2 for each of 256 t_1 values. For higher resolution spectra (6.4 Hz/point) the number of data points was increased to 2048 in t_2 for each of 450–512 t_1 values. A relaxation delay (d) of 0.8–1.0 s was incorporated into each pulse sequence and the number of scans per FID was 160–256, depending on the available time. The total accumulation time for a single 2D spectrum was in the range 15–24 h in both COSY and RELAY experiments. The data were zero-filled to 512–1024 points in t_1 and multiplied by a sine bell squared window function before Fourier transformation.

3. RESULTS AND DISCUSSION

Selective deuteration provides an unambiguous method for assigning resonances by residue type in the ^1H NMR spectrum of a protein. As reported previously [4], [γ-$^2H_6$]valine has been incorporated into *L. casei* dihydrofolate reductase enabling the $C_\gamma H_3$ resonances of valine residues in the isotopically normal enzyme to be identified by difference spectroscopy. Fig.1 illustrates the high-field region of the ^1H NMR spectra of the complexes of trimethoprim with isotopically normal dihydrofolate reductase and with the [γ-$^2H_6$]valine enzyme. The difference spectrum contains only the 32 methyl resonances from the 16 valine residues present in the enzyme. In this complex, methyl resonances from two valine residues, V_A (Val 61)

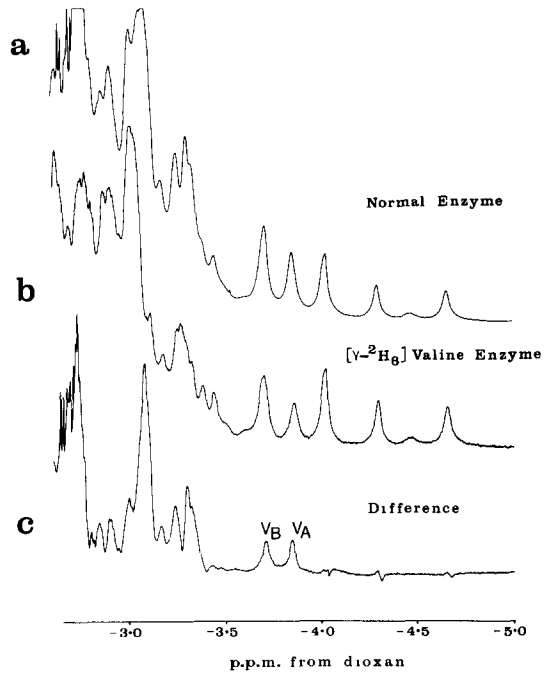

Fig.1. High-field region of the 500 MHz ^1H NMR spectra of (a) the dihydrofolate reductase-trimethoprim complex and (b) the corresponding complex of the [γ-$^2H_6$]valine-containing enzyme. (c) The difference spectrum, (a) – (b).

and V_B (Val 110 or 115; M.S. Searle, unpublished), are shifted to high field by the magnetic shielding effects of nearby aromatic rings, and only these two valine resonances can be individually resolved, even with the aid of the deuterated enzyme. The large group of valine methyl resonances between −2.5 and −3.4 ppm remains unresolved (fig.1). Thus in this case selective deuteration alone has not allowed us to make first-stage assignments for all the valine signals.

In the COSY spectrum of the isotopically normal enzyme-trimethoprim complex (fig.2A), valine and leucine residues can be identified by pairs of cross-peaks, corresponding to the two methyl groups of each residue, that are connected to a common ($C_\beta H$ or $C_\gamma H$) ω_1 frequency in the 2D matrix. For example, in fig.2A, two methyl resonances at −3.90 and −3.38 ppm give cross-peaks with a common ω_1 frequency of −2.20 ppm, and they thus arise from the same valine or leucine residue (in this case valine V_A; see below). Two kinds of ambiguity arise in interpreting this spectrum. First, in crowded regions of the spectrum where a number of different methyl resonances are coupled to $C_\beta H$ or $C_\gamma H$ resonances with very similar chemical shifts it may be difficult to 'pair up' the two methyl resonances from the same valine or leucine residue with certainty. Second, valine and leucine resonances cannot be distinguished by examination of the $-CH(CH_3)_2$ part of their spin systems at the resolution attainable for most protein COSY spectra, and the complexity of the region of the spectrum containing the $C_\beta H$ and $C_\gamma H$ resonances makes it very difficult to trace the spin systems any further in the COSY spectrum.

A combination of 2D correlation spectra with selective deuteration can be of considerable value in resolving these ambiguities. Because of the relatively high cost of preparing selectively deuterated enzymes, they are generally only available in small quantities, while the 2D NMR experiments are generally of rather low sensitivity, and thus require high-concentration samples. However, we have found that satisfactory COSY spectra can be obtained in 24 h from [γ-$^2H_6$]valine enzyme at a concentration of only 0.5 mM. The high-field part of this spectrum is shown in fig.2B. The high concentration of the sample used for fig.2A allowed us to record a relatively high resolution COSY spectrum; this is necessary in order to resolve all the cross-peaks in this region of the spectrum. The lower concentration of the [γ-$^2H_6$]valine enzyme sample meant that only a lower resolution COSY spectrum could be obtained. However, as can be seen from fig.2, this is quite adequate to establish which cross-peaks are present in both spectra and which are present only in fig.2A.

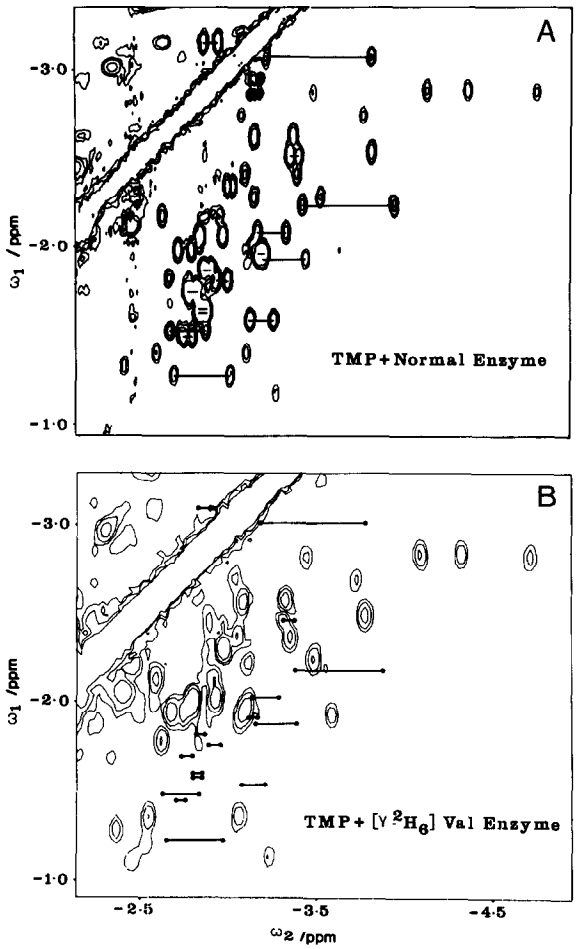

Fig.2. The high-field region of the 500 MHz ^1H 2D COSY spectra of (A) the dihydrofolate reductase-trimethoprim complex and (B) the corresponding complex of the [γ-$^2H_6$]valine containing enzyme. The pairs of $C_\gamma H_3$-$C_\beta H$ cross-peaks from valine residues identified by comparison of these two spectra (see text) are connected by horizontal lines in (A); these lines are also included in (B), the dots at either end showing the positions of the valine cross-peaks present in (A) but missing in (B).

We can thus identify all the cross-peaks arising from valine residues. For example, the pair of methyl cross-peaks with a common ω_1 frequency of -2.20 ppm is clearly missing in fig.2B, and thus must arise from a valine residue (V_A), while the pair with a common ω_1 frequency of -2.58 ppm is still present in fig.2B and must arise from a leucine (L_D). The valine methyl signals are indicated in fig.2 by horizontal lines linking the cross-peaks from the two methyl groups of the same residue. In the few cases where the $C_\beta H$ chemical shifts of two or more residues are similar, it is sometimes difficult to pair up the methyl resonances by comparison of the COSY spectra of the normal and deuterated enzyme.

The resolution of ambiguities in identification of methyl resonances arising from the same residue is one useful application of the homonuclear relayed coherence transfer (RELAY) experiment [9–13]. In this experiment cross-peaks are observed not only between pairs of protons that are scalar coupled but also between two protons that each have a scalar coupling interaction with a third nucleus. The spectrum from an AMX spin system will thus give rise to an AX cross-peak even when the spins A and X are not directly coupled. This experiment thus allows relayed shift correlation to be observed between the two $C_\gamma H_3$ resonances of the same valine residue. This is illustrated in fig.3, which shows the RELAY spectrum of the enzyme-trimethoprim-NADPH complex, with the cross-peaks of valine V_A (Val 61; M.S. Searle, unpublished) connected by dashed lines. The two cross-peaks at ω_1 -2.39, ω_2 -3.94 ppm and ω_1 -2.39, ω_2 -3.38 ppm, which are also observed in the COSY spectrum, have been identified as two valine $C_\gamma H_3$-$C_\beta H$ cross-peaks. Although they have a common ω_1 frequency, there are other cross-peaks with very similar ω_1 frequencies in the spectrum of this complex, and one cannot immediately be certain that the two indicated $C_\gamma H_3$-$C_\beta H$ cross-peaks do arise from the same residue. However, a RELAY cross-peak (not observed in the COSY spectrum) is observed at ω_1 -3.38, ω_2 -3.94 ppm; this $C_{\gamma 1}H_3$-$C_{\gamma 2}H_3$ cross-peak is unambiguous evidence that the two methyl resonances at -3.38 and -3.94 ppm do indeed arise from the same residue. Thus the RELAY experiment clearly provides a valuable aid to the pairing of the methyl cross-peaks in the COSY spectrum.

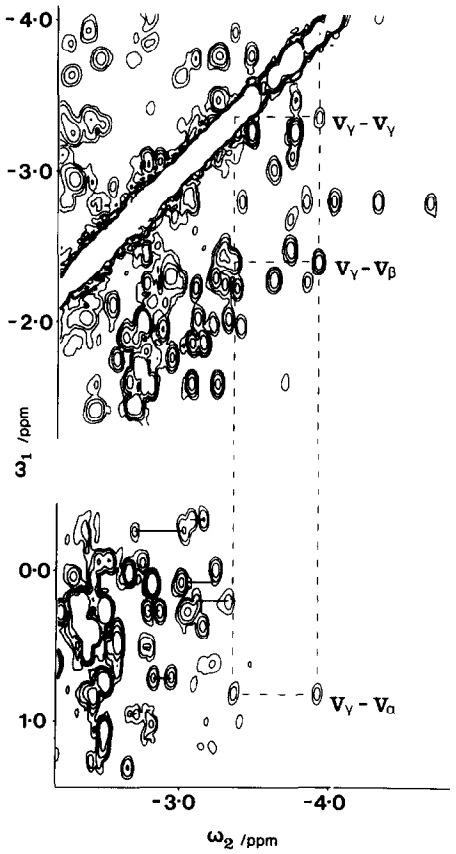

Fig.3. The high-field region of the 500 MHz ^1H 2D RELAY spectrum of the dihydrofolate reductase-trimethoprim-NADPH complex. The cross-peaks defining the spin system of valine V_A (Val 61) are connected by dashed lines; for details, see text. In the lower part of the figure, the pairs of valine $C_\gamma H_3$-$C_\alpha H$ RELAY cross-peaks are connected by horizontal lines.

The combination of the RELAY spectrum with the COSY spectrum of the [γ-$^2H_6$]valine enzyme has allowed us to identify all 32 valine methyl resonances in the spectrum of dihydrofolate reductase and to connect each pair originating from the same residue. By elimination, the remaining pairs of methyl cross-peaks must arise from leucine residues, and in this way all 28 leucine methyl resonances have been identified, and similarly connected in pairs. The chemical shifts of these valine and leucine resonances are summarised in table 1.

The sheer complexity of the spectrum of a protein of this size makes it impossible to trace the valine spin system step-wise from the $C_\gamma H_3$

Table 1

Chemical shifts (ppm from internal dioxan) of valine and leucine resonances of the dihydrofolate reductase-trimethoprim complex

Valine[a]	$C_\gamma H_3$	$C_\beta H$	Leucine[a]	$C_\delta H_3$	$C_\gamma H$
V_A (V61)	−3.90, −3.38	−2.20	L_A (L113)	−4.70, −3.43	−2.83
V_B (V115/V110)	−3.76, −3.16	−3.03	L_B (L118)	−4.31, −4.08	−2.84
V_C (V110/V115)	−2.89, −2.81	−3.12	L_C (L23)	−3.72, −3.03	−2.70
V_D	−3.35, −3.31	−2.48	L_D (L62)	−3.32, −3.11	−2.58
V_E	−3.28, −3.12	−2.05	L_E (L131)	−3.77, −3.31	−2.50
V_F	−3.40, −3.15	−1.90	L_F (L19)	−3.34, −3.05	−2.37
V_G	−3.15, −3.12	−1.92	L_G (L54)	−3.48, −3.11	−2.23
V_H	−2.86, −2.81	−1.84	L_H	−2.98, −2.95	−2.31
V_I	−2.95, −2.89	−1.78	L_I	−2.91, −2.59	−2.13
V_J	−2.78, −2.73	−1.71	L_J	−2.92, −2.80	−2.02
V_K	−2.83, −2.79	−1.60	L_K	−2.75, −2.68	−1.94
V_L	−2.83, −2.79	−1.61	L_L (L27)	−3.09, −3.14	−1.95
V_M	−3.21, −3.08	−1.55	L_M	−2.82, −2.62	−1.79
V_N	−2.83, −2.62	−1.50	L_N (L4)	−3.06, −2.54	−1.37
V_O	−2.75, −2.70	−1.47			
V_P	−2.97, −2.64	−1.25			

[a] Valine and leucine residues are denoted V_A, V_B, ... and L_A, L_B, ..., respectively; where a specific assignment to an individual residue is known it is given in parentheses ([15]; M.S. Searle, unpublished)

through the $C_\beta H$ to the $C_\alpha H$ resonance in the COSY spectrum. However, by supplementing the COSY experiment with a RELAY experiment, correlations can be extended directly from the $C_\gamma H_3$ to $C_\alpha H$ resonances for this residue. This is illustrated in fig.3 for Val 61; as shown by the dashed lines, RELAY cross-peaks are observed between the methyl signals at −3.94 and −3.38 ppm and the $C_\alpha H$ resonance at 0.86 ppm. The observation of RELAY cross-peaks for the valine residues is greatly aided by the selectivity of this experiment. The spectrum in fig.3 was obtained with τ_m = 30 ms, which is optimum for AMX_3 (threonine) and AMQ_3X_3 (valine) spin systems (with $T_2 \sim$ 20 ms) [10,11], and in fact we find that RELAY cross-peaks are seen *only* for these residues. A similar observation has recently been reported [11] on the *cro* protein. From the RELAY spectrum, the pattern of connectivities to the $C_\alpha H$ (and hence the complete spin system) has been traced for 14 of the 16 valine residues of dihydrofolate reductase; some of the pairs of $C_\gamma H_3$-$C_\alpha H$ RELAY cross-peaks are connected by solid horizontal lines in the lower part of fig.3. The low intensity of these RELAY cross-peaks for two of the valine residues could readily be accounted for if the $C_\alpha H$-$C_\beta H$ coupling constant were small (i.e. if the conformation about the C_α–C_β bond were g^- or t) in these residues. In a statistical survey of side-chain conformations in proteins [14], it was noted that valine residues show a strong preference for the g^+ conformation, in which the C_α and C_β hydrogens are *trans*. One would therefore expect that $C_\gamma H_3$-$C_\alpha H$ RELAY cross-peaks should be observed for the majority of valine residues in proteins.

The experiments reported here show that an appropriate combination of selective deuteration with 2D NMR methods allows the resolution and first-stage assignment of the resonances from a substantial proportion of the aliphatic residues in a protein having M_r 18500. It seems likely that further experiments of this kind with different selectively deuterated enzymes could complete the first-stage assignment of the spectrum. In view of the crucial importance of selective deuteration in extending these assignment procedures to larger proteins, it is encouraging that an adequate COSY spectrum can be obtained with only 3 mg (160 nmol) of protein. The method could be im-

proved by using COSY difference spectra, but this would require a somewhat better signal-to-noise ratio for the spectrum of the deuterated enzyme. Once the first-stage assignments have been made, subsequent assignment to individual residues in the sequence depends largely on NOE experiments. Using this approach resonances from some 20% of the residues of dihydrofolate reductase have been assigned [4,15], allowing a detailed comparison of the binding of different ligands, and a description of the conformational changes they produce.

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REFERENCES

[1] Wagner, G., Kumar, A. and Wuthrich, K. (1981) Eur. J. Biochem. 114, 375–384.
[2] Feeney, J., Roberts, G.C.K., Birdsall, B., Griffiths, D.V., King, R.W., Scudder, P. and Burgen, A.S.V. (1977) Proc. Roy. Soc. Lond. B 196, 267–290.
[3] Feeney, J., Roberts, G.C.K., Thomson, J., King, R.W., Griffiths, D.V. and Burgen, A.S.V. (1980) Biochemistry 19, 2316–2321.
[4] Birdsall, B., Feeney, J., Griffiths, D.V., Hammond, S., Kimber, B.J., King, R.W., Roberts, G.C.K. and Searle, M. (1984) FEBS Lett. 175, 364–368.
[5] Billimoria, J.D. and Cook, A.H. (1949) J. Chem. Soc., 2323–2329.
[6] Cook, A.H., Heilbron, I. and Levy, A.L. (1948) J. Chem. Soc., 201–206.
[7] Dann, J.G., Ostler, G., Bjur, R.A., King, R.W., Scudder, P., Turner, P.C., Roberts, G.C.K., Burgen, A.S.V. and Harding, N.G.L. (1976) Biochem. J. 157, 559–571.
[8] Wider, G., Macura, S., Kumar, A., Ernst, R.R. and Wuthrich, K. (1984) J. Magn. Reson. 56, 207–234.
[9] Eich, G., Bodenhausen, G. and Ernst, R.R. (1982) J. Am. Chem. Soc. 104, 3732.
[10] Bax, A. and Drobny, G. (1985) J. Magn. Reson. 61, 306–320.
[11] Weber, P.L., Drobny, G. and Reid, B.R. (1985) Biochemistry 24, 4549–4552.
[12] Wagner, G. (1983) J. Magn. Reson. 55, 151–156.
[13] King, G. and Wright, P.E. (1983) J. Magn. Reson. 54, 328–332.
[14] Janin, J., Wodak, S., Levitt, M. and Maigret, B. (1978) J. Mol. Biol. 125, 357–386.
[15] Hammond, S., Birdsall, B., Searle, M.S., Roberts, G.C.K. and Feeney, J. (1985) submitted.