Role of Tyrosines in the Combining Site of the Dinitrophenyl-Binding IgA Myeloma M315: Specific Nitration and High-Resolution Hydrogen-1 Nuclear Magnetic Resonance Studies†

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ABSTRACT: The binding site of the dinitrophenyl (Dnp) binding IgA myeloma M315 contains two tyrosines, Tyr-34_L and Tyr-33_H. These have been selectively nitrated. The pK_a values of the nitrotyrosine proteins alone and in the presence of a variety of haptens suggest that Tyr-33_H is close to the α-CH_2 side chains of the ligands—as originally proposed in the model of the combing site [Dwek, R. A., Wain-Hobson, S., Dower, S., Gettins, P., Sutton, B., Perkins, S. J., & Givol, D. (1977) *Nature (London) 266*, 31–37]. In contrast, pK_a values for the NO_2-Tyr-34_L protein are only affected by haptens with long flexible side chains, suggesting that Tyr-34_L may not be a contact residue of the Dnp ring in the manner originally proposed. High-resolution ^{1}H NMR studies show that the effects of nitration of either tyrosine on the protein structure are probably localized to the immediate environment of the nitro group. Further, the mode of binding of Dnp-glycine, as judged from the large upfield shifts on the hapten protons and the similarities of the 470-MHz ^{1}H protein difference spectra, is virtually unchanged in the Fv, NO_2-Tyr-34_L Fv, and NO_2-Tyr-33_H Fv fragments. Ring-current calculations using the new empirical factors for Trp residues [Perkins, S. J., & Dwek, R. A. (1980) *Biochemistry 19*, 245–258] show that the shifts on Dnp-glycine can be accounted for without any contribution from Tyr-34_L. The revised model of the combining site of M315, with only minimal adjustments of the positions of protein side chains, can account for the experimental data from a large range of haptens.

It is now well established that the structure of immunoglobulin domains are very similar. The basis for this similarity is the conservation of a framework structure—the immunoglobulin fold (Poljak et al., 1973; Davies et al., 1975a,b; Edmundson et al., 1975; Epp et al., 1975). Similarity is particularly marked when equivalent domains are compared (Padlan & Davies, 1975). We assumed that it would be possible to predict the structure of an immunoglobulin domain by aligning its amino acid sequence with that of an equivalent domain of known structure (Poljak et al., 1974; Padlan et al., 1976). The resulting model may then be tested against physiochemical data and modified accordingly (Dwek et al., 1977). If validated such a procedure provides an important complementary approach to crystallographic studies and is of particular use for immunoglobulins which cannot be crystallizied or for which high-resolution X-ray data cannot be collected.

The determination by X-ray crystallography of the structures of several antibody combining sites provides the essential starting point for the detailed understanding of the diversity of antigen binding sites. Spectroscopic techniques are not capable of providing the extensive structural information given by X-ray diffraction. The power of spectroscopic methods lies in their ability, in conjunction with the knowledge of structures determined by X-ray crystallography or model building, to define the nature of the interactions which are responsible for the specificity of the protein.

Our approach to studying combining sites has mainly involved using high-resolution NMR[1] (Dwek et al., 1977; Dower & Dwek 1979a,b; Gettins et al., 1981a). The very detailed studies we have undertaken have been necessary to make as rigorous as possible the NMR interpretations in establishing the general procedure of using physical data to refine predicted antibody sites.

In this context we have sought to test the structural conclusions obtained from NMR studies for proteins whose structures are well-defined by X-ray crystallography. The most important advance in this process has been the development of empirical ring-current factors to account for the chemical shifts produced by Trp rings (Perkins & Dwek, 1980).

We have recently used these factors as part of an NMR study (Gettins et al., 1982) in which the interactions, between hapten and proteins in the combining site of the phosphorylcholine binding protein M603, were found to be consistent with those suggested from the X-ray structure. This indicates that, in principle, the NMR parameters can be used to provide structural information on antibody combining sites.

The main test case of the spectroscopic and combined model building approach to studying antibody combining sites is clearly the Dnp binding IgA myeloma M315, which is probably the myeloma protein most studied by physical techniques and for which crystal data will soon be available (Aschaffenburg et al., 1979). This will allow a critical assessment of the techniques and also of the assumptions in the interpretation of the data from them. If, indeed, the immunoglobulin fold is conserved as we have assumed, and the deductions from the NMR experiments are valid, our approach may provide a general method for studying any antibody combining site.

According to our postulated model of the combining site of M315, the main interaction with the Dnp hapten is a stacking one between the Dnp ring and Trp-93_L. This is carried out in a highly aromatic environment involving Phe-34_H, Tyr-33_H, and Tyr-34_L as potential contact residues (Dwek et al., 1977). In this paper, we use the new ring-current factors

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[1] Abbreviations: NMR, nuclear magnetic resonance; Dnp, 2,4-dinitrophenyl; IgA, immunoglobulin A; Fv fragment, immunoglobulin fragment composed of the variable domains of a light chain (L) and a heavy chain (H); V_L, variable domain of a light chain; NO_2, nitro; DNNS, 2,4-dinitro-1-naphthol-7-sulfonic acid; Tris, tris(hydroxymethyl)aminomethane; $NaDodSO_4$, sodium dodecyl sulfate.

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for Trp residues to assess what changes this could make to a proposed structure of the binding site. We support our conclusions by studies on M315 in which Tyr-34_L and Tyr-33_H have been specifically nitrated.

Materials and Methods

The Fv fragment of protein 315 was prepared as described by Inbar et al. (1972). Solutions of this or the nitrated species for the NMR experiments were prepared by dissolving the freeze-dried protein in ^2H_2O (99.8% ^2H; Ryvan Chemical Co., Ltd., Southampton, U.K.).

Nitration of Tyr-33_H. This was performed by reacting the Fv fragment (4×10^{-5} M) in 0.1 M Tris-HCl, pH 8.2, with TNM (2.8×10^{-4} M) for 1 h at room temperature. The reaction was terminated by passing the mixture down a Dnp-lysine–Sepharose column. Bound protein was eluted with 0.05 M NH_3 or with Dnp-glycine (12.5 mg/mL; pH 7). Approximately 30% of the material did not bind to the column. Both the active and inactive fractions were subjected to Na-DodSO$_4$–polyacrylamide gel electrophoresis. The active material was indistinguishable from the Fv fragment, whereas the inactive material clearly contained covalent aggregates (results not shown) as is frequently observed in nitration of tyrosine residues by this method (Doyle et al., 1968; Boesel & Carpenter, 1970; Vincent et al., 1970). The above conditions gave a modification of 1 mol of nitrotyrosine per mol of Fv fragment, assuming $E^M_{428} = 4100$ for nitrotyrosine at pH 10 (Riordan et al., 1967). It has previously been shown that 80% of the modification is on Tyr-33_H and 20% is on Tyr-34_L (R. Zakut and D. Givol, personal communication).

Haptens were obtained from BDH Chemicals Ltd., Poole, U.K., except for Tnp-NH(CH$_2$)$_2$NH$_3^+$ which was a gift from Professor D. Givol (Weizmann Institute, Israel).

Absorbance measurements for the pH titrations were made on a Gilford Model 250 spectrophotometer, and the experimental conditions are given in the appropriate tables. The titration data were fitted to a one-proton titration curve by using a nonlinear least-squares program.

NMR spectra were recorded on either a Bruker 270-MHz spectrometer, a Bruker 300-MHz spectrometer, or the Oxford Enzyme Group 470-MHz spectrometer which uses a Nicolet 1180 computer and an Oxford Instruments magnet.

Model Building. Models of the combining site were constructed with standard Kendrew skeletal components (Cambridge Repetition Engineers) on a scale of 2 cm = 0.1 nm. Coordinates are available on request.

Ring-Current Calculations. Ring-current shifts were calculated as described in Perkins et al. (1977) and Perkins & Dwek (1980).

Results and Discussion

Perturbations of pK_a Values of NO_2-Tyr-33_H and NO_2-Tyr-34_L by Ligands. In a recent paper (Gavish et al., 1979) we described a novel and effective way for selective modification of Tyr-34_L by nitration of the variable region of the light chain and reassociation with the unmodified variable region of the heavy chain. In contrast Tyr-33_H can be selectively nitrated quite straightforwardly, using tetranitromethane, in the intact Fv fragment.

Nitration is a convenient method of modification of tyrosine residues, for several reasons: the modifying group is small, minimizing the possibility of large structural changes; the pK_a of the nitrotyrosine is about 7, allowing observation of the effect of the ionization of the phenolic group; the ionization of the phenolic group itself may be monitored spectrophotometrically, enabling perturbations of the pK_a to be followed;

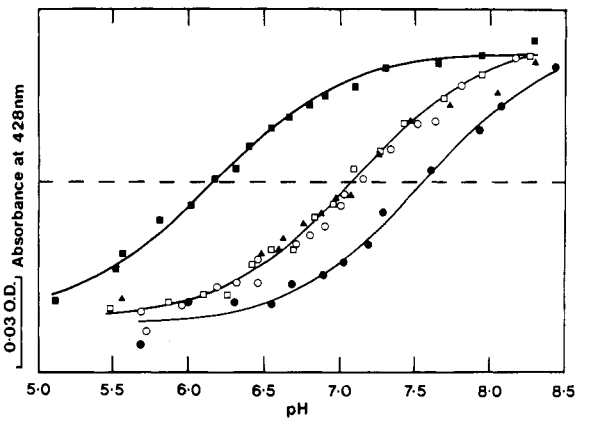

FIGURE 1: pH titrations of NO_2-Tyr-34_L in the NO_2-Tyr-34_L Fv fragment. Measurements were made at T = 293 K with solutions in 0.005 M potassium phosphate and 0.15 M NaCl. Protein concentrations were about 25×10^{-6} M. Ligands were present in a slight molar excess. Since the hapten chromophores ($\lambda_{max} \sim 360$ nm) contribute to the absorbance at 428 nm, the different curves have been plotted by using the same ordinate scale but offset such that the value of the OD_{428} shown by the dotted lines intersects each titration curve at the pK value. This allows direct visual comparison of relative pKs (the value of the OD_{428} at the pK was obtained from fitting the titration data to a one-proton titration curve by using a nonlinear least-squares program). (□) NO_2-Tyr-34_L alone; (■) +Dnp-lysine; (▲) +Dnp-glycine; (●) +Dnp-aminocaproate; (○) +Tnp-glycine.

Table I: pK_a Values and Relative Extinction Coefficients of NO_2-Tyr-33_H and NO_2-Tyr-34_L in the Absence and Presence of Various Nitrophenyl Ligands[a]

	NO_2-Tyr-34_L		NO_2-Tyr-33_H	
ligand	pK_a	rel change in extinction coefficient (428 nm)	pK_a	rel change in extinction coefficient (428 nm)
none	7.1	1.0	7.4	1.0
Dnp-NH_2			7.5	0.9
Tnp-NH_2			7.5	1.15
Dnp-$NHCH_2COO^-$	7.1	0.9	7.5	0.9
Tnp-$NHCH_2COO^-$	7.1	1.0	8.1	1.5
Tnp-$NHCH_2CH_2NH_3^+$			7.7	2.15
Dnp-$NH(CH_2)_4$-$CH(NH_3^+)COO^-$	6.2	0.9	7.0	1.25
Dnp-$NH(CH_2)_5COO^-$	7.5	1.0		

[a] Measurements were made in 0.01 M potassium phosphate and 0.15 M NaCl; T = 293 K. The protein concentrations were (25–50) $\times 10^{-6}$ M.

the nitrotyrosine may be reduced to aminotyrosine, to provide a point for the attachment of other spectroscopic probes if required.

The pH titration behavior of the different NO_2 Fv fragments, monitored at 428 nm, results in values of 7.4 and 7.1 for the pK_a values of NO_2-Tyr-33_H and NO_2-Tyr-34_L. The value for NO_2-Tyr-34_L is slightly higher then reported previously (Gavish et al., 1979).

The effects of various ligands on the pK_a of the nitrotyrosine were determined by using protein concentrations of 25–50 μM, with a slight excess of ligand. Titration curves for NO_2-Tyr-34_L are shown in Figure 1. The titration parameters for all the ligands, in terms of the pK_a and extinction coefficient relative to that of the nitrotyrosine in the uncomplexed NO_2-Fv fragment, are given in Table I.

Neither dinitroaniline nor trinitroaniline affects the ionization of NO_2-Tyr-33_H. Any observed effect may therefore be attributed to the side chain of the ligand. No such effect is seen with Dnp-glycine, but the presence of Tnp-glycine

causes an increase of the pK_a from 7.4 to 8.1 and a concomitant increase of the apparent extinction coefficient of the absorption band. An increase of the pK_a would be expected from the proximity of the negatively charged carboxyl group of the ligand side chain. The difference between the effects of Dnp-glycine and Tnp-glycine may then reflect either a difference in the modes of binding of the Dnp and Tnp rings (Dower et al., 1978) or a steric effect of the 6-nitro group on the conformation of the side chain. That the perturbations are not solely due to simple charge effects is shown by the presence of the positively charged hapten Tnp-aminoethylamine which, although is demonstrably perturbing the nitrotyrosine, causes an increase in the pK_a rather than the expected decrease.

However, regardless of the mechanisms by which the perturbations are mediated, these results are consistent with the proximity of Tyr-33_H to the α-CH_2 side chains of the ligands, as originally proposed in the model of the combining site (Dwek et al., 1977). Since neither Dnp-glycine nor Tnp-glycine alters the pK_a of NO_2-Tyr-34_L, it may be concluded that this residue is more distant from the α-CH_2 group.

The addition of Dnp-lysine reduces the pK_a values of both nitrotyrosine derivatives. Presumably the flexibility in the long side chain can result in interactions involving both the nitrotyrosines. Although the pK_a is reduced, it is difficult to separate the effects of the pertubations from the two charged end groups. However, the increase in pK_a in the presence of Dnp-aminocaproate, which is the same length as Dnp-lysine, is as expected. It is interesting to note that Tyr-34_L can be affinity labeled with *N*-(bromoacetyl)-*N'*-(dinitrophenyl)-ethylenediamine (Haimovich et al., 1972) which is also the same length as Dnp-lysine.

^1H NMR Studies. Specific chemical modification is a potential method of assigning resonances in the ^1H NMR spectra of proteins. The aromatic proton resonances of nitrotyrosine titrate upfield with increasing pH, thus allowing assignments and determination of pK_a values (Snyder et al., 1975). However, for both NO_2-Tyr-34_L Fv and NO_2-Tyr-33_H Fv no resonances, other than those of the histidine C-2(H) and C-4(H) protons, were observed to titrate over the pH range 5–8.

Another approach which may result in assignments for Tyr-34_L and Tyr-33_H resonances is a comparison, between the native and nitrated proteins, of the positions of peaks in the difference spectra produced upon binding of hapten. A careful titration at 470 MHz of all resonances at constant pH (as in Dower et al., 1977) revealed that on the whole the difference spectra are identical, indicating that nitration causes no significant change in the manner of ligand binding. However, it was found that a protein resonance in the Fv fragment, which shifts from 6.40 to 6.74 ppm in the presence of the hapten Dnp-glycine, is absent in the spectrum of the NO_2-Tyr-34_L Fv fragment. This resonance can thus be tentatively assigned to Tyr-34_L. At 270 MHz, a possible assignment of a resonance to Tyr-34_L at ca. 6.2 ppm was made on the basis of the effects of guanidine hydrochloride on the Fv spectrum (Gettins & Dwek, 1981). However, at 470 MHz, there is a marked increase in resolution of the broad spectral region between 6.2 and 6.6 ppm, splitting out more individual resonances which can then be more accurately followed in titrations.

Whatever the assignment problems, the ^1H NMR of native and nitrated proteins can be used to measure the extent of the structural perturbations caused by nitration. These spectra contain only about 1–2% of the original spectral intensity, so that modification of the tyrosines cause little or no perturbation of the protein structure beyond the immediate environment of the nitro group.

The pH titration behavior of the three histidine resonances provides further evidence that any perturbations are very localized. The pH titration curves for both the Tyr-34_L and Tyr-33_H nitrated species were found to be indistinguishable from those of the parent Fv fragments. Two of the histidines (His-102_H, pK_a = 7.1, and His-97_L, pK_a = 5.9; Dwek et al., 1977) are constituents of hypervariable loops which are in contact with the loops containing Tyr-33_H and Tyr-34_L, respectively, and are therefore excellent markers to sense possible structural changes.

The changes on the chemical shifts of Dnp-glycine on binding to the NO_2-Tyr-33_H Fv were determined by appropriate titrations as described in Dower et al. (1977). The fully bound shifts (in ppm) are the following: H(3), 1.3; H(5), 2.2; H(6), 1.4; and the CH_2, 0.7. These are very similar to the values found for NO_2-Tyr-34_L Fv (Gavish et al., 1979) and native Fv (Dower et al., 1977) and again indicate that the mode of binding of hapten to each species is similar.

In a previous paper (Dower et al., 1977), one of the criteria we used to place Tyr-34_L as a hapten contact residue was the observation that irradiation of a protein resonance at 6.6 ppm in a mixture of Fv and Dnp-O resulted in a change in intensity in the H(3) hapten resonance. Since it was assumed that the shift on the H(3) resonance came essentially from Tyr-34_L, the change in intensity was interpreted as an Overhauser effect arising from the proton(s) on Tyr-34_L. No such specific effect was observed with Dnp-glycine so either the mode of binding of Dnp-O^- is slightly different or there may have been effects of cross-relaxation (Kalk & Berendsen, 1976) involving several protein protons. Certainly, at 300 MHz we can observe considerable cross-relaxation effects, making any interpretation in terms of an Overhauser effect ambiguous.

Ring-Current Calculations. The evidence suggesting that the Dnp ring interacts with a tryptophan side chain has been presented previously (Jackson & Dwek, 1981). This includes induced circular dichroism spectra of Dnp and Tnp ligands on binding to M315 (Freed et al., 1976; Orin et al., 1976) and the red shift in the ultraviolet absorption spectrum of the bound ligands. The more recent binding studies on Fv fragment in which all the tryphophans are deuterated (Gettins & Dwek, 1981) provide further strong evidence for the involvement of tryptophan with the Dnp. The tryptophan ring system is therefore expected to be a major contributor to the observed large upfield chemical shifts on the hapten proton resonances. Recently we have reexamined (Perkins & Dwek, 1980) the ring-current shifts from tryptophan rings and concluded that the empirical intensity factors need to be increased; otherwise underestimates of the shift occur. A comparison of calculated shifts 0.33 nm above the plane of the tryptophan ring (the closest approach for a Dnp and tryptophan stacking interaction) using the intensity factors of Perkins et al. (1977) and Perkins & Dwek (1980) is shown in Figure 2. As can be seen, considerable differences are found between the two treatments, with the latter predicting values in this plane to be up to 1.2 ppm higher.

The implications of this are as follows: In our previous analysis (Perkins et al., 1977; Dower et al., 1977) to account for the observed chemical shift changes of the hapten protons on binding to Fv, it was necessary to position the Dnp ring at 0.33 nm over the Trp ring and surround it by a minimum of two additional aromatic side chains. By use of the intensity factors of Perkins & Dwek (1980), a Trp ring and a single additional aromatic residue become the minimum requirement.

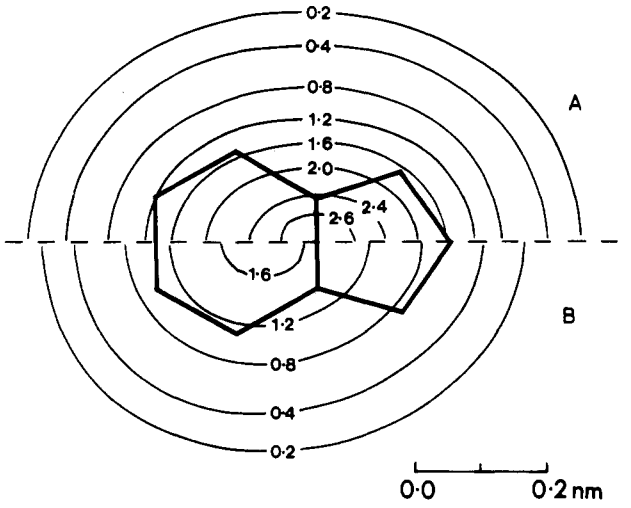

FIGURE 2: Comparison of the calculated current contours (in ppm) from a tryptophan residue at 0.33 nm above the plane of the ring by using the treatments in (A) Perkins & Dwek (1980) and in (B) Perkins et al. (1977).

Table II: Comparison of Calculated and Observed Chemical Shifts of Various Resonances of Hapten Rings on Binding to the Fv Fragment of M315[a]

hapten	proton	shift change exptl	calcd Trp	calcd Phe	calcd total	pH*
DNNS[b]	H(3)	0.84	0.83	0.0	0.83	7.0
	H(5)	1.07	0.40	0.6	1.00	
	H(6)	1.72	0.17	1.7	1.87	
	H(8)	0.65	0.55	0.0	0.55	
Tnp-glycine[c]	H(3) + H(5)	2.6	1.3	0.9	2.2	7.05
Dnp-aspartate[d]	H(3)	1.68	1.6	0.0	1.6	6.9
	H(5)	2.3	0.6	1.8	2.4	
	H(6)	1.3	0.9	0.5	1.4	

[a] Measurements were made at 270 MHz, $T = 303$ K, in the presence of 0.15 M NaCl. [b] From Wain-Hobson (1977). [c] From Dower et al. (1978). [d] From Dower et al. (1977).

The additional ring must be in proximity to H(5) and H(6) rather than H(3) to produce the necessary shifts. With these two rings it is possible to construct a site that accounts for the observed chemical shift changes on binding of a wide variety of different haptens. The arrangements which are compatible with the experimental data for Dnp-aspartate (Dower et al., 1978), Tnp-glycine (Dower et al., 1977), and DNNS (Wain-Hobson, 1977), which are representative of the entire range of haptens studied, are given in Figure 3. As is shown in Table II the calculated shifts agree well with the observed values.

The arrangement of these rings can then be built into the combining site of M315 provided a suitable model is available. There are of course other constraints such as the proximity of Tyr residues to the hapten side chains. In this respect the recently predicted model of the combining site of M315 of Stanford & Wu (1981) places Tyr-33_H well removed from the hapten side chain, ~1.3 nm, a point at variance with the experimental results presented here. Further, to build the geometries shown in Figure 3 into their site requires major alterations in the α-carbon backbone. By contrast the model of M315 as already discussed in Dwek et al. (1977) requires only adjustments in positions of the hapten and side chains of Trp-93_L and Tyr-34_L to be compatible with the results above and is therefore the only one considered here.

In this model Trp-93_L, Phe-34_H, but not Tyr-34_L are now required to shift the hapten ring protons. Tyr-34_L is still, of course, in close proximity to the hapten but is now placed more peripheral to the site. Tyr-33_H remains placed so that its ring currents can shift the hapten side-chain protons and in a position which is also consistent with the pK_a mapping studies described above. The other major difference between this model and our previous one is that the orientation of the side chain of Trp-93_L is altered.

One other result that can be included in extending this model of the combining site is that reported in Hardy & Richards (1978) of an upfield shift on binding of the proton of the p-CHF_2 group in the hapten $F_2HCC_6H_4NH(CH_2)COOH$. The shifts from Trp-93_L and Phe-34_L (the nearest rings) would only account for <0.5 ppm, so the presence of another ring at the back of the site can be inferred. The side chain of Phe-105_H is close enough to explain the required shift, while being too far from the hapten ring protons (0.6 nm) to affect them significantly (Figure 4).

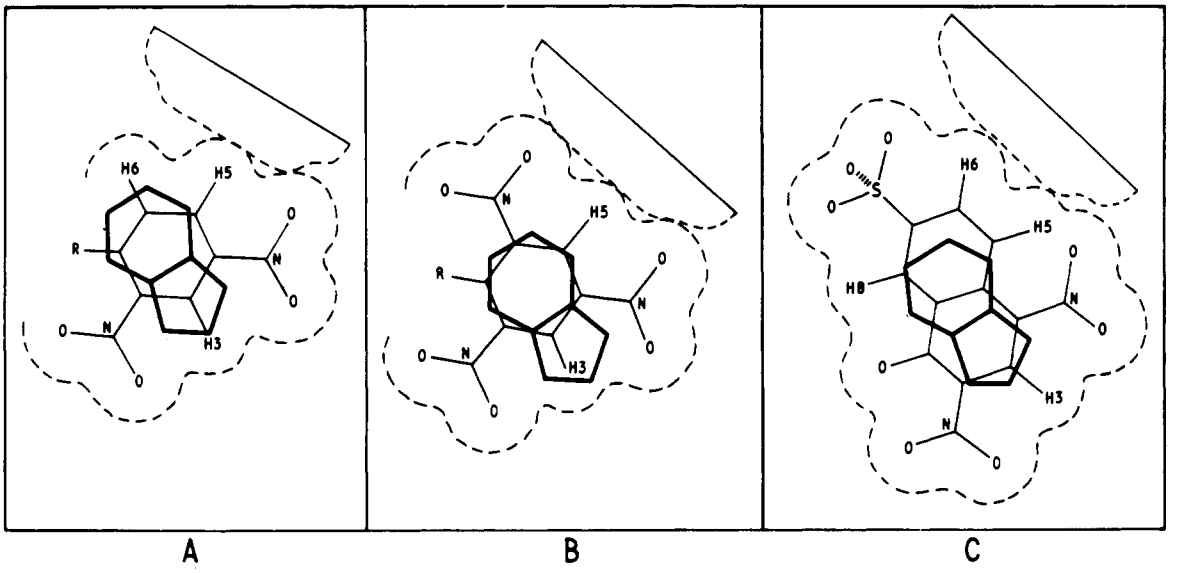

FIGURE 3: Positioning of aromatic rings to account for the experimental shifts (see Table II) on DNNS, Dnp-glycine, and Tnp-glycine. The hapten ring is positioned 0.33 nm from and parallel to the Trp ring. van der Waals surfaces (dashed lines) of the hapten and adjacent aromatic ring are indicated: (A) Dnp-glycine, (B) Tnp-glycine, and (C) DNNS. R = $-CH_2COO^-$.

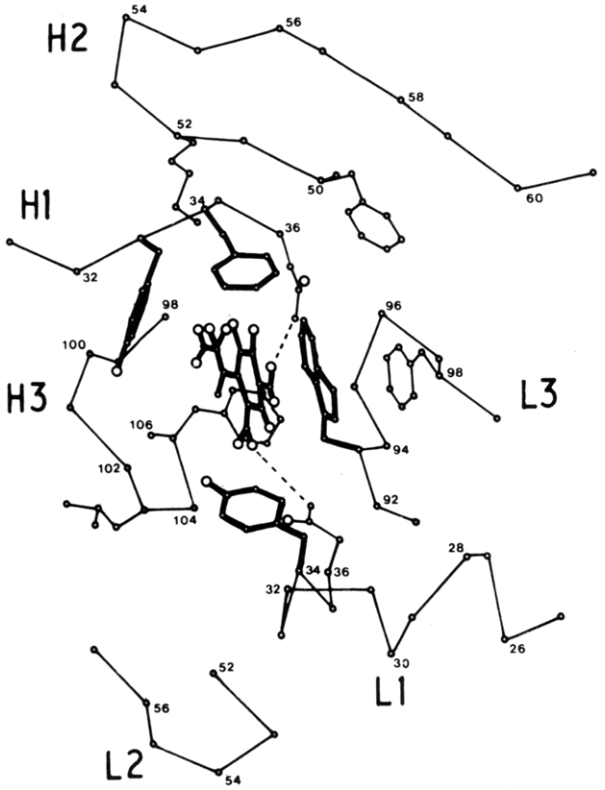

FIGURE 4: Postulated combining site of M315 containing Dnp-glycine. The three hypervariable regions of the heavy (H) chain and light (L) chain are indicated. The residues in contact with the hapten are Trp-93_L, Phe-34_H, and at the back of the site Phe-105. The dispositions of Tyr-33_H and Tyr-34_L are shown. Tyr-33_H can interact with the α-CH_2 protons. The dotted lines indicate possible polar interactions (or hydrogen bonds) between Asn-36_L and Asn-36_H.

Table III: Comparison of Calculated with Experimental Shifts (ppm) on Dnp-Glycine Using the Coordinates of the Final Model in Figure 4[a]

Trp-93_L	1.08	0.60	1.24	0.26
Tyr-33_H		0.01	0.17	0.56
Phe-34_H		1.52	0.55	0.16
Phe-105_H	0.17	0.13		
total	1.25	2.26	1.96	0.98
exptl[b]	1.21	2.20	1.77	1.00
calcd − exptl	+0.06	+0.06	+0.19	−0.02

[a] Only aromatic rings <6 Å from the proton of interest were considered (Dwek, 1973). [b] From Dower et al. (1977).

The presence of hydrogen bonds to the 2- and the 4- but not the 6-nitro groups has been inferred previously (Dwek et al., 1977; Dower et al., 1978; Gettins et al., 1978; Hardy & Richards, 1978), although some doubt has been cast on the generality of these for all haptens (Gettins et al., 1981a,b). From the model, however, it is possible to construct the side chains of Asn-36_L and Asn-36_H such that they can if required participate in polar interactions with the 2- and 4-nitro groups of suitably oriented haptens. The model of the combining site which then emerges is shown in Figure 4. The calculated contributions, using the model coordinates, to the shifts on Dnp-glycine from aromatic rings within 0.6 nm of the hapten protons are listed in Table III.

Protein 315 is known to bind a wide variety of haptens (Gettins & Dwek, 1977). In our final model, haptens of differing size and shape may be accommodated in the site in a way which is compatible with the experimental data with only minimal adjustments in the positions of the protein side chains as shown in Figure 3. This implies that antibody specificity is essentially a shape problem with the site being essentially preformed. This is in contrast with the results for the binding of Dnp ligands to the light chain dimer $(V_L)_2$ from M315 (Jackson et al., 1981) in which we concluded that the size and shape of the combining site is not optimal for the ligands requiring substantial movements of protein side chains in the combining site and resulting in weaker binding of ligands to the $(V_L)_2$ compared with Fv.

Our model of the combining site of M315 now awaits critical comparison with that from the X-ray crystallographic data currently under analysis in Oxford (Aschaffenburg et al., 1979).

Acknowledgments

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Nuclear Overhauser Assignment of the Imino Protons of the Acceptor Helix and the Ribothymidine Helix in the Nuclear Magnetic Resonance Spectrum of *Escherichia coli* Isoleucine Transfer Ribonucleic Acid: Evidence for Costacked Helices in Solution†

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ABSTRACT: In a previous study we showed that, in the low-field nuclear magnetic resonance (NMR) spectrum of *Escherichia coli* $tRNA_1^{Val}$, the hydrogen-bonded imino protons of the four Watson–Crick base pairs in the dihydrouridine helix could be assigned on the basis of their proximity to the imino proton of s^4U8 by means of sequential Nuclear Overhauser (NOE) connectivity (Hare & Reid, 1982). In the present paper we have used the nearest-neighbor NOE technique to assign all the imino proton resonances of the acceptor helix and the ribothymidine helix of *E. coli* $tRNA_1^{Ile}$. As reference points we used the GU-type base pairs located at positions 5 and 49 in this molecule which are readily identifiable in the NMR spectrum by virtue of containing two imino protons in the same base pair. From UG5 the imino protons of base pairs 4,3,2,1 and 6,7 could be assigned by through-space NOE connectivity. Similarly the imino protons of 50,51,52,53 were assigned by their spatial relationship to GΨ49. NOE connectivity also revealed a base pair stacked on the external side of GC53 which, by analogy with the crystal structure of yeast phenylalanine tRNA, is presumed to be the tertiary pair T54–A58. This was confirmed by NOE connectivity from the thymine methyl resonance. In addition to assigning 17 of the imino resonances in the low-field NMR spectrum of isoleucine tRNA, these NOE studies show that the acceptor helix and the ribothymidine helix are stacked on each other in solution in that base pairs 7 and 49 are directly connected in space.

One of the most useful applications of NMR is the ability of this technique to elucidate the three-dimensional folding of polymers from the spatial proximity of assigned resonances in the linear sequence via the nuclear Overhauser effect (NOE).[1] The NOE proximity technique makes use of the marked distance dependence of the negative NOE in polymers. If a given proton in a NMR spectrum is individually saturated by selective radio-frequency irradiation at its resonance frequency, its principal mode of recovery is by cross-relaxation with neighboring protons provided the correlation time is long compared to the reciprocal of the NMR frequency (this condition is met in large, slowly tumbling rigid polymers). A consequence of this cross-relaxation ("mutual spin flips") is that the neighboring protons become cross-saturated, and this is the basis of the negative NOE observed in polymers (Noggle

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[1] Abbreviations: tRNA, transfer ribonucleic acid; NMR, nuclear magnetic resonance; NOE, nuclear Overhauser effect; DSS, 4,4-dimethylsilapentane-1-sulfonate; T, ribothymidine; D, dihydrouridine; Ψ, pseudouridine; EDTA, ethylenediaminetetraacetic acid.

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