Resonance Raman-Spectroscopic Studies of the Hapten Features Involved in the Binding of 2,4-Dinitrophenyl Haptens by the Mouse Myeloma Proteins MOPC 315 and MOPC 460

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The binding of four dinitrophenyl haptens to the mouse myeloma proteins MOPC 315 IgA (immunoglobulin A) and MOPC 460 IgA was studied by resonance Raman spectroscopy. Isotopic substitution with ^{15}N and ^2H was used to assign features in the resonance Raman spectra of the free haptens. Changes in each of these features on binding to the proteins could then be attributed to interactions of the proteins' binding sites with either the *p*-NO₂ or the *o*-NO₂/amine regions of the haptens. The interactions between a given hapten and MOPC 315 IgA are often quite distinct from those between the same hapten and MOPC 460 IgA. Moreover, for both antibodies the nature of the R side chain in a Dnp-NHR (Dnp, 2,4-dinitrophenyl) compound appears to modify the interactions between the Dnp chromophore and the protein. Thus, with the haptens studied, there is no unique set of contacts between the Dnp group and the binding site. The contacts expected between ϵ -2,4-dinitrophenyl-L-lysine and the site on MOPC 315 IgA, on the basis of a recent model for this site [Dwek, Wain-Hobson, Dower, Gettins, Sutton, Perkins & Givol (1977) *Nature (London)* 266, 31-37] were not detected. However, the contacts between this hapten and the site on MOPC 460 IgA were closer to those predicted by the model for MOPC 315 IgA.

Since the discovery that the IgA protein secreted by the mouse myeloma cell line MOPC 315 has a high affinity for ϵ -Dnp-L-lysine and related compounds (Eisen *et al.*, 1968), a large volume of work has led to a detailed picture of the structure and properties of this protein. Though neither the intact protein, nor fragments prepared from it, crystallize in suitable forms for X-ray structural analysis, a three-dimensional structure was proposed for the binding-site region on the basis of the structures determined for other antibody Fab fragments taken with the sequences of the MOPC 315 IgA chains (Padlan *et al.*, 1977). This proposed structure has been refined by extensive n.m.r. and e.s.r. studies, which have been reviewed by Dwek (1977).

Resonance Raman spectroscopy can give detailed information on the structure of coloured ligands while they are in the binding sites of proteins (Carey & Schneider, 1978). Thus it may serve as a valuable complement to other physical techniques such as micro-calorimetry by indicating the particular regions

of the ligand that interact strongly with the binding site. Major ligand-site interactions are detected by the changes in the vibrational properties of the ligand that occur on binding to the protein. The suitability of Dnp compounds for resonance Raman work was shown in a previous investigation of rabbit antibodies to the Dnp determinant (Carey *et al.*, 1973). In the present work, the resonance Raman spectra of Dnp compounds used in a kinetic study (Pecht *et al.*, 1972) were characterized, and the compounds were then used to probe the Dnp-binding sites of MOPC 315 IgA and the Dnp-binding IgA from another myeloma line, MOPC 460. For both binding sites a major result is the finding that the Dnp compounds do not all interact in the same fashion.

Materials and Methods

The IgA proteins from myelomas MOPC 315 and MOPC 460 were kindly provided by Dr. David Givol and Dr. Frank F. Richards respectively, in reduced monomeric form.

Haptens

The Dnp compounds used were obtained commercially or prepared by reaction of fluorodinitro-

Abbreviations used: Dnp, 2,4-dinitrophenyl; IgA, immunoglobulin A. Subscripts H and L after residue numbers refer to the heavy and light chain respectively.

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benzene with the appropriate amine. Haptens labelled with ^{15}N were prepared as follows.

2-Nitro-4-[^{15}N]nitro-*N*-methylaniline. 2-Nitrochlorobenzene (0.9 g; 5.7 mmol) was stirred at room temperature in reagent grade conc. H_2SO_4 (5 ml) and H^{15}NO_3 (1 ml; 95.8 atom %; ICN Pharmaceuticals, Cleveland, OH, U.S.A.) was added rapidly. The exothermic reaction raised the temperature to about 70°C . The reaction mixture was heated to 100°C , by using a heat gun, and then allowed to cool to room temperature when it was poured on ice (about 100 g). After the ice had melted, the solid dinitrochlorobenzene [no 2-nitro derivative could be seen by t.l.c. on silica gel (hexane)] was collected by suction and air-dried for 4 h. The material was dissolved in methanol (10 ml) through which methylamine was bubbled for 10 min. A yellow crystalline solid began forming after a few minutes. The reaction mixture was left overnight, and the product was collected and dried. Yield 0.94 g (84 % from *o*-nitrochlorobenzene); m.p. $177\text{--}178^\circ\text{C}$ (lit. $179\text{--}180^\circ\text{C}$; Hollingsworth, 1959). The n.m.r. spectrum of the product was identical with that of authentic material and mixed m.p. showed no depression. The material was homogeneous by t.l.c. on silica gel (hexane/benzene, 1:1, v/v) (Found: C, 42.13; H, 3.66; N, 21.54; $\text{C}_7\text{H}_7\text{N}_3\text{O}_4$ requires C, 42.43; H, 3.56; N, 21.72 %). A further 0.11 g (10 %) of material was obtained on removal of methanol, suspension in water, collection and drying. This material was estimated to be an approx. 3:1 mixture of 2,6-dinitro- and 2,4-dinitro-*N*-methylaniline. These could be readily separated by preparative t.l.c. to yield the pure 2-nitro-6-[^{15}N]nitro-*N*-methylaniline, if desired.

2-[^{15}N]Nitro-4-nitro-*N*-methylaniline. 4-Iodonitrobenzene (1.25 g; 5 mmol) was suspended with stirring in conc. H_2SO_4 (5 ml) to which was added H^{15}NO_3 (1 ml). The reaction mixture was heated rapidly to 80°C with a heat gun, allowed to cool, and poured on ice (200 g). The solid product was collected and dried to give 1.26 g (86 %) of crude dinitroiodobenzene. This material was dissolved by warming in methanol (12 ml) while methylamine gas was bubbled through the solution for 10 min. The solution was allowed to cool to room temperature and the resultant solid recrystallized from methanol to give 0.58 g (59 % overall yield from 4-iodonitrobenzene), m.p. $176\text{--}178^\circ\text{C}$ (Found: C, 42.08; H, 3.56; N, 21.51; $\text{C}_7\text{H}_7\text{N}_3\text{O}_4$ requires C, 42.43; H, 3.56; N, 21.72 %).

Mass spectroscopy indicated both the 2-[^{15}N]nitro- and the 4-[^{15}N]nitro- derivatives to be over 90 % enriched in ^{15}N (parent peaks at m/e 198 for each). The ^{15}N enrichment was further demonstrated by the expected peak shifts in the i.r. and Raman spectra.

2,4-Dinitro-[^{15}N]aniline. 2,4-Dinitrochlorobenzene was dissolved in methanol and the resultant solution treated with $^{15}\text{NH}_3$ in a stoppered flask for 7 days. The reaction mixture was concentrated and

purified by preparative t.l.c. (silica gel; benzene) to give a yellow solid whose behaviour in a number of t.l.c. systems was identical with that of authentic 2,4-dinitroaniline. The structure was confirmed by a mass-spectroscopic comparison of the ^{15}N -substituted and authentic unsubstituted compounds.

Resonance Raman spectroscopy

The Raman spectrometer and sampling conditions have been described elsewhere (Kumar *et al.*, 1976). Approx. $10\ \mu\text{l}$ of protein solution was required to fill the Raman capillary cell for each binding experiment. Spectral conditions are given in the Figure legends. For the bound haptens, laser power at 441.6 nm was varied from 15 to 50 mW to provide the maximum signal-to-noise ratio in the absence of photochemical decomposition. Heating effects in the tube were minimal as evidenced by the absence of a thermal lens effect (Leite *et al.*, 1967). Laser illumination at 441.6 nm of Dnp haptens bound to MOPC 315 IgA (but not to MOPC 460 IgA) resulted in fluorescence. Therefore KI (see captions) was added to decrease the fluorescent background. KI quenches fluorescence by Van der Waals rather than charge-transfer interactions (Watkins, 1974) and addition of KI did not change the resonance Raman spectra of the free haptens (Kumar & Carey, 1975). Hence the KI should have little effect on the resonance Raman spectra of the bound haptens. To test for any possible changes in the nature of the binding, the spectra of ϵ -Dnp-L-lysine bound to MOPC 315 IgA in the presence and absence of KI were recorded. To the extent the fluorescent background allowed, the peak positions were identical (Table 1).

Since the changes on binding are small, great pains were taken to ensure that the results were reproducible. Peak positions were calibrated by using discharge lamps and are thought to be accurate to $\pm 1\text{ cm}^{-1}$. Concentrations were adjusted to ensure that, in the binding experiments, the haptens were present predominantly in the protein-bound form, and no evidence for the unbound forms was found in the resonance Raman spectra. Preliminary data from MOPC 315 IgA were in good agreement with the bulk

Table 1. Effect of KI on peak positions for ϵ -Dnp-L-lysine bound to MOPC 315 IgA

Values without KI are the average of 14 individual scans, and those with KI are the average of four individual scans.

Peak position (cm^{-1})	
Without KI	0.35 M-KI
1384	1382
1339	1337
(Not resolved)	1315

of the data recorded 1 year later on a second sample of protein, providing an additional test of reproducibility.

Results

Spectroscopic assignments

In order to understand the nature of the interactions between the haptens and the proteins, the resonance Raman spectra of the haptens alone had to be investigated to allow assignment of spectral features to groups in each molecule. Studies of the

Dnp-NHR compounds have been reported (Kumar & Carey, 1975). A major conclusion of this work was that the -NHR and -NO₂ groups give rise to a highly characteristic resonance Raman spectrum in the 1200–1400 cm⁻¹ region. The *o*-NO₂ and amino functions are best considered as one unit, the 'ortho structure', and some Raman intensity enhancement is due to a change in this ortho structure in going from the electronic ground state to the excited state.

Fig. 1. Pre-resonance Raman spectra of Dnp-NH₂ and Dnp-NHCH₃: ²H effects

(a) Dnp-NH₂ (approx. 1 mM) in ²H₂O (+20% C²H₃CN) and H₂O (+20% C²H₃CN). (b) Dnp-NHCH₃, (approx.) 1 mM) in ²H₂O (+16% C²H₃CN) and H₂O (+16% C²H₃CN). Spectral conditions in both parts: 457.9 nm excitation, 90 mW, 9 cm⁻¹ spectral slit. Features attributable to solvent are indicated by S.

Fig. 2. Pre-resonance Raman spectra of Dnp-NHCH₃: ¹⁵N effects

Spectra of unlabelled Dnp-NHCH₃ (b) and Dnp-NHCH₃ separately labelled with ¹⁵N at the *p*-NO₂ (a) and *o*-NO₂ (c). Concentrations were approx. 50 mM in C²H₃CN, which was chosen in view of its peak positions, and did not cause deuterium exchange. Spectral conditions were 514.5 nm excitation, 90 mW, 9 cm⁻¹ spectral slit. Features attributable to solvent are indicated by S. In (c), 6* and 12* indicate 6 and 12 cm⁻¹ decreases in the 1370 and 1276 cm⁻¹ features respectively.

Further assignments can be made by comparing the spectra of ^{15}N -labelled compounds and spectra in $^2\text{H}_2\text{O}$ solutions with the spectra of the unlabelled compounds. Although the previous spectroscopic study emphasized the coupled and 'mixed' nature of the resonance Raman features, it is possible to identify bands associated with the ortho structure and others associated with the $p\text{-NO}_2$ group, from the isotopic substitution experiments. Moreover it appears that perturbations of the ortho structure do not affect measurably the $p\text{-NO}_2$ modes, and vice versa. It is therefore possible to monitor independently perturbations of the ortho structure and of the $p\text{-NO}_2$ group in binding studies.

On dissolving the haptens in $^2\text{H}_2\text{O}$, the labile protons of the amine groups are replaced by ^2H . The effects of this substitution on the spectra of Dnp-NH $_2$ and Dnp-NHCH $_3$ are shown in Fig. 1. The ϵ -Dnp-L-lysine gave results (not shown) essentially identical with those obtained with Dnp-NHCH $_3$. In the key 1200–1400 cm^{-1} region both compounds in Fig. 1 show similar changes. On deuteration a fairly broad feature near 1280 cm^{-1} moves to near 1295 cm^{-1} and a shoulder at 1354 cm^{-1} ($-\text{NH}_2$) and a peak at 1315 cm^{-1} ($-\text{NHCH}_3$) move, probably to higher wavenumbers. For Dnp-NH $_2$ the 1392 cm^{-1} peak is replaced, on deuteration, by a doublet at 1365 and 1380 cm^{-1} . For Dnp-NHCH $_3$ the position of the 1370 cm^{-1} feature is unchanged on deuteration, and it is thought that its intensity is increased by the contribution from the shifted 1315 cm^{-1} feature. Crucially the band near 1340 cm^{-1} for both compounds remains unperturbed.

This band is therefore associated with the $p\text{-NO}_2$ group, a conclusion substantiated by ^{15}N substitution.

Fig. 2 shows the changes occurring in the resonance Raman spectrum of Dnp-NHCH $_3$ on separately substituting ^{15}N in the o - and $p\text{-NO}_2$ groups. In the $o\text{-NO}_2$ case, the 1370 and 1276 cm^{-1} bands decrease on ^{15}N substitution by 6 and 12 cm^{-1} respectively and the 1340 cm^{-1} band does not alter. In the $p\text{-NO}_2$ case, the 1340 and 1318 cm^{-1} bands are replaced by an intense feature at 1308 cm^{-1} on ^{15}N substitution, and the 1370 cm^{-1} band does not alter.

Substitution of ^{15}N in the amino group of Dnp-NH $_2$ caused no frequency or intensity changes, with the possible exception of a slight change in a shoulder at 1354 cm^{-1} .

Drawing these observations together it is possible to make the following assignments. For Dnp-NHCH $_3$ the 1370, 1317 and 1275 cm^{-1} bands are associated with the ortho ($-\text{NHCH}_3$, $-\text{NO}_2$) structure, whereas the 1340 cm^{-1} feature is associated with the $p\text{-NO}_2$ group. Similarly, by analogy, for Dnp-NH $_2$, the 1340 cm^{-1} peak has contributions from the $p\text{-NO}_2$ group, whereas the 1392, 1354 and 1282 cm^{-1} bands are associated with the ortho structure. Based on ^{15}N substitution in Dnp-NH $_2$ the spectra do not seem to have a major contribution from the phenyl-N bond in the Ph-NH $_2$ or Ph-NHCH $_3$ groups. The close resemblance between the spectra of Dnp-NHCH $_3$ and ϵ -Dnp-L-lysine allows the Dnp-NHCH $_3$ assignments to be transferred to the chromophore of the lysine derivative with some confidence. The assignments are given in Table 2.

Table 2. Dnp hapten spectral features and effects of binding to MOPC 315 and MOPC 460 IgA

All measurements are in cm^{-1} , and were taken in phosphate buffer. Abbreviations: NC, no perceptible change; NC ν , Int $\downarrow$, no change in band frequency but a diminution in relative intensity; o, feature from the $-\text{NHR}/o\text{-NO}_2$ ortho structure; p, feature associated with the $p\text{-NO}_2$ group; D, ortho structure feature sensitive to deuteration; broad, feature too broad for exact wavelength to be determined; sh, shoulder.

Hapten	Unbound	Bound to MOPC 315	Bound to MOPC 460
Dnp-NH $_2$	1393 o, D	NC	NC
	1354 sh, o, D	NC	NC
	1338 p	1331	1325
	1280 o, D	1268	Broad
Dnp-NHCH $_3$	1371 o	NC	NC ν , Int $\downarrow$
	1340 p	NC	NC ν , Int $\downarrow$
	1316 o, D	1308	1305
	1272 o, D	broad	NC
ϵ -Dnp-L-lysine	1375 o	1382	1384
	1338 p	NC	1327
	1315 o, D	NC	1302
	1275 o, D	Broad	Broad
Dnp-N(CH $_3$) $_2$	1367 sh	NC	NC
	1330	1323	1322
	1310	1306	1303

Fig. 3. Pre-resonance Raman spectra of Dnp-NHCH₃ and ϵ -Dnp-L-lysine binding to MOPC 315 IgA. -NHCH₃ is Dnp-NHCH₃ (0.1 mM) in H₂O. -NHCH₃+MOPC 315 is Dnp-NHCH₃ (35 μ M) bound to MOPC 315 IgA (80 μ M). Dnp-lysine is ϵ -Dnp-L-lysine (0.3 mM) in H₂O. Dnp-lysine+MOPC 315 is ϵ -Dnp-L-lysine (0.15 mM) bound to MOPC 315 IgA (0.2 mM). All solutions were in 0.15 M-NaCl/0.01 M-phosphate buffer, pH 6.9, with MOPC 315 IgA solutions containing 0.35 M-KI to quench fluorescence. Spectral conditions were 441.6 nm excitation, 40 mW, spectral slit approx. 9 cm⁻¹.

Effects of binding to MOPC 315 and MOPC 460 IgA

The changes in the resonance Raman spectra of the Dnp haptens on binding to MOPC 315 and MOPC 460 IgA are shown in Figs. 3–6 and summarized in Table 2. In general the frequency shifts and intensity changes are small. They are, however, reproducible and outside experimental error (see the Materials and Methods section).

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Fig. 4. Pre-resonance Raman spectra of Dnp-NH₂ and Dnp-N(CH₃)₂ binding to MOPC 315 IgA. -NH₂ is Dnp-NH₂ (0.5 mM) in H₂O. -NH₂+MOPC 315 is Dnp-NH₂ (0.1 mM) bound to MOPC 315 IgA (0.2 mM). -N(CH₃)₂ is Dnp-N(CH₃)₂ (80 μ M) in H₂O. -N(CH₃)₂+MOPC 315 is Dnp-N(CH₃)₂ (0.1 mM) bound to MOPC 315 IgA (0.12 mM). All solutions were in 0.15 M-NaCl/0.01 M-phosphate buffer, pH 7.0, with MOPC 315 IgA solutions having 0.25 M-KI added to quench fluorescence. Spectral conditions: 441.6 nm excitation, approx. 30 mW, 9 cm⁻¹ spectral slit.

Two generalizations are immediately apparent from Table 2. The interactions between a given hapten and MOPC 315 IgA are often quite distinct from those between the same hapten and MOPC 460 IgA. For example, with ϵ -Dnp-L-lysine on binding to MOPC 315 IgA the protein-hapten contacts only perturb the 1375 cm⁻¹ band, whereas the 1375 cm⁻¹

Fig. 5. Pre-resonance Raman spectra of Dnp-NHCH₃ and ϵ -Dnp-L-lysine binding to MOPC 460 IgA. -NHCH₃ is Dnp-NHCH₃ (50 μ M) in H₂O. -NHCH₃ + MOPC 460 is Dnp-NHCH₃ (50 μ M) bound to MOPC 460 IgA (0.15 mM). Dnp-lysine is ϵ -Dnp-L-lysine (0.15 mM) in H₂O. Dnp-lysine + MOPC 460 is ϵ -Dnp-L-lysine (0.1 mM) bound to MOPC 460 IgA (0.3 mM). All solutions were in 0.15 M-NaCl/0.01 M-phosphate buffer, pH 7.2. Spectral conditions: 441.6 nm excitation, approx. 20 mW, 9 cm⁻¹ spectral slit.

and the 1338 and 1315 cm⁻¹ features are perturbed when this hapten binds to MOPC 460 IgA. The second and more important generalization is that for both antibodies, the nature of the R side chain in a Dnp-NHR compound appears to modify the contacts between the chromophore and the protein. Thus, for MOPC 315 IgA, the 1338 cm⁻¹ feature of Dnp-NH₂, ascribed to the *p*-NO₂ group, goes to lower wavenumber in the bound form, whereas for Dnp-

Fig. 6. Pre-resonance Raman spectra of Dnp-NH₂ and Dnp-N(CH₃)₂ binding to MOPC 460 IgA. -NH₂ is Dnp-NH₂ (0.1 mM) in H₂O. -NH₂ + MOPC 460 is Dnp-NH₂ (50 μ M) bound to MOPC 460 IgA (0.15 mM). -N(CH₃)₂ is Dnp-N(CH₃)₂ (0.5 mM) in H₂O. -N(CH₃)₂ + MOPC 460 is Dnp-N(CH₃)₂ (0.13 mM) bound to MOPC 460 IgA (0.2 mM). All solutions were in 0.15 M-NaCl/0.01 M-phosphate buffer, pH 7.2. Spectral conditions: 441.6 nm excitation, 15 mW for Dnp-NH₂ and 50 mW for Dnp-N(CH₃)₂, 9 cm⁻¹ spectral slit.

NHCH₃ and ϵ -Dnp-L-lysine analogues there is no evidence for a perturbation affecting the *p*-NO₂ group. Furthermore, the 1316 cm⁻¹ peak in Dnp-NHCH₃, associated with the ortho structure and sensitive to ²H substitution, shifts by 8 cm⁻¹ on binding, whereas the analogous 1315 cm⁻¹ band in ϵ -Dnp-L-lysine is unaffected by binding. Again the 1371 cm⁻¹ peak in the Dnp-NHCH₃ case, which has some *o*-NO₂ character but shows no dependence on ²H substitution (above), does not shift on binding, whereas the analogous peak in ϵ -Dnp-L-lysine does.

Similar trends are noted for MOPC 460 IgA. The peak near 1340cm^{-1} associated with the *p*-NO₂ group in Dnp-NHCH₃ is not affected by binding to the protein, but the same feature does move when ϵ -Dnp-L-lysine or Dnp-NH₂ is bound. This suggests that in the latter two compounds the contact between the *p*-NO₂ group and the protein perturbs the -NO₂ to a greater extent than in Dnp-NHCH₃. It is noteworthy that the *p*-NO₂ group in ϵ -Dnp-L-lysine appears to have a significant contact in MOPC 460 IgA, but not in MOPC 315 IgA.

Spectroscopically, Dnp-N(CH₃)₂ represents a different hapten. It cannot form the hydrogen bond in the ortho structure and, moreover, steric hindrance is likely to twist the *o*-NO₂ group out of the plane of the phenyl ring. Tentatively we assign the 1330 and 1310cm^{-1} peaks to features associated with *o*- and *p*-NO₂ groups, respectively, although in the absence of isotopic substitutions the assignments carry much less weight than for the foregoing haptens. Both the assigned features are altered when Dnp-N(CH₃)₂ is bound by the proteins, indicating significant contacts with both NO₂ groups occurs in the binding sites on MOPC 315 and MOPC 460 IgA.

Discussion

In Table 3 the major contacts that were detected by the resonance Raman technique, when the four Dnp haptens were bound by MOPC 315 and MOPC 460 IgA, are indicated. It is evident that the interactions with ϵ -Dnp-L-lysine are different for the two proteins, with strong interaction at the *p*-NO₂ group occurring with MOPC 460 IgA but not with MOPC 315 IgA. The other three haptens are each bound similarly by the two proteins, though the spectral changes are usually larger with MOPC 460 IgA. More importantly, for both proteins the four haptens appear to have different contacts with the sites, indicating that at the level of atomic contacts, quite small changes in -NHR (e.g. -NH₂ $\rightarrow$ -NHCH₃) seriously change the Dnp-protein interactions.

Table 3. Major Dnp-hapten interactions with binding sites on MOPC 315 IgA and MOPC 460 IgA

Splitting of the ortho structure features into amine and *o*-NO₂ contributions is only approximate; see the text.

Hapten	MOPC 315 IgA	MOPC 460 IgA
Dnp-NH ₂	<i>p</i> -NO ₂ *	<i>p</i> -NO ₂
Dnp-NHCH ₃	Amine group	Amine group
ϵ -Dnp-L-lysine	<i>o</i> -NO ₂	Amine group <i>o</i> - and <i>p</i> -NO ₂
Dnp-N(CH ₃) ₂	<i>o</i> - and <i>p</i> -NO ₂	<i>o</i> - and <i>p</i> -NO ₂

* A shift in the sensitive 1280cm^{-1} peak (Table 2) indicates weak interactions about the ortho structure.

In constructing their model of the site on MOPC 315IgA, Haselkorn *et al.* (1974) pointed out that it was assumed that no changes in the binding of the Dnp portion occurred when -NHR was altered. Their model is not invalidated by our finding that this assumption is untenable, since it was used to obtain a general description of the site, and not to assign energetic contributions to different regions of the site. In n.m.r. experiments, Dower *et al.* (1977) found the aromatic protons of Dnp-O⁻, Dnp-glycine and Dnp-L-aspartate experienced similar changes in chemical shift on binding to MOPC 315 IgA and concluded they were being bound in a similar fashion. Since we have not examined any of these haptens by resonance Raman spectroscopy, we cannot confirm or refute their conclusion.

The variation in binding affinities of different Dnp compounds (greater than 10^3 in K_{ass}) has pointed to a large role for the R group in Dnp-NHR haptens. The resonance Raman results show that this arises not only from direct interaction between the site and the R group but also from modulation of the binding about the Dnp group.

In seeking to understand the causes of the observed frequency shifts, we proceed on the basis of four tenets: (1) Raman peak positions are a property of the ground electronic state of the Dnp chromophore, (2) hydrophobic and charge-transfer interactions will not cause frequency shifts, (3) distortion within the π electron frame and interactions between charged amino acid side chains and chromophores will change peak positions, (4) strong hydrogen bonds in a protein environment may change peak positions.

Rules (2) and (3) were set out in an earlier publication on the binding of Dnp haptens by rabbit antibodies (Carey *et al.*, 1973), and subsequent experience with chromophoric ligands in enzyme-inhibitor and enzyme-substrate (Carey & Schneider, 1978) situations has reinforced their validity. Hydrophobic forces may be important in the overall energetics of binding; however, in general they do not perturb vibrational peak positions. Similarly there is good evidence that charge transfer interactions of the type occurring in Dnp antibody sites are not responsible for Dnp vibrational peak shifts (Carey *et al.*, 1973; Larkindale & Simkin, 1972). One modification to the original concept has to be made, namely the inclusion of strong hydrogen bonds. In a protein environment, strong hydrogen bonds to protein groups may be capable of changing Dnp vibrational states. In general terms, for a shift of several cm^{-1} to occur on binding of a hapten the forces involved must be of medium strength and fairly localized (Kumar *et al.*, 1976).

Most of the frequency shifts observed on binding are to lower wavenumbers. For the feature associated with the *p*-NO₂ group this is consistent with a decrease in bond order concomitant with an increase in

importance of canonical forms of the type

where the extra positive charge may be delocalized over the rest of the hapten. This effect would follow from the presence of a positively charged amino acid side chain near the oxygen atoms or a strong hydrogen bond to the nitro group. The situation with regard to those peaks assigned to the 'ortho structure' is more complex; vibrationally they are highly coupled, with the added complication of steric interactions between the -NO₂ and -NHR groups competing with contacts with the proteins.

Although most models of the Dnp hapten structure assume there is a strong hydrogen bond between the *o*-NO₂ and -NHR groups, this is probably not the case (Kumar & Carey, 1975). Although its quantitative nature has been the source of much discussion (Dyall, 1961; Moritz, 1962), it does seem certain that such an interaction would be much weaker than the intramolecular hydrogen bond in *o*-nitrophenol (Pauling, 1960). The hapten's intramolecular hydrogen bond is important in the excited electronic state, but that does not concern us here.

In view of these complexities one can only tentatively assign 'ortho' frequency shifts to the effects of particular contacts. Nevertheless, since ϵ -Dnp-L-lysine is the strongest hapten for the proteins, the results obtained with it are worth considering further. The peak near 1373 cm⁻¹ obtained for ϵ -Dnp-L-lysine and Dnp-NHCH₃ is ascribed to the ortho structure (from the behaviour of the 2-¹⁵NO₂ compound) but is not sensitive to deuteration. For ϵ -Dnp-L-lysine binding to MOPC 315 IgA, the peak is perturbed, whereas for Dnp-NHCH₃ it is not. Conversely the 1316 cm⁻¹ peak, an ortho structure vibration that is sensitive to deuteration, is unperturbed when Dnp-lysine is bound, but the equivalent peak for Dnp-NHCH₃ shifts to 1308 cm⁻¹. Thus although detectable changes caused by contacts about the ortho structure are seen for both compounds, their precise nature seems to be different. For ϵ -Dnp-L-lysine the contact affects the mode more associated with the -NO₂ group, whereas for Dnp-NHCH₃ the contact affects the mode sensitive to ²H substitution.

The simple site model derived for MOPC 315 IgA on the basis of kinetic experiments (Haselkorn *et al.*, 1974) can help explain some of the observations. This model identifies four 'subsites of interaction' within the binding site, namely the Dnp subsite, S₁, hydrophobic subsites, S₂ and S₃, and a positive subsite, S₄. A major role for subsite S₂ could explain the above results if hydrophobic forces generated at this point with haptens such as ϵ -Dnp-L-lysine and Dnp-NHCH₃ were sufficiently attractive that the hapten is drawn toward subsite S₂ in MOPC 315 IgA so that the

contacts about the Dnp portion, in particular those about the *p*-NO₂ group, are changed from those in Dnp-NH₂. The retention of *p*-NO₂ contacts in Dnp-N(CH₃)₂ would arise from the rather different ortho structure in this hapten causing poorer interaction with subsite S₂, or a very different Dnp orientation (see below). The thermodynamic measurements of Johnston *et al.* (1974) also point to a major role for subsite S₂; for example, Dnp-glycine is a poor hapten, perhaps owing to the placement of the charged carboxylate group in the hydrophobic environment of subsite S₂.

The binding of ϵ -Dnp-L-lysine as revealed by the resonance Raman experiments can be compared with the effects predicted by the MOPC 315 IgA binding-site model of Dwek *et al.* (1977) and Dower *et al.* (1977). This work has implicated particular residues as being important for the binding of the Dnp portion of the haptens, but a definite scheme for the interaction of this group with the amino acid residues could not be deduced. The resonance Raman results presented here suggest that at the level of atomic contacts there is not one unique scheme for the Dnp-binding region. The orientations of the site residues may vary or different residues may interact with the Dnp, depending on the nature of the R group. For ϵ -Dnp-L-lysine, which has the greatest affinity for MOPC 315 IgA of the Dnp compounds studied, the interactions postulated for the *p*-NO₂ group on the basis of the n.m.r. work were hydrogen-bonding to Tyr-34_L and Asn-36_L. Depending on the orientation of the group the *o*-NO₂ group might also interact with these hydrogen-bond donors. The positive charge of Arg-95_L was also thought to interact with the side chain of the hapten, and Trp-93_L with the aromatic ring. Although the charge interaction with Arg-95_L might give effects detectable by resonance Raman spectroscopy, the charge-transfer interaction with Trp-93_L will not, though it is responsible for other spectroscopic effects, such as induced circular dichroism (Rockey *et al.*, 1971) and fluorescence quenching (Eisen *et al.*, 1968).

The Raman spectra indicate that the hydrogen-bond interaction with the *p*-NO₂ group may take place with Dnp-NH₂ and Dnp-N(CH₃)₂, but not with ϵ -Dnp-L-lysine. The only compound in which simultaneous contact with both NO₂ groups of the hydrogen-bond donors may occur is Dnp-N(CH₃)₂. The Arg-95_L contributes a positive charge, which might be the cause of the changes shown in the *p*-NO₂ group vibrations of Dnp-NH₂ or Dnp-N(CH₃)₂, or those of the *o*-NO₂ group of ϵ -Dnp-L-lysine and Dnp-N(CH₃)₂. Since Dnp-glycine is a poor hapten, charge interaction of its carboxylate group with Arg-95_L is apparently not occurring, hence Arg-95_L is not close to the -NHR part of the ortho structure.

No site model is available for MOPC 460 IgA, since its amino acid sequence has not been determined

in full; what sequence data are available (Haimovich *et al.*, 1972) point to differences in the hypervariable regions of the light chains. E.s.r. experiments have indicated that MOPC 460 IgA has a site similar in dimensions to that of MOPC 315 IgA (Willan *et al.*, 1977). The affinity of MOPC 460 IgA for Dnp ligands is overall lower than that of MOPC 315 IgA. Despite this, the observed shifts in vibrational peak positions in the resonance Raman spectra were greater for MOPC 460 IgA than for MOPC 315 IgA. A simple explanation would be a lesser role in binding of the S₂ region in MOPC 460 IgA. The pattern of interactions shown by the spectra are actually more like those predicted by the MOPC 315 model (Dwek *et al.*, 1977; Dower *et al.*, 1977) than MOPC 315 IgA itself showed, with three of the four haptens having significant interaction about the *p*-NO₂ group, and simultaneous *o*- and *p*-NO₂ interactions occurred in two of the haptens.

Traditionally, work using hapten analogues to probe the combining sites of antibodies raised with chemically defined antigenic determinants has tended to view the site as a relatively rigid structure. However, there is spectroscopic evidence for conformational changes in the combining site on hapten binding, e.g. the c.d. work of Holowka *et al.* (1972). Taken with the present data, this leads to a view of hapten binding by a site as a mutual-fit process, with the actual conformation of the site and the major hapten-site contacts varying subtly with changes in the hapten structure. Such a situation is in accord with current views of protein binding sites in general (Burgen *et al.*, 1975; Williams, 1977).

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