The variability of nitro group–protein interaction in the 2,4-dinitrophenyl-binding antibodies M315, M460 and X25 investigated by resonance Raman spectroscopy

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Pre-resonance Raman spectroscopy was used to study the interactions of the nitro groups of dinitrophenyl haptens with three dinitrophenyl-binding antibody fragments: M315 Fv, M460 Fab' and X25 Fab'. The observed changes in frequency of modes associated with the nitro moieties are compared with solvent-induced changes for the model hapten 2,4-dinitroaniline. These comparisons demonstrate a specific interaction via the $\text{H}_2\text{N}-\text{C}-\text{C}-2-\text{NO}_2$ and $4-\text{NO}_2$ groups with the protein. The interaction with the $4-\text{NO}_2$ group appears to be absent for ϵ -N-2,4-dinitrophenyl-L-lysine bound to M315 Fv fragment in contrast with either 2,4-dinitrophenylaspartate or 2,4-dinitrophenylglycine bound to M315 Fv fragment, despite the much tighter binding of the lysine derivative. The implications of this for M315 Fv fragment in terms of the antibody specificity are discussed. Comparisons of the effect of binding to M460 Fab' and X25 Fab' fragments also revealed significant differences in the shifts of the nitro group vibrations of 2,4-dinitrophenyl-lysine and 2,4-dinitroaniline.

On a simple model of an antibody combining site, the total energy of association of a hapten can be considered as the sum of the individual contributions of each complementary group. The affinity of dinitrophenyl (Dnp) haptens for Dnp-binding antibodies is considered as the sum of a constant Dnp contribution and a side-chain contribution. The nature of the binding of Dnp haptens has been investigated by thermodynamic studies (Gettins *et al.*, 1978), by ^{15}N n.m.r. (Gettins *et al.*, 1980), by ^1H n.m.r. (Dower *et al.*, 1977), by ^{19}F n.m.r. (Kooistra & Richards, 1978; Hardy & Richards, 1978) and by R.R. spectroscopy (Kumar *et al.*, 1978). Of particular importance in testing this binding model was the evidence from R.R. spectroscopy that the nature of the R side chain in a Dnp-NHR hapten modifies the interaction between

the Dnp moiety and the protein. In addition, Kumar *et al.* (1978) showed that the interactions of ϵ -Dnp-L-lysine with MOPC 315 IgA are quite distinct from those with MOPC 460 IgA. Thus the R.R. results call for a more sophisticated binding model for Dnp haptens.

Evidence both from n.m.r. (Dower *et al.*, 1977) and R.R. spectroscopy (Kumar *et al.*, 1978) has been cited in support of hydrogen-bonding between some Dnp-haptens and antibody proteins. These authors reported R.R. spectra of Dnp-NH₂, Dnp-NHCH₃, Dnp-N(CH₃)₂ and Dnp- ϵ -L-lysine bound to MOPC 315 IgA and MOPC 460 IgA. N.m.r. spectra of Dnp-L-aspartate, Dnp-glycine, Dnp-O⁻ and 5,7-dinitronaphthalene-2-sulphonate bound to MOPC 315 and X25 antibodies have been reported in detail, and less-detailed investigations of ϵ -Dnp-L-lysine and Dnp-NH₂ have also been reported (Dower *et al.*, 1977; Wain-Hobson, 1977).

In the present study we have extended the R.R. work of Kumar *et al.* (1978) on protein M315 to include the haptens studied in detail by n.m.r. (Dnp-glycine, Dnp-L-aspartate and 5,7-dinitronaphthalene-2-sulphonate). Additionally we have studied the binding of the haptens Dnp-NH₂ and Dnp- ϵ -L-lysine to another Dnp-binding protein: X25. We

Abbreviations used: Dnp, 2,4-dinitrophenyl; IgA, immunoglobulin A; Fab' fragment, light chain and N-terminal half of heavy chain; Fv fragment, variable region of heavy chain and light chain; subscripts H and L after residue numbers refer to heavy and light chains respectively; R.R., resonance Raman.

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have also investigated the solvent-dependence of the R.R. spectrum of Dnp-NH₂ in an attempt to deduce the nature of its interactions in solution and hence its binding to antibody proteins.

Materials and methods

Haptens

Dnp-NH₂, ϵ -Dnp-L-lysine, Dnp-L-aspartate, Dnp-glycine and 5,7-dinitronaphthalene-2-sulphonate were all purchased from BDH Chemicals (Poole, Dorset, U.K.). Hapten solutions of concentration 10 mM were employed for the solvent-dependence experiments unless the solutions were saturated at lower concentration. The solvents were distilled before use. For the protein-binding experiments 3 mM solutions were employed.

Antibody fragments

The Fv fragment of protein M315 was prepared by the modification of the method of Inbar *et al.* (1972) described by Gettins *et al.* (1980). The Fab' fragments of proteins M460 and X25 were prepared by pepsin digestion of IgA monomer at pH 4.5 (Inbar *et al.*, 1971). The use of Fv and Fab' fragments enabled concentrations of 3 mM to be obtained rather than those of 0.16–0.4 mM for the IgA as employed by Kumar *et al.* (1978). Protein solutions were made by dissolving the freeze-dried antibody in 50 mM-sodium phosphate buffer, pH 6.9, containing 150 mM-NaCl and 350 mM-KI to quench unwanted protein fluorescence (Watkins, 1974). Hapten solutions, unless stated otherwise, were made in the same buffer, but without KI, which adversely affects the solubility of some of the haptens studied and whose presence does not change the Raman spectra of the unbound ligands (Kumar & Carey, 1975).

Raman spectroscopy

Carey and co-workers have shown with rabbit anti-Dnp antibodies (Carey *et al.*, 1973) and more recently with proteins M315 and M460 (Kumar *et al.*, 1978) that the nitro groups and NHR group of 2,4-dinitroaniline can be selectively studied while bound to antibody, by using the pre-resonance enhancement effect on Raman spectra (Johnson & Peticolas, 1976).

Pre-resonance laser Raman experiments were performed on a SPEX Ramalog 5 spectrometer with illumination from a Spectra Physics 164 CW argon ion laser. Light of wavelength 476.5 nm was used to excite spectra from a thin-walled capillary containing about 10 μ l of solution (cf. 441.6 nm excitation used by Kumar *et al.*, 1978). The absorption maxima of the haptens are in the range 340–360 nm (Ashman & Metzger, 1969).

The spectrometer was calibrated before each run

by using the 459 cm⁻¹ band of CCl₄. Excellent reproducibility, to within 1 cm⁻¹, was obtained. A spectral slit of 8 cm⁻¹ was used. Output powers in the range 50–100 mW for the protein solutions and up to 160 mW for the haptens were used and did not give rise to overheating.

Band assignments in R.R. spectra

The R.R. spectrum of Dnp-NH₂ shows four bands between 1250 and 1400 cm⁻¹, which have been assigned to vibrations involving the NO₂ and NH₂ groups (Kumar & Carey, 1975; Kumar *et al.*, 1978). Kumar *et al.* (1978) showed by isotopic substitution that the band at 1337 cm⁻¹ arises from the 4(*p*)-NO₂ group, whereas the other three are associated with the H₂N-C-C-NO₂ ensemble. Thus effects on the 4-NO₂ group can be observed independently, whereas changes in the 2-NO₂ group and the NH₂ group cannot be distinguished. All the other Dnp-NHR compounds examined by Kumar *et al.* (1978) and by us exhibit a band within 7 cm⁻¹ of 1337 cm⁻¹. We here follow the argument of Kumar *et al.* (1978) that this band can be assigned to the 4-NO₂ group. However, limitations to the separability of the vibrational modes are pointed out below. Since changes in R.R. intensities are not readily interpreted, we indicate only major effects in the schemes.

Results

Effect of solvent on the R.R. spectra of Dnp-NH₂

The R.R. spectra of Dnp-NH₂ observed between 1250 and 1400 cm⁻¹ in 11 solvents with a wide range of dielectric constants and hydrogen-bonding characteristics are shown schematically in Fig. 1. The two highest-frequency bands of the H₂N-

Fig. 1. Solvent-dependence of the R.R. bands of 2,4-dinitroaniline between 1250 and 1400 cm⁻¹. A dash indicates that the band is obscured.

C–C–NO₂ ensemble (~ 1380 and $\sim 1350\text{cm}^{-1}$) show appreciable solvent-dependence with a range of 18 and 10cm^{-1} respectively. The shifts of these bands correlate approximately with dielectric constant and hydrogen-bonding strength, and imply that this ensemble interacts significantly with the solvent via Van der Waals and/or hydrogen-bond forces. The lowest-frequency band of the H₂N–C–C–NO₂ ensemble ($\sim 1280\text{cm}^{-1}$) and the band of the 4-NO₂ group ($\sim 1337\text{cm}^{-1}$) show much smaller shifts (range 6 and 2cm^{-1} respectively) in solvents ranging from benzene through acetonitrile to water. However, much larger shifts to low frequency (range 18cm^{-1} and 11cm^{-1} from CCl₄ respectively) are observed in carbonyl solvents. These effects are maximized in *NN*-dimethylformamide and *NN*-dimethylacetamide. The origin of these shifts in carbonyl solvents is unclear, but they do provide a measure for comparison with the shifts observed on protein binding.

Binding of Dnp-NH₂ to antibody proteins

The simplest hapten investigated by us and Kumar *et al.* (1978) was Dnp-NH₂. Fig. 2 shows the Raman spectra before and after binding to M315 Fv

fragment (see Fig. 4 of Kumar *et al.*, 1978). Fig. 3 summarizes the data for Dnp-NH₂ binding to M315 Fv, M460 Fab' and X25 Fab' fragments together with the band maxima for H₂O and CCl₄ solution. Our measurements of the free hapten in aqueous buffer agree with the published ones to within 1cm^{-1} . However, there are small differences in two bands between our measurements of M315 Fv fragment and those by Kumar *et al.* (1978) of M315 IgA, which may result from the better signal-to-noise ratio in our experiments from use of the more soluble Fv fragment.

The magnitude of shifts in frequencies on protein binding depends considerably on the choice of reference solvent. For this reason we have shown the frequencies of Dnp-NH₂ in two very different solvents in Fig. 3(a). Most of our conclusions are drawn relative to the frequencies observed in H₂O; they are not on an absolute scale.

This limitation is least important for the 4(*p*)-NO₂ vibration of Dnp-NH₂, which shifts about 6cm^{-1} to low frequency relative to a range of solvents on binding to M315 Fv fragment. The shifts of the other bands are less than 5cm^{-1} relative to H₂O or CH₃OH, but range up to 15cm^{-1} relative to CCl₄ or

Fig. 2. R.R. spectra of 2,4-dinitroaniline before and after binding to the Fv fragment of protein M315

C_6H_6 . The position of the 4- NO_2 band is not matched by the position in solvents such as H_2O or CCl_4 , suggesting that we are observing a specific interaction not available in these solvents. The position of all three bands of the $NH_2 \cdots 2-NO_2$ groups are considerably close to those of the H_2O than CCl_4 . Since these shifts are quite substantial ($>14\text{ cm}^{-1}$ from CCl_4 , $<6\text{ cm}^{-1}$ from H_2O), this represents strong evidence for a polar and probably hydrogen-bonding environment for this moiety. At this level the conclusions are equally applicable to the fragments of proteins X25, M460 and M315.

Comparison of binding effects of X25 Fab', M460 Fab' and M315 Fv fragments

Similar comparisons between spectra in different solvents are not possible for Dnp-substituted amino acids because of their insolubility in non-aqueous solvents. An alternative approach is to analyse the shifts produced by a given hapten on a variety of proteins. Comparison of the band positions in the spectra of Dnp- NH_2 bound to X25 Fab', M460 Fab' and M315 Fv fragments (Fig. 3) shows no qualitative differences. The most conspicuous differ-

ences between the proteins lie in the large low-frequency shift of the 4- NO_2 band and the 1280 cm^{-1} band of Dnp- NH_2 bound to M460 Fab' fragment.

The behaviour of Dnp-lysine (Fig. 3b) on binding to the three different protein fragments shows a much more marked variation. The 4- NO_2 band ($\sim 1337\text{ cm}^{-1}$) shifts to high frequency relative to H_2O on binding to M315 Fv fragment, shifts to low frequency on binding M460 Fab' fragment and does not shift at all on binding X25 Fab' fragment. The largest shift in the 2- NO_2 bands on binding to X25 Fab' fragment is in the 1280 cm^{-1} band ($+5\text{ cm}^{-1}$), whereas the largest shift for M315 Fv fragment is in the 1370 cm^{-1} band ($+8\text{ cm}^{-1}$). M460 Fab' fragment induces sizable shifts in the 1311 cm^{-1} (-9 cm^{-1}) band. These shifts are not readily interpretable at a molecular level, but do highlight the diversity of the interactions of Dnp-lysine.

Results on the binding of Dnp-glycine were obtained only for M315 Fv and M460 Fab' fragments (Fig. 3c). No major differences were observed in the shifts.

Binding of Dnp haptens to particular proteins

M315 Fv fragment. The shifts relative to H_2O observed in the R.R. spectra of five haptens on binding M315 Fv fragments are summarized in Fig. 4. [The results obtained by Kumar *et al.* (1978) are also indicated.] All the haptens except Dnp-lysine show a low-frequency shift in the 4- NO_2 ($\sim 1330\text{ cm}^{-1}$) band on binding M315 Fv fragment. Dnp-lysine is also unique in inducing a substantial ($+8\text{ cm}^{-1}$) shift in the high-frequency band of the 2- NO_2 group. The binding of Dnp-lysine therefore appears distinct from that of the group of haptens Dnp-glycine, Dnp-aspartate, Dnp- NH_2 and 5,7-dinitronaphthalene-2-sulphonate.

M460 Fab' fragment. Data on the binding of Dnp-glycine, Dnp-lysine and Dnp- NH_2 to M460 Fab' fragment are given in Fig. 5(a). Unlike the binding to M315 Fv fragment, these three haptens produce similar effects on all bands except the high-frequency band of the 2- NO_2 group, for which the shifts show appreciable variations in sign and magnitude.

X25 Fab' fragment. The binding to X25 Fab' fragment of two haptens, Dnp- NH_2 and Dnp-lysine, was compared (Fig. 5b). Like the binding to M315 Fv fragment, these haptens differ in their behaviour. For instance, no shift is observed in the 4- NO_2 band of Dnp-lysine, whereas a considerable shift (-6 cm^{-1}) is observed for Dnp- NH_2 .

Discussion

The present work differs from that of Kumar *et al.* (1978) in two important respects. Firstly, we have studied the solvent-dependence of the R.R. spectrum

Fig. 3. Comparison of the band positions of the R.R. spectra of (a) 2,4-dinitroaniline, (b) Dnp-lysine when bound to Fv fragment of protein M315 and Fab' fragments of proteins X25 and M460 and (c) Dnp-glycine when bound to Fv fragment of proteins M315 and Fab' fragment of protein M460

Broken lines refer to IgA (data from Kumar *et al.*, 1978). b indicates broad band.

Fig. 4. Comparison of the band positions of the R.R. spectra of different haptens when bound to F.v. fragment of protein M315

Broken lines refer to IgA (data from Kumar *et al.*, 1978). Abbreviation: DNNS, 5,7-dinitronaphthalene-2-sulphonate.

Fig. 5. Comparison of the band positions of the R.R. spectra of selected haptens when bound to Fab' fragments of proteins M460 and X25

A dash indicates that band is obscured.

The comparisons made in the Results section show that the $\text{NH}_2\text{--C--NO}_2$ unit in Dnp-NH_2 bound to the three antibody proteins is certainly in a polar environment and is probably hydrogen-bonded. However, they do not distinguish protein-bound H_2O from the effect of polar side chains close to this group of the hapten. The postulated model for protein M315 has at least two polar groups (asparagine-36_L and tyrosine-34_L) that could form hydrogen bonds to the 2- NO_2 group of the hapten. In proteins M460 and X25 a histidine residue corresponds in position to asparagine-36_L of protein M315; position 34_L is occupied by a tyrosine residue in protein M460 and by an asparagine residue in protein X25 (Barstad *et al.*, 1978).

By contrast with the interpretation of the solvent-dependence of the 2- NO_2 vibrations, the frequency of the 4- NO_2 R.R. band is independent of solvent except in carbonyl solvents. We have considered four interpretations of the shifts observed on binding to proteins: (1) charge-transfer interactions with amino acid residues, e.g. tryptophan (Dower *et al.*, 1977); (2) a similar interaction to that found in the carbonyl solvents; (3) breaking the intramolecular hydrogen bond at the 2- NO_2 position, which allows the nitro group to twist and form a hydrogen bond to the protein; (4) formation of a strong hydrogen bond that cannot be simulated in any of the solvents used.

The first possibility is excluded by the early experiments of Carey *et al.* (1973) on the indole-1,3,5-trinitrobenzene complex. On the basis of the postulated model for protein M315 (Dower *et al.*, 1977), we note that, in keeping with the second possibility, there are peptide carbonyl groups at about 0.4 nm from the hapten. The third possibility is inconsistent with the work of Gettins *et al.* (1978), who showed that the 2- NO_2 group remains planar on binding to protein M315. The fourth interpretation would imply that the stronger hydrogen bond in the protein arises from the fixed geometry of the site, which placed the nitro group next to a hydrogen-bonding group. Indeed, the postulated model has asparagine-36_H or asparagine-36_L, which could interact with the 4- NO_2 group. In this respect it is noteworthy that the X-ray results for a 2,4,6-trinitrophenyl derivative, when bound to the light-chain dimer protein REI (Epp *et al.*, 1975; O. Epp, personal communication), indicate hydrogen bonds to all the nitro groups from tyrosine and asparagine residues.

The possibility of hydrogen bonds, favoured by Carey *et al.* (1973), has the drawback that the shifts of the 4- NO_2 group cannot be mimicked in hydrogen-bonding solvents. By contrast, the shifts in carbonyl solvents can stimulate the effect of protein binding, although the nature of the complex formed in these solvents with the hapten is not known.

of Dnp-NH_2 , allowing us to scale the shifts observed on binding to proteins. Secondly, we have closed the gap between the haptens used by Kumar *et al.* (1978) and those used in n.m.r. studies.

The ^1H n.m.r. results on several of the haptens have been interpreted in terms of small differences in relative positions of the hapten and protein side chains rather than of fundamental changes in the interactions involved (Dower *et al.*, 1977). Ironically it is for ϵ -N-Dnp-L-lysine that there is R.R. evidence for the absence of an interaction with the 4- NO_2 group. This hapten has a binding constant 100 times greater than those of the glycine and aspartate derivatives (Wain-Hobson *et al.*, 1977) and is the hapten most frequently used by immunologists in structural studies on Dnp-binding antibodies. Its high affinity for protein M315 may explain its anomalous behaviour in relation to the other haptens examined in the present work, since the side chain may require a slight change in Dnp position (with a decrease in ΔG from this subsite but a net increase overall) to be able to form a favourable hydrophobic interaction between the side chain and the protein (Haselkorn *et al.*, 1974).

From the postulated model of protein M315 and the n.m.r. results on Dnp-glycine, it was suggested that the two nitro groups were hydrogen-bonded to groups on the protein. If the changes seen with the R.R. technique for Dnp- NH_2 are also indicative of hydrogen-bonding, the result on protein M315 for Dnp-lysine would imply that hydrogen-bonding groups, though available, are not essential for the binding of nitroaromatic ligands. In line with other studies on protein M315 in this laboratory, this leads to the conclusion that antibody specificity for Dnp ligands is provided, not so much by discrete directional interactions, such as hydrogen bonds to the nitro groups, but by factors often considered to be non-specific. These would include the transfer of a relatively non-polar molecule from water to an aromatic combining site, i.e. 'hydrophobic' interactions, and the overall shape of the site in terms of its ability to provide effective Van der Waals interactions with the ligands.

An explanation based on the different shapes of the binding sites of proteins M460, X25 and M315 (Willan *et al.*, 1977) may account for the differences in behaviour of ϵ -N-Dnp-L-lysine with the three proteins. Proteins M460 and X25 have K_d values of $3.3\ \mu\text{M}$ (Jaffe *et al.*, 1971) and $5\ \mu\text{M}$ (Sharon & Givol, 1976) respectively, and might have been expected to behave similarly on the basis of affinity but differently from protein M315, with its K_d of $0.1\ \mu\text{M}$. Willan *et al.* (1977), in comparing the dimensions of the combining sites of these three proteins by e.s.r. studies on nitroxide spin-labelled Dnp haptens, found that the entrance to the Dnp subsite, presumably the volume occupied by the lysine side chain, was much wider in protein M460 than in either protein X25 or protein M315. Since the contribution to binding of the side chain and its concomitant ability to alter the Dnp ring position

may depend partly on the degree of constraint imposed by the protein, the apparently normal Dnp binding of ϵ -N-Dnp-L-lysine to protein M460 could arise from the much greater freedom of movement of the attached lysine side chain.

In conclusion, different Dnp-binding antibodies interact similarly when probed by a hapten without side chain (Dnp- NH_2). The addition of side chains to the hapten can cause alterations in the Dnp-binding mode that may reflect an optimization of binding energy. Consequently any studies aimed at investigating antibody combining sites by using haptenic determinants attached to long side chains may give misleading results by not fully probing the true site of hapten-antibody complementarity. This may explain the controversial results obtained by Richards and co-workers on different sites of interaction of menadione and Dnp in protein M315 (Manjula *et al.*, 1976; Rosenstein & Richards, 1976).

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