Melchers, F. (1973) *Biochemistry 12*, 1471.
Morrison, S. L. (1978) *Eur. J. Immunol. 8*, 194.
Morrison, S. L. (1979) *J. Immunol. 123*, 793.
Morrison, T. G., & Lodish, H. F. (1975) *J. Biol. Chem. 250*, 6955.
Morrison, T. G., & McQuain, C. (1977) *J. Virol. 21*, 451.
Morrison, T., Stampfer, M., Baltimore, D., & Lodish, H. (1974) *J. Virol. 13*, 62.
Nakamura, K., & Compans, R. W. (1978) *Virology 84*, 303.
Preud'Homme, J. L., Birshtein, B. K., & Scharff, M. D. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72*, 1427.
Rabbitts, T. H., Forster, A., Dunnick, W., & Bently, D. L. (1980) *Nature (London) 283*, 351.
Raff, M. C. (1976) *Cold Spring Harbor Symp. Quant. Biol. 41*, 159.
Roberts, B. E., & Paterson, B. M. (1973) *Proc. Natl. Acad. Sci. U.S.A. 70*, 2330.
Sakano, H., Rogers, J. H., Huppi, K., Brack, C., Traunecker, A., Maki, R., Wall, R., & Tonegawa, D. (1979) *Nature (London) 277*, 627.
Schibler, U., Marcu, K. B., & Perry, R. P. (1978) *Cell (Cambridge, Mass.) 15*, 1495.
Schilling, J., Clevinger, B., Davie, J. M., & Hood, L. (1980) *Nature (London) 283*, 35.
Schwinghamer, M. W., & Shephard, R. J. (1980) *Anal. Biochem. 103*, 426.
Sharon, J., Morrison, S. L., & Kabat, E. A. (1980) *Somatic Cell Genet. 6*, 435.
Sikorav, J.-L., Auffray, C., & Rougeon, F. (1980) *Nucleic Acids Res. 8*, 3143.
Southern, E. M. (1975) *J. Mol. Biol. 98*, 503.
Stark, G. R., & Williams, J. G. (1979) *Nucleic Acids Res. 6*, 195.
Szybalski, W., Szybalska, E. H., & Ragni, G. (1962) *Natl. Cancer Inst. Monogr. No. 7*, 75.
Tucker, P. W., Marcu, K. B., Newell, N., Richards, J., & Blattner, F. R. (1979a) *Science (Washington, D.C.) 206*, 1303.
Tucker, P. W., Marcu, K. B., Slightom, J. L., & Blattner, F. R. (1979b) *Science (Washington, D.C.) 206*, 1299.
Yamawaki-Kataoka, Y., Kataoka, T., Takahaski, N., Obata, M., & Honjo, J. (1980) *Nature (London) 283*, 786.
Weitzman, S., & Scharff, M. D. (1976) *J. Mol. Biol. 102*, 237.

Antibody Specificity: A 270-MHz Hydrogen-1 Nuclear Magnetic Resonance Study of the Binding of Dinitrophenyl Compounds to the V_L Dimer of Protein 315†

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ABSTRACT: The binding of dinitrophenyl (DNP) compounds to the V_L dimer of protein 315 (IgA/λ_2) results in small perturbations of about ten resonances in the aromatic region of the 270-MHz ^1H NMR spectrum of the protein. From a comparison of the chemical shifts of these resonances with the chemical shifts of resonances which are affected by the binding of DNP compounds to the Fv fragment of protein 315, it is concluded that the conformation of combining site residues in the V_L domain of the Fv fragment is maintained in the V_L dimer. The binding of DNP compounds to the V_L dimer is therefore considered to reflect the retention of structural features important in determining the specificity of the Fv fragment and not the fortuitious creation of a binding site. These findings provide a structural explanation for the observation that antibodies with a λ_2 light chain are contained in the anti-DNP response of BALB/c mice [Cotner, T., & Eisen, H. N. (1978) *J. Exp. Med. 148*, 1388]. The ligand resonances undergo large upfield chemical shift changes following binding to the V_L dimer. These changes are interpreted in terms of ring-current effects from aromatic residues. It is shown that the same conformation of the combining site aromatic residues of the V_L domain is capable of explaining the chemical shift changes of the ligand resonances which are observed on binding to the V_L dimer and to the Fv fragment. The chemical shifts of the aromatic resonances of DNP-aspartate and DNP-glycine, when bound to the V_L dimer, are found to differ. Ring-current calculations show that the positions of the DNP rings may differ by about 1.5 Å relative to Trp-93_L. A difference of about 0.5 Å is calculated for binding to the Fv fragment. It is suggested that the specificity of protein 315 for DNP compounds is determined largely by the size and shape of a predominantly nonpolar combining site.

Many factors which are important in determining the structural basis of antibody specificity and diversity have now been recognized. The comparison of a large number of antibody V-region sequences (Wu & Kabat, 1970) and the determination of the structures of several antibody V domains by X-ray crystallography (Poljak et al., 1973; Schiffer et al., 1973; Segal et al., 1974; Epp et al., 1974; Wang et al., 1979), have confirmed the idea that the structural mechanism used to generate the diversity of antigen binding sites is a conserved framework, the immunoglobulin fold, to which the hypervariable sequences, forming the antigen binding site, are attached. It has therefore been considered feasible to construct models of immunoglobulin combining sites for which no X-ray data exist on the basis of their presumed homology with known structures (Poljak et al., 1974; Padlan et al., 1976). Further aspects of antibody specificity may then be investigated by use of these models.

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A model of the combining site of the dinitrophenyl (DNP)[1] binding mouse myeloma protein 315 has been constructed according to the above principles (Padlan et al., 1976) and has been refined by high-resolution ^1H NMR studies (Dwek et al., 1977; Dower et al., 1977; R. J. Leatherbarrow & R. A. Dwek, unpublished results). It was subsequently found that the L-chain dimer and the V_L dimer of protein 315 retain affinity for the DNP group (Schechter et al., 1976; Gavish et al., 1977). The analysis, by ^1H NMR, of the binding of DNP ligands to the V_L dimer may be used as a test of the model of the intact combining site since the structure of the V_L domain residues determined from ^1H NMR studies of the intact Fv fragment should also be capable of explaining the results obtained with the V_L dimer. This comparison relies on the assumption that the structure of the combining site residues of the V_L domain is unaffected by dimerization either with a V_H domain or with another V_L domain.

These experiments also served to investigate two other ideas. First, it has been suggested that dimers of like chains are particularly able to form cavities which can bind nonpolar molecules and therefore that the binding of DNP compounds by such dimers may be fortuitous (Stevenson, 1974). A comparison of the structures of the combining sites of the V_L dimer and of the Fv fragment of protein 315 should show to what extent this conclusion is justified in the case of protein 315. Second, an extensive analysis of partial amino acid sequences of the V_H and V_L regions of many mouse myeloma proteins with antigenic specificities revealed a marked correlation between specificity and V_H, rather than V_L, sequences (Barstad et al., 1978). This relationship extended to the three DNP binding proteins, 315, 460, and 25, which have closely related V_H sequences but very different V_L sequences. The structural studies described in this paper allowed a further investigation of the relationship between primary sequence and binding specificity.

Materials and Methods

The V_L domain was prepared from the Fv fragment of protein 315 on DEAE-cellulose in 8M urea (Hochman et al., 1973). Solutions 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.) with the addition of a small volume of 1 M [$^2H_4$]acetic acid (99.5% ^2H, Ryvan), to a final acetate concentration of 0.025 M. The pH (uncorrected for the ^2H isotope effect), was adjusted to 4.8 by the addition of NaO^2H or ^2HCl (99+% ^2H, obtained from Aldrich Chemical Co., Gillingham, U.K.). 2,4-Dinitrophenyl-L-aspartic acid (DNP-aspartate) and 2,4-dinitrophenylglycine (DNP-glycine) were obtained from B.D.H. Chemicals Ltd., Poole, U.K. The following extinction coefficients were used: V_L domain, $E_{280}^{0.1\%} = 1.0$; DNP-aspartate, $E_{360}^{M} = 17\,400$; DNP-glycine, $E_{360}^{M} = 15\,890$.

^1H NMR spectra were recorded at 270 MHz by use of a modified Bruker spectrometer with an Oxford Instrument Co. superconducting magnet and an internal ^2H field-frequency lock. Free induction decays were sampled at 4096 points by use of quadrature detection and Fourier transformed over 8192 points. Signal-to-noise ratios were increased by convolution. Sweep widths of 4000 Hz were employed, with a pulse angle of 70° and a delay of 130–150 μs between the main pulse and the start of accumulation. Overall interpulse times were 0.52 s. Chemical shift values are reported as parts per million (ppm) downfield from the high-field singlet resonance of sodium 4,4-dimethyl-4-silapentanesulfonate (DSS), used as an external standard. The chemical shifts of perturbed resonances are reproducible within about 0.03 ppm. The probe temperature was 298 ± 1 K.

Ring current shifts were calculated by the ring current contour map approach and/or use of Johnson–Bovey tables (Dower et al., 1977).

The α-carbon coordinates of protein REI were kindly provided by Professor R. Huber, Max Planck Institute of Biochemistry, Munich. The coordinates of protein Mcg were obtained from the Protein Data Bank, University Chemical Laboratory, Cambridge. Superposition of protein structures was achieved by use of the Fortran IV program SUPERB, written for the Oxford ICL 1906A computer by Dr. J. Thornton and kindly made available by Dr. K. Wilson, Department of Molecular Biophysics, Oxford. The program uses a nonlinear least-squares routine.

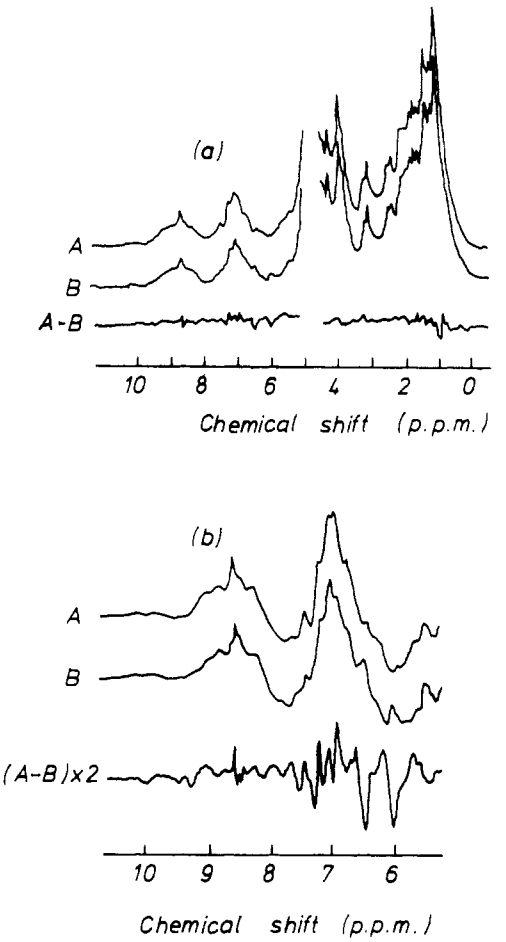

FIGURE 1: Effect of binding of DNP-aspartate on the 270-MHz ^1H NMR spectrum of the V_L dimer. Scans (2000) were recorded at T = 298 K with solutions in ^2H_2O–0.25 M [^2H]acetate. (a) Whole spectrum. (b) Aromatic region. (A) V_L dimer alone (0.85 mM). (B) V_L dimer (0.80 mM) with equimolar DNP-aspartate.

Results and Discussion

Observation of the Binding of Ligands by ^1H NMR. The 270-MHz ^1H NMR spectra of the V_L dimer, at pH 4.8, in the absence of ligand or saturated with DNP-aspartate and the resulting difference spectrum are shown in Figure 1. The difference represents only 2–3% of the total intensity, with approximately equal perturbations of the aromatic and aliphatic regions, despite the presence of 1100 unexchangeable

[1] Abbreviations used: NMR, nuclear magnetic resonance; DNP, 2,4-dinitrophenyl; IgA, immunoglobulin A; Fv fragment, immunoglobulin fragment composed of the variable domains of a light chain and a heavy chain; V_L, variable domain of a light chain; V_H, variable domain of a heavy chain.

Table I: Chemical Shifts (δ) and Changes in Chemical Shifts (Δδ) of Protein Resonances Perturbed on the Binding of Ligands to the Fv Fragment of Protein 315 and to the V_L Dimer[a]

V_L + DNP-aspartate, pH* 4.8		V_L + TNP-glycine, pH* 4.8		V_L + DNP-glycine, pH* 4.8		Fv + DNP-aspartate,[b] pH* 6.9		Fv + TNP-glycine,[c] pH* 6.7		Fv + TNP-aspartate,[c] pH* 7.05	
(δ)	(Δδ)	(δ)	(Δδ)	(δ)	(Δδ)	(δ)	(Δδ)	(δ)	(Δδ)	(δ)	(Δδ)
		5.63	+0.18	5.64	−0.13					5.65	−0.13
6.15	+0.23	6.17	+0.30	6.14	+0.27	6.16	+0.25	6.20	+0.14	6.20	+0.07
6.23	+0.30	6.24	+0.37	6.21	+0.36	6.33	+0.17	6.31	+0.15	6.31	+0.05
6.36	−0.08	6.36	−0.12	6.38	−0.11	6.37	−0.29	6.35	−0.17	6.35	−0.22
6.58	+0.16	6.62	+0.14	6.62	+0.55	6.62	−0.08				
6.73	+0.18	6.75[h]		6.72[h]							
6.90[g]	<+0.15	6.94[h]		6.87[h]		6.90	+0.19	6.88	+0.15	6.88	+0.20
								6.43	−0.05		
						6.95[d]	+0.07[d]	6.93[d]	<+0.05[d]	7.01[d]	<+0.05[d]
7.06[g]	<+0.15	7.07[h]		7.04	−0.17?	7.00[i]		7.04[i]		7.04[i]	
		7.22	+0.07	7.22	+?	7.20[j]		7.23[j]		7.23[j]	
7.45	−0.15	7.46	no shift	7.45	−0.20	7.42[k]		7.43[k]		7.43[k]	
		7.64	+0.14	7.63	+0.13						
8.53[e]	−0.06[e]	8.56[e,i]		8.55[e]	−0.21[e]						
8.67[e]	+0.03[e]	8.68[e]	+0.07[e]	8.67[e]	+0.03[e]						
						7.82[e]	+0.06[e]	7.76[e]	<+0.05[e]	7.87[e]	+0.06[e]
								8.07[f]	no shift	8.07[f]	−0.07[f]

[a] Measurements were made at 270 MHz in ^2H_2O. Solutions of the V_L dimer contained 0.025 M sodium acetate, T = 298 K. Protein concentrations were ~0.8 mM. Solutions of the Fv fragment contained 0.15 M NaCl, T = 303 K. Protein concentrations were ~1.3 mM. pH* values are given in the table. Shifts were measured from DSS, used as an external standard. + and − denote upfield and downfield shift changes, respectively. Assignments to His-H_2 resonances and probable assignments to His-H_4 resonances are indicated. Blank positions in the table do not imply a clear absence of perturbation. [b] Data from Dower et al. (1977). [c] Data from Dower et al. (1978). [d] His-102_H. [e] His-97_L. [f] His-102_H. [g] Or broadens. [h] Broadens ? [i] Broadens. [j] Decreases. [k] Increases.

aliphatic ^1H nuclei compared with 104 aromatic ^1H nuclei per dimer. The small intensity of the difference spectrum and the proportionately large perturbation of aromatic rather than aliphatic resonances are also observed on the binding of ligands to the Fv fragment (Dower et al., 1977). The small intensity of the difference spectrum implies that any conformational changes on binding are limited to the combining site. It is in marked contrast to the large changes observed on the binding of ligands to some proteins, for example, *N*-acetylglucosamine to lysozyme (Dwek, 1977). The difference spectrum of the aromatic region (Figure 1b), representing about 20 out of 104 nuclei, suggests a combining site containing several aromatic residues. The resonances of the ligand are in fast exchange and undergo large upfield chemical shift changes on binding to the V_L dimer. The titration of the ligand resonances may be seen in Figure 2. These large upfield chemical shift changes are also observed with the Fv fragment of protein 315 (Dower et al., 1977) and are characteristic of a highly aromatic environment of the ligand nuclei. The titration curves of the aromatic protein and ligand resonances are shown in Figure 3, and the titration behavior of the protein resonances is summarized in Table I. Inspection of Figure 3 shows that at equimolar concentrations of V_L dimer and ligand the chemical shift changes of all the protein resonances are 70–80% of their total chemical shift changes. This demonstrates that binding at only one site per V_L dimer is affecting the protein resonances, in agreement with the equilibrium dialysis results, at pH 5.0, of Gavish et al. (1978). If binding were occurring at two sites, many or all of the protein resonances would have titrated over less than 50% of their total range. The number of binding sites (n) and dissociation constant (K_D) for the binding of DNP-aspartate were obtained from the titration curve of the protein resonance at 6.23 ppm, the most easily observable resonance, by use of an iterative least-squares procedure which systematically varied n and K_D. The analysis gave n = 1.03 and K_D = 0.05 mM. These values may then be used to calculate the chemical shifts of the resonances of the bound ligand. The theoretical curves generated by using the calculated values (given in Table II) and a K_D of 0.05 mM are superimposed on the data given in Figure 3.

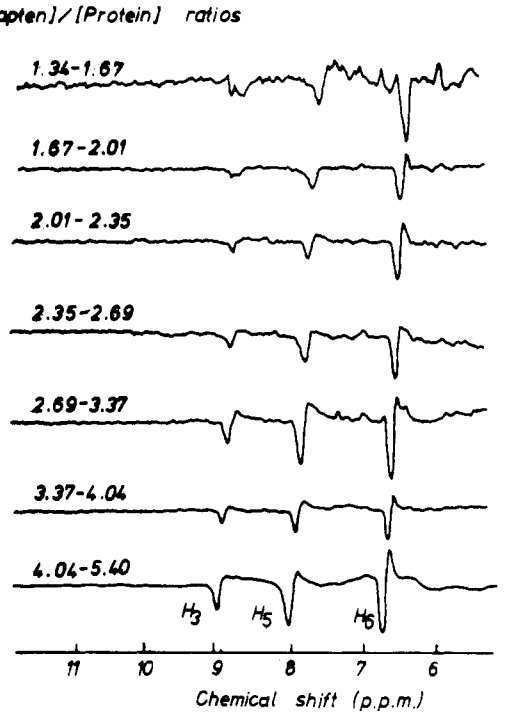

FIGURE 2: Chemical shift changes of the resonances of the aromatic ^1H nuclei of DNP-aspartate following binding to the V_L dimer. Measurements were made at 270 MHz, T = 298 K, with solutions in ^2H_2O–0.025 M [^2H]acetate, pH* 4.8. The protein concentrations were ~0.75 mM. The differences were taken between successive additions of hapten. The chemical shifts of the resonances of the free ligand are H_3 9.12 ppm, H_5 8.29 ppm and H_6 6.97 ppm.

Similar experiments were also carried out with the ligand DNP-glycine. The aromatic regions of the ^1H NMR spectra at 270 MHz of the V_L dimer in the absence of ligand or saturated with DNP-glycine and the resultant difference spectrum are shown in Figure 4. For comparison, the difference with DNP-aspartate is also shown. It is clear from the two difference spectra that the ligands are not affecting

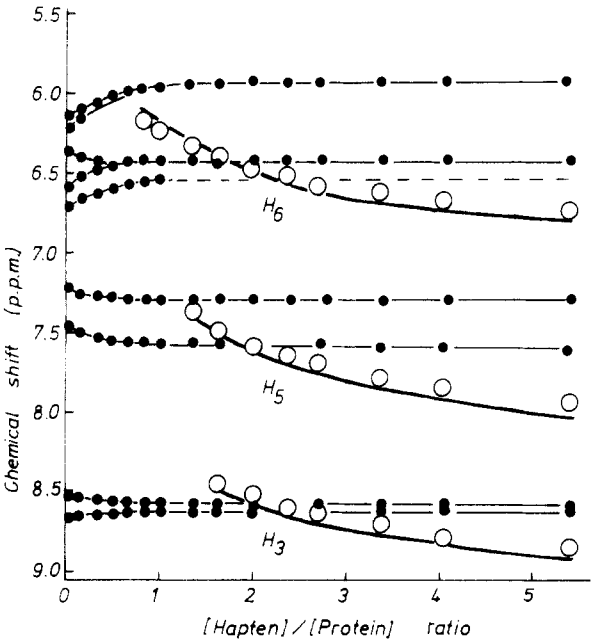

FIGURE 3: Chemical shift changes of the resonances of aromatic ^1H nuclei following the binding of DNP-asparatate to the V_L dimer. Measurements were made at 270 MHz, T = 298 K, with solutions in ^2H_2O–0.025 M [^2H]acetate, pH* 4.8. The initial protein concentration was 0.85 mM. The chemical shifts of the resonances of the free ligand are H_3 9.12 ppm, H_5 8.29 ppm, and H_6 6.97 ppm. The solid curves for the ligand resonances were calculated by assuming K_D = 0.05 mM, n = 1.0, and the chemical shift changes are given in Table II.

Table II: Upfield Chemical Shift Changes of the ^1H Resonances of DNP-glycine and DNP-aspartate on Binding to the V_L Dimer of Protein 315[a]

resonance	DNP-glycine (Δδ)	DNP-aspartate (Δδ)
H_3	1.6	1.2
H_5	2.7	1.5
H_6	3.0	1.1
α-CH_2	1.2	

[a] Measurements were made at 270 MHz; T = 298 K in ^2H_2O containing 0.025 M [^2H]acetate, pH* 4.8. Estimated errors are ±10% on the values given.

the protein resonances in identical manners. The titration curves of the affected resonances are shown in Figure 5, and the data are summarized in Tables I and II. Blank positions in Table I do not imply that the resonances are not perturbed, since resonance overlap often makes the titration of resonances difficult to follow. Examination of the titration curves of the protein resonances again showed that only one binding site per V_L dimer was detectable. The best fit procedure, applied to the resonance at 6.23 ppm, gave n = 0.97 and K_D = 0.07 mM, very similar to the results obtained with DNP-aspartate. The chemical shift changes of the resonances of DNP-glycine were calculated as described above for DNP-aspartate. The extrapolated chemical shifts of the resonances of bound DNP-glycine provide good fits to the complete titration data and are clearly substantially different from the values obtained for DNP-aspartate (Table II).

Retention of Combining Site Conformation in the Separated V_L Domain. Comparison of the resonances of aromatic ^1H nuclei which are perturbed by the binding of ligands to the V_L dimer and to the Fv fragment reveals several similarities (Table I). Many perturbed resonances have almost the same chemical shifts in the spectra of both proteins. These equivalent resonances are often perturbed in a similar manner—

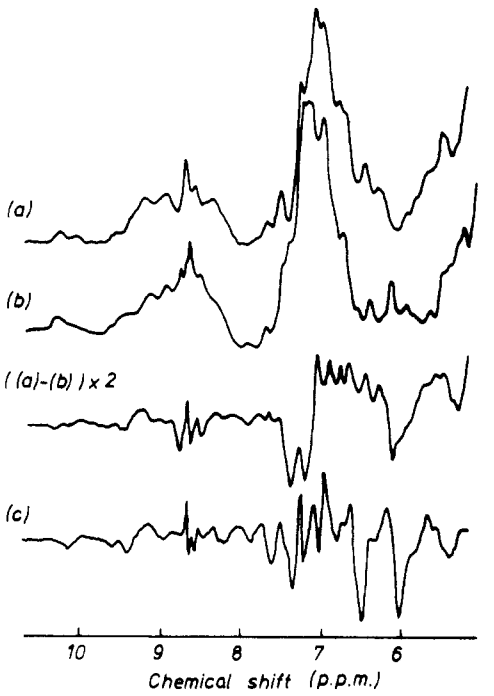

FIGURE 4: Comparison of the effects of the binding of DNP-glycine and DNP-aspartate on the aromatic region of the 270-MHz ^1H NMR spectrum of the V_L dimer. Scans (2000) were recorded at T = 298 K, with solutions in ^2H_2O–0.25 M [^2H]acetate, pH* 4.8. (a) V_L dimer (0.76 mM). (b) V_L dimer (0.70 mM) with approximately equimolar DNP-glycine. (c) Difference obtained with DNP-aspartate (as for Figure 3).

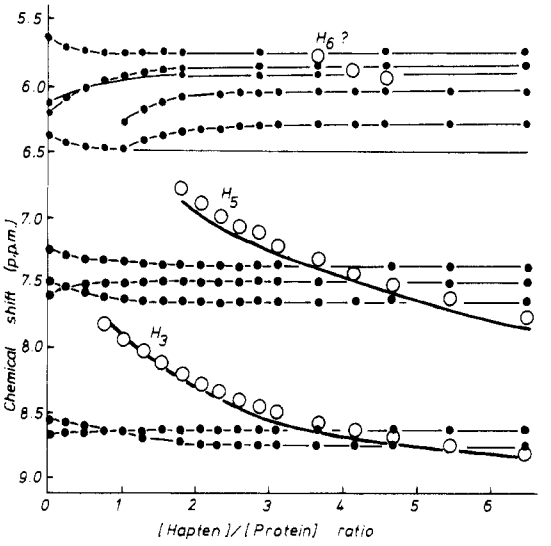

FIGURE 5: Chemical shift changes of the resonances of aromatic ^1H nuclei following the binding of DNP-glycine to the V_L dimer. Measurements were made at 270 MHz, T = 298 K, with solutions in ^2H_2O–0.025 M [^2H]acetate, pH* 4.8. The initial protein concentration was 0.76 mM. The chemical shifts of the resonances of the free ligand are H_3 9.12 ppm, H_5 8.29 ppm, and H_6 6.97 ppm. The solid curves for the ligand resonances were calculated by assuming K_D = 0.07 mM, n = 1.0, and the chemical shift changes are given in Table II.

either upfield or downfield—on hapten binding. Coincidences under the main aromatic envelope (6.5–7.4 ppm) could be fortuitous, but this is unlikely to be true of resonances outside the envelope. The clearest examples are provided by the resonances at 5.64, 6.16, and 6.36 ppm which reflect specific tertiary structure interactions.

The coincidences of the chemical shifts of resonances affected by binding of ligand to either Fv fragment or V_L dimer indicate that the conformation of the combining site residues

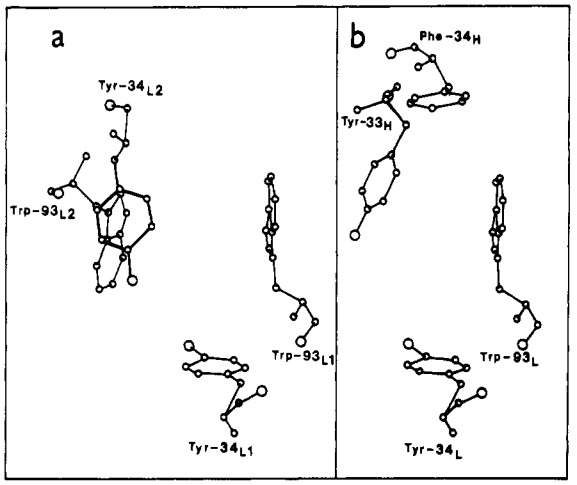

FIGURE 6: (a) Combining site of the V_L dimer of protein 315. The model was constructed by using the coordinates of protein REI, as described in the text. The side chains of Tyr-34_L points out toward the viewer. (b) Combining site of the Fv fragment of protein 315. The side chains of Tyr-34_L and Tyr-33_H point out toward the viewer whereas that of Phe-34_H points back into the site.

of the V_L domain remains essentially the same in both cases. Indeed these resonance positions may be concluded to arise from intrachain rather than interchain interactions since replacement of a V_H domain by another V_L domain has little effect on their positions. That similar resonances are involved in each case also implies that the haptens bind in a similar position with respect to the V_L domain whether in the Fv fragment or in the V_L dimer. If the shifts of the resonances of the V_L dimer itself are examined, it is found that the protein resonances involved are the same for both haptens and that the shift changes, with the exception of that for the resonance at 6.62 ppm, are very similar. Comparison with the shift changes of the Fv fragment when different haptens bind reveals a similar degree of variation. Thus it is suggested that the binding of DNP compounds by the V_L dimer reflects the retention of structural features important in determining the specificity of the intact Fv fragment. This is an important conclusion in the light of suggestions that binding of ligands such as DNP compounds by isolated chains may be predominantly fortuitous (Stevenson, 1974). It is probable that the stacking of the DNP group with Trp-93_L is a major interaction in both proteins.

Possible Structure of the DNP Binding Site of the V_L Dimer. A model of the combining site of the V_L dimer of protein 315 was constructed by taking the coordinates of the V_L domain of our current model of the Fv fragment (R. J. Leatherbarrow & R. A. Dwek, unpublished results) and applying to them the appropriate rotation and translation matrices relating the monomers of the REI or Mcg dimers. Because the coordinate systems of the three proteins are different, it was necessary to transform the coordinate system of protein 315 into that of proteins REI or Mcg, using the SUPERB program. The matrices relating the α-carbon coordinates of protein 315 to monomer 1 of protein REI were obtained by superimposing the homologous L_2 and L_3 hypervariable loops. The L_1 and L_2 loops were used to relate the coordinates to monomer 2 of protein Mcg. Since construction of the model relies on the homology between hypervariable loops and since the mode of dimerization of V_L domains may not be invariant (Wang et al., 1979), the model must be regarded only as a qualitative aid to the interpretation of the NMR data. The important features of the combining site are shown in Figure 6, with the corresponding region of the Fv fragment for comparison. The predicted site is larger than that proposed for the Fv fragment although, like that for Fv, it is highly aromatic. The sides of the site are comprised of the side chains of Trp-93_L which are positioned approximately parallel and 8 Å apart. The two Tyr-34_L side chains border the site and are ~9 Å apart. This structure was used as a starting point for the ring current calculations.

Table III: Experimental and Calculated Chemical Shift Changes of Ligand ^1H Resonances on Binding to the V_L Dimer[a]

	calculated shift change (ppm)						
	DNP-glycine				DNP-aspartate		
	H_3	H_5	H_6	CH_2	H_3	H_5	H_6
Trp-93_L (monomer 1)	1.55	0.95	0.80	0.05	1.10	1.30	1.05
Trp-93_L (monomer 2)	0.10	1.65	2.40	1.10	0.30	0.40	0.25
	1.65	2.60	3.20	1.15	1.40	1.70	1.30
	experimental shift change (ppm)						
	1.6	2.7	3.0	1.2	1.2	1.5	1.1

[a] The orientations of the Trp-93_L residues in Figures 7 and 8 were used.

Any proposed geometry of the combining site residues has to be able to explain both the very large upfield chemical shift changes observed for all four ^1H resonances of DNP-glycine and the much smaller changes observed for DNP-aspartate (Table II). The ring current theory used to interpret the chemical shift changes on the hapten is a modification of the Johnson–Bovey treatment (Johnson & Bovey, 1958) described by Perkins et al. (1977). One recent alteration to this treatment has been included. This involves increasing the strength of the ring current field due to tryptophan. The rationale for this alteration has been described by Perkins & Dwek (1980). It is expected that the DNP ring will stack between the two Trp-93_L residues. Evidence for a stacking interaction with a tryptophan in the combining site of the L-chain dimer has been obtained by CD (Freed et al., 1976) and by UV difference spectroscopy (Gavish et al., 1978).

It is found that all the observed shift changes can be accounted for by involvement of just the two Trp-93 aromatic rings. However, in order to explain the large differences in the chemical shifts of DNP-aspartate and DNP-glycine on binding, there needs to be a relative difference in position between the DNP ring and one or both tryptophan rings of around 1.5 Å. (This is in contrast to a difference of only ~0.5 Å estimated for binding of these haptens to the Fv fragment (Dower et al., 1977; R. J. Leatherbarrow & R. A. Dwek, unpublished results). One explanation consistent with this and also with the similar perturbations to the protein spectrum is described below. Rotation of the side chain of Trp-93 of monomer 1 by approximately 25° into the site as shown in Figure 7 allows explanation of the chemical shift changes found for DNP-aspartate. The DNP ring is assumed to lie parallel to and 3.3 Å from this tryptophan ring. The larger shifts on binding of DNP-glycine may be accounted for by an additional rotation of the side chain of Trp-93 of monomer 2 by approximately 30° into the site so that the two indole rings are parallel and 6.6 Å apart. The DNP ring is positioned parallel to and 3.3 Å from both these rings, as shown in Figure 7. This results in increased ring current shifts to H_5 and H_6 while relatively little is contributed to H_3. It is worthwhile noting that this arrangement is not possible for DNP-aspartate due to the bulky nature of the branched side chain. A comparison of calculated and experimental shifts is given in Table III.

Implications of the DNP Binding Activity of the V_L Dimer

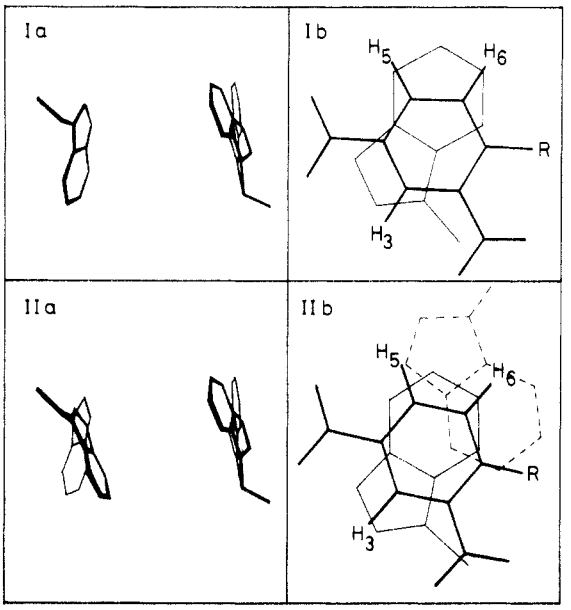

FIGURE 7: Possible geometries of the DNP rings relative to the two tryptophan residues in the combining site of the V_L dimer. (I) DNP-aspartate; (II) DNP-glycine. (a) Orientation of tryptophans. Lightly drawn residues indicate original positions as in Figure 6. Heavily drawn residues represent orientation after repositioning of side chains to explain chemical shifts on haptens as described in the text. (Ib) Position of DNP-aspartate in the site Ia. The tryptophan ring of monomer 1 is parallel to and 3.3 Å from the DNP ring. (Tryptophan monomer 2 (not shown) is at an angle to the DNP ring.) (IIb) Position of DNP-glycine in site IIa. The ring of tryptophan monomer 1 is shown solid, with that of monomer 2 dotted. Both tryptophans are 3.3 Å from and parallel to the DNP ring.

for the Mechanism of the Generation of Antibody Diversity. It has been concluded from the NMR experiments described above that the binding of DNP compounds to the V_L dimer is not fortuitous, but reflects the conservation of structural features of the L chain of importance in determining the affinity of the intact protein 315 for the nitrophenyl group. A predominant role for either H chain or L chain in determining antibody specificity may not be a general feature of antibody diversity. Nevertheless, an extensive study of partial sequence data from mouse myeloma proteins showed a distinct relationship between the binding specificity and the subgroup to which the V_H region could be assigned (Barstad et al., 1978). This relationship included the three DNP binding proteins 315, 460, and 25. It was also pointed out by these authors that more variation in the V_L and V_H regions may be permitted for small moieties such as the DNP group than for larger ligands. This is substantiated by the finding that either the V_H domain or V_L domain of protein 315 can combine with the V_L domain to give specificity for the DNP group. Nevertheless, it would be expected from the sequence comparisons of proteins 315, 460, and 25 that the V_H region would be of predominant importance in determining the specificity of these proteins for the DNP group. This does not appear to be the case for protein 315. The importance of the V_L domain in determining the specificity of protein 315 explains the observation that antibodies containing L chains of the uncommon λ_2 subtype, the same subtype as protein 315, are induced by DNP-keyhole limpet hemocyanin in BALB/c mice (Cotner & Eisen, 1978). The putative germ-line gene for the λ_2 V region in BALB/c mice has been sequenced (Tonegawa et al., 1978). The predicted amino acid sequence differs at only four positions from the V_L sequence of protein 315. None of these changes, at positions 38 (Ile in protein 315 becomes Val), 94 (Phe → Tyr), 95 (Arg → Ser), and 96 (Asn → Thr) is expected greatly to alter the binding properties of the V_L domain. It would therefore be expected that this putative λ_2 germ-line V-region gene, and closely related mutants, would be represented in the anti-DNP response of BALB/c mice. It is, of course, possible that particular V_H–V_L pairs are preferentially expressed. For example, both the V_λ and V_H loci are found to determine the responsiveness of mice to α(1,3)-dextran (Geckeler et al., 1976). The sequence results (Barstad et al., 1978) suggest that the V_H locus is important in determining the specificity of anti-DNP antibodies. However, in the case of protein 315, the V_H region would appear merely to complement a specificity provided largely by the V_L region. Consistent with this conclusion is the observation that the "private" idiotype of protein 315, associated with the V_H region, is not detectable in immune sera from BALB/c mice (Zeldis et al., 1979).

Significance of the Different Hapten Chemical Shift Changes on Binding of DNP-glycine and DNP-aspartate. It appears that in protein 315 the L chain is largely responsible for providing the DNP binding subsite, i.e., a tryptophan side chain in an appropriately hydrophobic environment. However, the much weaker binding to the V_L dimer compared to the Fv fragment implies a role for the H chain. It would appear likely that this involves provision of a suitable size/shape for the site together with the possibility of more specific interactions such as hydrogen bonds. It can be inferred that weaker binding of haptens to the V_L dimer results from, at least in part, less optimal shape of the site. Interactions of a hapten with the topology of a site are, however, difficult to examine. With protein 315 the shape and size of the combining sites of both the Fv fragment and V_L dimer are expected to be determined predominantly by aromatic residues. In such a case NMR can provide a sensitive means of monitoring these nondirectional interactions because of ring current effects. The shifts incurred on binding of different haptens to the Fv fragment are approximately the same, indicating similar (although not identical) interactions with the aromatic groups forming the site. Binding of DNP-glycine and DNP-aspartate to the V_L dimer, however, results in significantly different shifts which suggests differing orientations with respect to the aromatic groups in the site. Even so, the net interactions involved are energetically equivalent, as is demonstrated by the similar binding constants. Thus it seems that the weaker binding in V_L compared with Fv is accompanied by a less well-defined set of interactions with the aromatic "walls" of the site. It is possible, therefore, that our results reflect the less than optimal size/shape produced upon replacement of the V_H by another V_L domain. The specificity of antibodies for the nitrophenyl group may therefore largely be determined by the size and shape of a predominantly nonpolar combining site. An "ideal" antibody may also maximize the use of specific directional interactions, including hydrogen bonds. As suggested many years ago by Karush (1962), what is required is a high-affinity antibody to determine the upper limits of the interaction. This suggestion may now be realized by using the cell fusion technique developed by Kohler & Milstein (1975). It should then be possible to define the relative importance of these forces more precisely.

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References

Barstad, P., Hubert, J., Hunkapiller, M., Goetze, A., Schilling, J., Black, B., Eaton, B., Richards, J., Weigert, M., & Hood,

L. (1978) *Eur. J. Immunol. 8*, 497.
Cotner, T., & Eisen, H. N. (1978) *J. Exp. Med. 148*, 1388.
Dower, S. K., Wain-Hobson, S., Gettins, P., Givol, D., Jackson, W. R. C., Perkins, S. J., Sunderland, C. A., Sutton, B. J., Wright, C. E., & Dwek, R. A. (1977) *Biochem. J. 165*, 207.
Dower, S. K., Gettins, P., Jackson, R., & Dwek, R. A. (1978) *Biochem. J. 169*, 179.
Dwek, R. A. (1977) *Contemp. Top. Mol. Immunol. 6*, 1.
Dwek, R. A., Wain-Hobson, S., Dower, S., Gettins, P., Sutton, B., Perkins, S. J., & Givol, D. (1977) *Nature (London) 266*, 31.
Epp, C., Colman, P., Fehlhammer, H., Bode, W., Schiffer, M., Huber, R., & Palm, W. (1974) *Eur. J. Biochem. 45*, 513.
Freed, R. M., Rockey, J. H., & Davis, R. C. (1976) *Immunochemistry 13*, 509.
Gavish, M., Dwek, R. A., & Givol, D. (1977) *Biochemistry 16*, 3154.
Gavish, M., Dwek, R. A., & Givol, D. (1978) *Eur. J. Immunol. 8*, 42.
Geckeler, W., Blomberg, B., de Preval, C., & Cohn, M. (1976) *Cold Spring Harbor Symp. Quant. Biol. 41*, 743.
Hochman, J., Inbar, D., & Givol, D. (1973) *Biochemistry 12*, 1130.
Johnson, C. E., & Bovey, F. A. (1958) *J. Chem. Phys. 29*, 1012.
Karush, F. (1962), *Adv. Immunol. 2*, 1.
Kohler, B., & Milstein, C. (1975) *Nature (London) 256*, 495.
Padlan, E. A., Davies, D. R., Pecht, I., Givol, D., & Wright, C. E. (1976) *Cold Spring Harbor Symp. Quant. Biol. 41*, 627.
Perkins, S. J., & Dwek, R. A. (1980) *Biochemistry 2*, 245.
Perkins, S. J., Dower, S. K., Gettins, P., Wain-Hobson, S., & Dwek, R. A. (1977) *Biochem. J. 165*, 223.
Poljak, R. J., Amzel, L. M., Avey, H. P., Chen, B. L., Phizackerley, R. P., & Saul, F. (1973) *Proc. Natl. Acad. Sci. U.S.A. 70*, 3305.
Poljak, R. J., Amzel, L. M., Chen, B. L., Phizackerley, R. P., & Saul, F. (1974) *Proc. Natl. Acad. Sci. U.S.A. 71*, 3440.
Schechter, I., Ziv, E., & Licht, A. (1976) *Biochemistry 15*, 2785.
Schiffer, M., Girling, R. L., Ely, K. R., & Edmundson, A. B. (1973) *Biochemistry 12*, 4620.
Segal, D. M., Padlan, E. A., Cohen, G. H., Rudikoff, S., Potter, M., & Davies, D. R. (1974) *Proc. Natl. Acad. Sci. U.S.A. 71*, 4298.
Stevenson, G. T. (1974) *Biochem. J. 139*, 369.
Tonegawa, S., Maxam, A. M., Tizard, R., Bernhard, O., & Gilbert, W. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 1485.
Wang, B. C., Yoo, C. S., & Sax, M. (1979) *J. Mol. Biol. 129*, 657.
Wu, T. T., & Kabat, E. A. (1970) *J. Exp. Med. 132*, 211.
Zeldis, J. B., Riblet, R., Konigsberg, W. H., Richards, F. F., & Rosenstein, R. W. (1979) *Mol. Immunol. 16*, 657.

Solubilization and Characterization of Two Rat Brain Membrane-Bound Aminopeptidases Active on Met-Enkephalin†

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ABSTRACT: Two aminopeptidases which hydrolyze Met-enkephalin at the Tyr–Gly bond have been solubilized from rat brain membranes and resolved by ion-exchange chromatography. These aminopeptidases are designated MI and MII based on the order in which they are eluted during ion-exchange chromatography. The two aminopeptidases can be distinguished kinetically; aminopeptidase MI hydrolyzes L-arginine β-naphthylamide 17 times faster than L-alanine β-naphthylamide, while only a 1.7-fold difference is exhibited by aminopeptidase MII. Aminopeptidase MII exhibits a higher affinity for amino acid β-naphthylamides, Met-enkephalin, Leu-enkephalin, and the inhibitor puromycin as compared to aminopeptidase MI. Greater than 90% of aminopeptidase MII activity is lost upon dialysis against ethylenediaminetetraacetate (EDTA) but can be reconstituted with $CoCl_2$ and $MnCl_2$. In contrast, aminopeptidase MI loses only 30% of its activity when dialyzed against EDTA. In addition to cleaving the Tyr–Gly bond of Met-enkephalin, aminopeptidase MII also cleaves the Tyr–Gly bond of α- and γ-endorphin. Hydrolysis of Met-enkephalin by intact membranes derived from whole rat brain occurs primarily by cleavage at the Tyr–Gly bond, with this activity attributable to aminopeptidase MII.

Recent studies suggest that the opioid peptides Met-enkephalin (Tyr-Gly-Gly-Phe-Met) and Leu-enkephalin (Tyr-Gly-Gly-Phe-Leu) may serve as neurotransmitters (Frederickson, 1977; Smith et al., 1976). Since neurotransmitters are generally inactivated shortly after they are released, recent attention has been focused on the mechanism of inactivation of Met- and Leu-enkephalin. Three mechanisms for the inactivation of enkephalins in brain have been observed: (a) cleavage at the Gly–Phe bond by a membrane bound dipeptidyl carboxypeptidase (Malfroy et al., 1978, 1979; Sullivan et al., 1978; Swerts et al., 1979; Guyon et al., 1979; Gorenstein & Snyder, 1979); (b) cleavage at the Gly–Gly bond by a membrane-bound dipeptidyl aminopeptidase (Gorenstein & Snyder, 1979), and (c) cleavage at the Tyr–Gly bond by soluble (Craves et al., 1978; Dupont et al., 1977; Craviso & Musacchio, 1978; Hambrook et al., 1976; Marks et al., 1977; Meek et al., 1977) and membrane-bound (Jacquet et al., 1976; Knight & Klee, 1978) aminopeptidases.

The soluble aminopeptidase which cleaves the Tyr–Gly bond of enkephalins has been identified as an arylamidase and has

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