STRATEGIES FOR SPECTRAL ASSIGNMENT IN THE ^{1}H NMR SPECTRA OF A 25 000 M_r MURINE ANTIBODY FRAGMENT: (i) in vivo deuteration and (ii) use of a denaturant

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1. Introduction

The Fv fragment of M315 is a murine antibody fragment of M_r 25 000, which possesses high affinity for nitroaromatic haptens. It has been studied extensively by high resolution ^{1}H NMR in an attempt to understand the interactions and perturbations on antigen binding [1–3]. Large upfield changes in chemical shift of the hapten resonances were used to deduce a structure for the combining site. However, because no assignments, other than histidines, had been made, data on the changes in chemical shift of antibody resonances could only be used in a very qualitative way to state that different DNP and TNP haptens bind very similarly to the protein.

In a protein of this size spectral assignment is extremely difficult because no spin–spin coupling is visible. This results from the large linewidths and the overlap of many resonances. The most satisfactory spectral simplification is to replace selected amino acids by deuterated analogues, since, unlike, e.g., ^{19}F substitution, the resulting chemically modified system can be described realistically as unperturbed.

Extensive rather than specific deuteration to simplify the ^{1}H NMR spectrum has been widely applied [4–8]. These studies have, however, all been confined to bacterial proteins, where a reasonably high yield of the desired protein can be expected. We report here the first instance of specific deuteration in a mammalian system. Tryptophan deuterated at all 5 aromatic ring protons is incorporated into the Fv fragment of M315 by feeding the mice on a diet in which tryptophan is replaced by its deuterated analogue. This amino acid was chosen because it is an essential one, it can be deuterated relatively easily and it is believed to be a hapten contact residue [1].

As a second potential aid to spectral assignment of the ^{1}H NMR spectrum of M315 Fv, we report the effects of the denaturant guanidine–HCl on the spectrum. Denaturation of antibodies occurs at ~2 M guanidine–HCl [9]. However, at much lower concentrations, this denaturant (and also urea) acts as an inhibitor of antigen–antibody precipitation [10, 11]. This has been interpreted as preferential binding of the denaturant in the combining site. We make use of this finding to perturb selective resonances from residues in or near to the combining site.

Abbreviations: DNP, 2,4-dinitrophenyl; Fv fragment, variable region of light and heavy chains; NMR, nuclear magnetic resonance; ppm, parts per million; TNP, 2,4,6-trinitrophenyl

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2. Materials and methods

2.1. *Deuterated tryptophan*

L-Tryptophan, deuterated at all 5 aromatic proton positions, was prepared as in [12]. The 4 water-exchangeable protons of L-tryptophan were initially deuterated with 99.8% ^2H_2O and the tryptophan extracted before reaction with 98% CF_3COO^2H at 310 K for 8 h. The degree of incorporation at each position was determined from the integrated area of the residual ^{1}H NMR resonances at 270 MHz. Four cycles of deuteration, with removal of $CF_3COO^2H/^1H$ and addition of fresh CF_3COO^2H to the viscous residue, resulted in 91–95% incorporation of deuterium.

2.2. *Preparation of antibody fragments*

Six-week old BALB/c D_2CF_1 mice were fed on the synthetic diet given in table 1. The protein component

Table 1
Synthetic diet (a) containing tryptophan- and lysine-deficient protein (zein) and supplemented with L-lysine and deuterated L-tryptophan

(a)	Zein	20%
	Sucrose	25%
	Maize starch	38%
	Arachis oil	10%
	Minerals[a]	5%
	Vitamins[b]	1%
	L-Lysine	1%
	d_5-Tryptophan	0.2%

[a] see (b); [b] see (c)

(b)	Sodium chloride	104 g	(c) Calcium pantothenate	1.2 g
	Magnesium sulphate	166 g	Pyridoxine	0.5 g
	Disodium hydrogen phosphate	204 g	Riboflavin	0.5 g
	Potassium dihydrogen phosphate	272 g	Thiamine	1.0 g
	Calcium hydrogen phosphate	324 g	Nicotinic acid	1.0 g
	Calcium lactate	789 g	Folic acid	0.1 g
	Ferric citrate	2 g	*p*-Amino benzoic acid	1.0 g
	Potassium iodide	200 g	Biotin	0.02 g
	Cupric sulphate	2 g	Inositol	20 g
	Manganous chloride	2 g	Glucose	974 g

Water was added to give a very stiff doughlike consistency. Constituents are given as percentage of dry weight

of this is the maize-derived substance zein, which is deficient in tryptophan and lysine [13,14] and could therefore be supplemented with L-tryptophan deuterated at all 5 ring positions. The absence of tryptophan from the commercial zein was confirmed by the appearance of the 270 MHz ^{1}H NMR spectrum in 8 M deuterated urea. During the last 4 weeks of feeding the MOPC315 tumour was induced and ascites fluid collected at 3 day intervals after the initial draining, which was 10 days after induction [15]. Fv containing deuterated tryptophan was produced from this ascites in the same way as Fv from normal ascites [16].

2.3. *^{1}H NMR*

Spectra were recorded in the Fourier transform mode at 270 MHz or 470 MHz on the Oxford Enzyme Group instruments. Residual intensity due to solvent water protons was removed by a selective saturating pulse of 0.4 s duration applied before the non-selective pulse and with a delay of 0.8 ms between the pulses to minimise breakthrough.

2.4. *Other materials*

CF_3COO^2H and ^2H_2O were purchased from Ryvan Chemical Co. Southampton. L-Tryptophan and zein were obtained from BDH, Poole, Dorset. Guanidine–HCl was purchased from BDH and deuterated prior to addition to antibody by dissolution in ^2H_2O and freezedrying. This process was performed twice.

3. Results

3.1. *Production of deuterated tryptophan Fv*

Enrichment of the IgA myeloma M315 with deuterotryptophan was achieved by exogenous administration of deuterotryptophan into the tryptophan-deficient diet at the same time as the tumour was implanted. Ascites fluid was drained over the following period from 12–30 days. The Fv fragment was prepared in the normal way. The extent of incorporation of deuterated tryptophan into Fv was estimated to be ~50%. This was calculated from a comparison of the integrated areas of the ^{1}H NMR spectra of deuterated and normal Fv samples following digestion with pepsin at 37°C and pH 5 for 48 h. Since identification of tryptophan resonances in the intact Fv fragment involves noting those resonances which are absent when compared with the non-deuterated protein, a period of 50 days feeding with the deuterated diet prior to tumour induction was used to increase the incorporation. Only a small amount of protein was obtained in this experiment and a deuterium analysis was not performed, but the extent of incorporation, as estimated from the difference spectra, was >50%. This sample was used for subsequent NMR experiments described here.

3.2. *^{1}H NMR spectra*

In fig.1 the aromatic regions of normal and deuterated Fv are compared. The main differences in intensity are in the region 6.9–7.7 ppm (which compares with the free positions for tryptophan aromatic protons of 7.2–7.7 ppm) and at 8.5 ppm, where there is a decrease in the deuterated sample. Importantly,

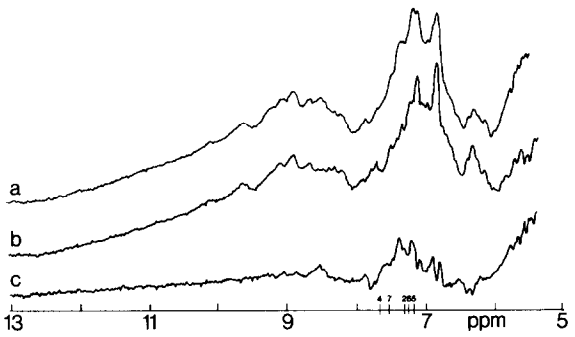

Fig.1. Aromatic region 470 MHz ^{1}H NMR spectra of (a) normal Fv and (b) Fv with deuterated tryptophan residues. (c) = (a) – (b). The free positions of L-tryptophan aromatic resonances are shown numbered. Spectra were recorded at 310 K ± 1 K. Sweep-width was 8000 Hz, collected in 8192 data points. Repetition time was 2.5 s with solvent suppressed by a selective saturating pulse between the end of observation and the next 90° pulse. Spectra are the average of 512 transients. Quadrature detection was employed. Protein concentrations were 0.8 mM and pH* 6.95.

there is no decrease in the region of highfield-shifted resonances at 6.1–6.5 ppm, which is invariably and strongly perturbed by hapten binding [2]. A small sharpening in the deuterated sample spectrum at 6.3 ppm may be due to a reduction in the efficiency of relaxation for this proton arising from substitution of a deuteron for a proton in a proximal tryptophan residue. The difference spectra obtained after addition of DNP–L-aspartate to both deuterated and non-deuterated Fv are shown in fig.2 and show many similarities, with the differences being mainly in the same regions as in fig.1.

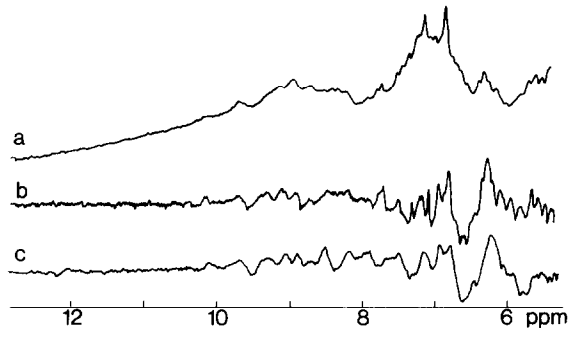

Fig.2. 470 MHz ^{1}H NMR spectrum of deuterated Fv (a) as in fig.1(b) and the difference spectrum after addition of 1:1 DNP–aspartate (b). (c) is the difference spectrum between normal Fv and DNP–aspartate at 470 MHz for comparison.

3.3. *Addition of guanidine–HCl*

Deuterated guanidine–HCl was added in aliquots to Fv up to 2 M. Initially this results in a gradual alteration of the ^{1}H NMR spectra of Fv until near 2 M, when a marked transition to a random coil-type spectrum occurs. This also coincides with significant precipitation of the protein. The most striking changes (fig.3) are to the C(2) protons of His 102_H and 97_L, which have pK_a-values of 6.9 and 5.9, respectively [1]. Both resonances move continuously to lower field, consistent with a decrease in their pK_a-values. Such an increase would result in conversion of more of the molecules to the protonated form at this pH. The greater change in chemical shift for His 97_L (compared with His 102_H) is due to a larger fraction existing as the uncharged form at pH 6.9 in the absence of guanidine–HCL. Both the continuity of the change and its direction suggest an ionic strength effect on surface residues, which is consistent with the proposed structure of M315 Fv [1] in which both of these histidine side chains are accessible to solvent. (A third histidine residue, 44_L, is also located near the surface of the fragment, but has pK_a 8.2 and is least affected, with only a very small shift to low field discernible over this range of denaturant concentrations. This is in agreement with the above hypothesis.)

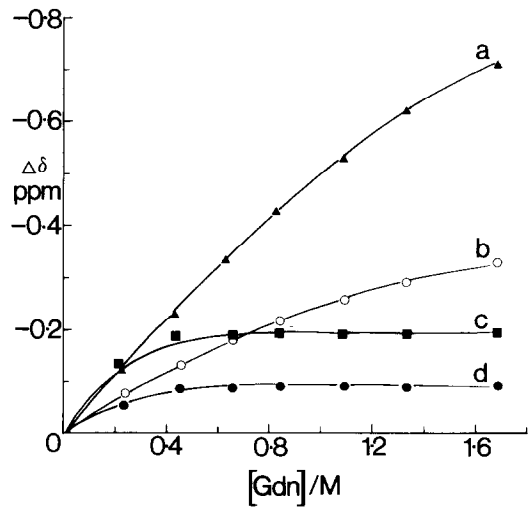

Fig.3. Changes in chemical shift (Δδ) in ppm of 4 resonances in Fv upon addition of guanidine–HCl (Gdn.). (a) and (b) are the C(2) protons of histidines 97_L and 102_H, respectively, which have normal pK_a-values of 5.9 and 6.9 in native Fv. Resonance (c) originates at 7.30 ppm and (d) at 6.19 ppm in native Fv.

In marked contrast is the behaviour of two resonances originating at 6.19 ppm and 7.30 ppm, both of which are shifted downfield upon addition of the first 2 aliquots of guanidine–HCl, but do not subsequently titrate. Apart from the histidine C(2) and C(4) protons (not shown) of residues 97_L and 102_H, these two resonances are the only obvious features in the aromatic region difference spectra up to 0.4 M guanidine–HCl.

4. Discussion

These preliminary experiments show that incorporation of deuterotryptophan by mice into the myeloma protein M315 is feasible. Increasing the length of time of feeding the mice with the special diet containing the deuterotryptophan should lead to greater incorporation. However, the levels of deuteration achieved here are sufficient to illustrate that resonances from one or more tryptophan residues, with protons which have chemical shifts of 8.5 ppm and of between 7.1 and 7.7 ppm, appear in the hapten difference spectrum of Fv 315. The resonance at 8.5 ppm is in the region commonly found for amide protons. However, the invariance of the Fv spectrum up to 1 year after dissolution in ^2H_2O and the reproducibility of the DNP–L-aspartate – non-deuterated Fv difference spectrum make it most unlikely that the absence of a peak at 8.5 ppm for the deuterated Fv is due to different degrees of amide exchange for the two samples. This is consistent with the proposed model, based on extensive NMR, UV and fluorescence measurements [1,2], which has Trp 93_L in the binding site. This residue plays a major role in the binding of DNP ligands; the indole ring forming a stacked complex with the DNP ring. Several of the resonances between 7.1–7.7 ppm are in fact shifted upfield on hapten binding (Leatherbarrow and Dwek, unpublished). This would also be consistent with the expected perturbations on the protons of Trp 93_L from the DNP ring.

The deuteration result can also be used to show which protons do not arise from tryptophan residues. In particular the group of resonances between 6.1–6.5 ppm, which is strongly perturbed on hapten binding, is present in the deuterated sample. These resonances must arise from either phenylalanine or tyrosine residues. The second method of assignment is of assistance in confirming this. This involves monitoring the effects on the ^1H NMR spectrum of Fv on titrating in guanidine–HCl. Apart from perturbing the histidine residues, the most noticeable features are specific effects on the resonances from only two (groups of) protons which are at 6.19 and 7.30 ppm, suggesting that no general conformational change occurs at these levels of denaturant. Since the denaturant is thought to bind at low concentrations in the antibody combining site [10,11], these results suggest that the proton resonances at these positions arise from a residue in the site. That these resonances are perturbed in step makes it probable that they arise from the same residue – possibly a tyrosine, which if flipping rapidly would give 2 resonances each of an intensity corresponding to 2 protons. Further support for assignment to a tyrosine residue comes from the difference spectrum between Fv and Fv in which Tyr 34_L has been nitrated. This shows a reduction in intensity at 6.20 ppm and possibly at ~7.2 ppm (Leatherbarrow and Dwek, unpublished). The current model of the combining site of M315 has Tyr 34_L positioned as a contact residue on the periphery of the DNP subsite (Leatherbarrow and Dwek, unpublished). We therefore assign these resonances to the aromatic protons of Tyr 34_L.

Clearly a combination of methods such as described here may have more general application in the assignment of proton resonances in the NMR spectra of large proteins.

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