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Interaction of Saccharide Haptens with Myeloma Proteins. A 270-MHz Proton Nuclear Magnetic Resonance Study†

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ABSTRACT: 270-MHz proton NMR is used to study U61, E109, and J539, three homogeneous IgA mouse myeloma proteins which have specificities for saccharides. Three histidine resonances are observed to titrate in U61 and E109 and two in J539. These two as judged by their pK_a values (4.8 and 7.4), titration range, and chemical shifts are common to all three proteins. On the basis of work on light chains, these are assigned to His-198$_L$ (pK_a = 4.8) and His-189$_L$ (pK_a = 7.4). Neither of these is perturbed on hapten binding. The third histidine (pK_a = 6.7) common in both U61 and E109 is assigned to His-53$_H$ or His-58$_H$, both in the hypervariable region. This histidine is perturbed slightly on hapten binding in U61. Difference spectra show that comparatively few protein resonances are perturbed on binding hapten, suggesting that accompanying conformational changes are limited to the combining site. The difference spectra of U61 and E109 on addition of inulotriose suggest very similar binding sites which contain aromatic residues. Difference spectra of J539 binding to the mono-, di-, and trigalactosides suggest that the first subsite is nonaromatic, unlike the second and possibly the third. The binding of *p*-nitrophenyl β-D-galactopyranoside to J539 is consistent with a second aromatic subsite. The exchange rate for this hapten between J539 and the free solution is 2545 s^{-1}, and the chemical shifts of the anomeric proton and those of the H(3) and H(6) protons of the nitrophenyl ring are all upfield. *m*-Nitrophenyl acetate binds weakly to J539. It is suggested that the importance of aromatic residues in binding sites for D sugars arises in part from the sugars adopting a $^4C_1$ D conformation in which there is a planar hydrophobic face which can interact with planar rings on aromatic side chains. The specificity for sugar antigens is provided by having many subsites which favor polymeric carbohydrate antigens and discriminate against smaller hydrophobic ligands which bind to an individual subsite but with relatively low affinity.

Saccharides form a widely distributed and very important class of naturally occurring antigens. As well as being encountered in food and in the environment, polysaccharides are major constituents of bacterial cell walls and provide the antigenic determinants responsible for the differentiation of blood groups. This importance is reflected in the large number of homogeneous mouse myeloma proteins found to have specificity for saccharide ligands (Glaudemans, 1975; Glaudemans et al., 1975).

Although polysaccharide antigens with molecular weights of several million exist, the region of the macromolecule recognized by the combining site is still very restricted. A hexasaccharide of alternating D-glucose and D-glucuronic acid residues has been postulated as the determinant in *S. pneumoniae* type III polysaccharide (Mage & Kabat, 1963). Determinants as small as disaccharides may exist (Kabat, 1968). There is, however, considerable scope for antigenic

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differences even with such small determinants both in composition of the constituent sugars and in the configuration of their linkages. Cross-reactions are frequent because of the occurrence of common di- or trisaccharide as part of a larger determinant (Heidelberger et al., 1965; Furukawa et al., 1972; Heidelberger & Dutton, 1973).

Previous NMR studies on antibodies have concentrated on myeloma proteins with specificity for nitroaromatics (Dwek et al., 1977; Morris et al., 1980; Dower et al., 1978) and phosphorylcholine (Gettins et al., 1977; Goetze & Richards, 1977). More recently these methods have been applied to a ^{13}C NMR study of a heterogeneous antibody population raised against polyalanine (Geller et al., 1980), though the heterogeneity limited the usefulness of the observations. Diversity, however, is the essence of the immune response, and it is therefore important to be able to compare different types of combining sites. In this paper, we extend and explore the use of ^1H NMR to study three homogeneous proteins (U61, E109, and J539) which have specificities for saccharide.

J539 is a galactose-binding mouse IgA, which was found to precipitate with *p*-azophenyl β-D-galactoside bovine δ-globulin (Sher & Tarikas, 1971). It is one of six mouse IgAs, all possessing the VHIB and VK4 isotypes, which have specificity for β(1–6)-linked D-galactose polymers (Glaudemans et al., 1975). Binding studies indicate that the combining site is large enough to accommodate four sugar units (Jolley & Glaudemans, 1974), with the terminal nonreducing galactose residue contributing most to the binding energy followed by the second and then the third and fourth residues. The latter residue contributes very little. In Gal_2[1] the ring oxygen and hydroxyls of the first galactose residue contribute 54.5% of the binding energy and the presence of bulky substituents on C(6)′, C(1), C(2), C(3), or C(4) does not interfere with affinity, suggesting that the galactose disaccharide is bound on only one side and does not penetrate into a cavity. Recently it has been concluded that J539 has a groove-type site (Roy et al., 1981).

A crystallographic determination of the structure of J539 is currently in progress (Navia et al., 1979), but no satisfactory derivative with a hapten has yet been obtained. However, on the basis of a proposed model of the V regions (Feldmann et al., 1981), a binding site has been suggested for the (1–6)-β-galactohexoses. This involves areas in the complementarity determining regions from the first and third hypervariable regions of the light chain and from the second hypervariable region of the heavy chain (which includes His-52_H). The use of NMR difference spectra (Dwek et al., 1975) on addition of the hapten and of pH titrations should help in testing the proposed site.

The other two sugar binding proteins used in this study are E109 and U61 which precipitate with the antigen inulin, a homopolysaccharide of β(2–1)-linked fructose units terminated with a single glucose residue (Glaudemans et al., 1975). Complete V_H sequences are known for both proteins (Vrana et al., 1978) and show only four differences, all outside the hypervariable regions (which make up the combining site). The most noticeable feature of the hypervariable loops is that the length of the third hypervariable loop is only one residue long. Sequences are available for the light chains (Vrana et al., 1979) and only show one difference (Ser in U61 for Asn in E109 at position 30_L) in the first 35 residues. However, binding studies on recombinant antibody composed of the constituent H and L chains of the two proteins in all permutations (Streefkerk et al., 1978) indicate that the differences between E109 and U61 (Streefkerk & Glaudemans, 1977) arise from light chain variations. The use of ^1H NMR difference spectra resulting from addition of hapten allows a structural fingerprint to be outlined and a comparison of the similarity of residues making up the combining site in the two proteins.

Materials and Methods

Fab′ Fragments. The Fab′ fragments of J539, E109, and U61 were all prepared from IgA by using the method of Inbar et al. (1971).

Saccharides. Methyl β-D-galactopyranoside and 2-nitrophenyl β-D-galactopyranoside were purchased from Sigma Chemical Co. Galactosyl (1–6)-β-D-galactopyranoside and galactosyl-(1–6)-β-D-galactosyl (1–6)-β-D-galactopyranoside were prepared as described previously (Aspinall et al., 1958). Inulotriose was isolated from the hydrolysate of inulin (Das et al., 1979a).

NMR. Protein samples were prepared by dissolving freeze-dried antibody in 99.8% D_2O containing 10 mM sodium phosphate, 150 mM NaCl, and 0.02% sodium azide at pH* 6.9, unless otherwise stated. The pH was adjusted with DCl and NaOD. Protein concentrations were checked by measurement of absorbance at 280 nm, using an extinction coefficient $E^{1\%}_{1cm}$ of 14.0 (Inbar et al., 1971). Normal 270-MHz ^1H spectra and difference spectra were obtained as described in Dower et al. (1978). The histidine titrations were monitored from the pH difference spectra (Dwek et al., 1975). However, certain spectra were also generated by using a Carr–Purcell sequence incorporating the Meiboom–Gill modification (Campbell et al., 1975) to produce relative enhancement of those resonances with long T_2 values. Samples were run at 303 K and typically required the averaging of 4000 pulses. pH values are reported as measured and are uncorrected for deuterium isotope effects. Assignments of the aromatic proton spectrum of *o*-nitrophenyl β-D-galactoside were achieved by time-shared homonuclear decoupling on a 20 mM sample at pH 7 in D_2O. Line widths where required were estimated by fitting the spectra to simulated Lorentzian lines of known line width.

Results

Histidine Markers. The resonances of the C(2) and C(4) protons of histidines, by virtue of their chemical shifts, titration behavior as a function of pH in the range 5–8, and commonly long T_2 values, are generally the ones most easily identifiable in the ^1H NMR spectra of large protein molecules, such as the 55 000-dalton Fab′ fragments considered here. Histidine titrations can most easily be followed by using difference spectroscopy as in Dwek et al. (1975) or using a Carr–Purcell.

For J539, only two histidine C(2) protons are discernible by using a Carr–Purcell–Meiboom–Gill pulse sequence with an overall delay of 20 ms between the 90° pulse and commencement of data acquisition. The chemical shift of these resonances vs. pH is shown in Figure 1a. From this, pK_a values of 7.4 and approximately 4.8 were determined. The accuracy of the latter value is poor because of the small fraction of the titration range that it was possible to cover. Addition of galactosyl (1–6)-β-galactopyranoside to the Fab fragment to produce the 1:1 complex resulted in no perturbation of the pK_as and chemical shift of these two histidine resonances (Figure 1a).

Both U61 and E109 have three histidine C(2) proton resonances that can be monitored in the Fab′ NMR spectra

[1] Abbreviations used: H, heavy chain, L, light chain; Gal_2, 6-*O*-β-D-galactopyranosyl-D-galactose; Gal_3, corresponding triose.

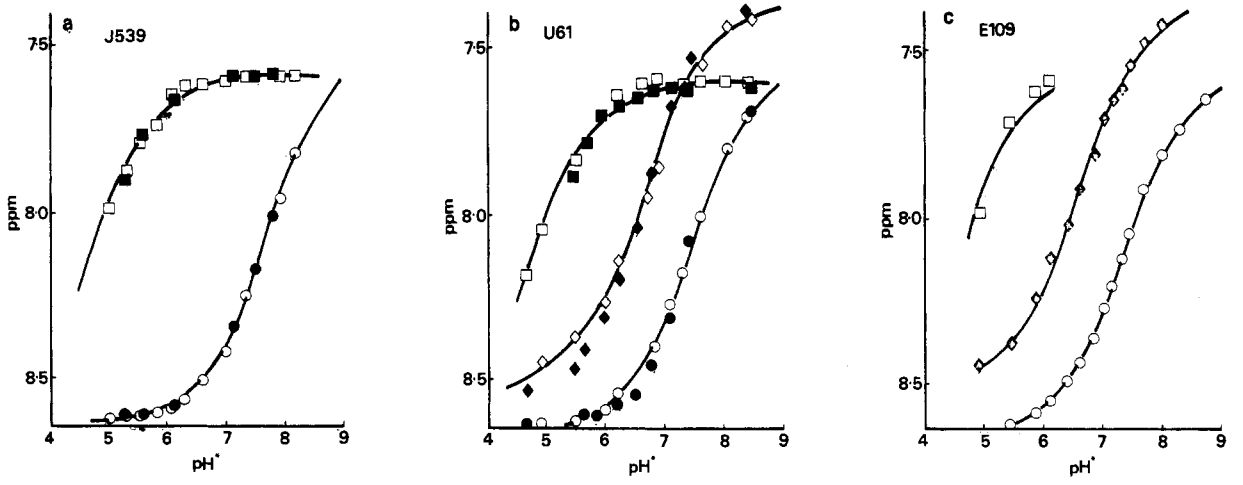

FIGURE 1: (a) 270-MHz ^{1}H proton NMR histidine titrations at 303 K of the C(2)H resonance shifts vs. pH for Fab's of J539 (1 mM) (open symbols) plus digalactose (filled in symbols); (b) U61 (1.5 mM) alone (open symbols) plus inulotriose (closed symbols); (c) E109 (1.5 mM). Spectra for J539 and U61 were obtained by using a Carr–Purcell B sequence with τ = 20 ms while those for E109 by normal single pulse experiments. The curves shown are theoretical fits to Hendersen–Hasselbalch behavior (Dwek, 1973). An upper error limit on the pK_a values is ±0.1 unit for all except the titration curve for E109, whose pK_a of 4.8 is only very approximate.

Table I: Known Histidine Residues on the Fab' Fragments of J539, U61, and E109 [a]

			Heavy Chain			
J539	His-52				His-96	His-145
U61		His-53	His-58			His-145
E109		His-53	His-58	His-90		His-145
			Light Chain			
J539	His-34 [b]		His-189	His-198		
U61		His-91	His-189	His-198		
E109		His-91	His-189	His-198		

[a] The constant domain data were taken from other mouse myelomas (Svasti & Milstein, 1972; Francis et al., 1974). [b] Rudikoff (1981).

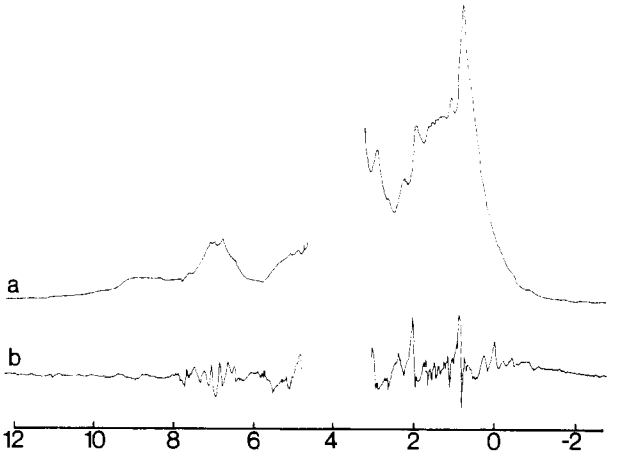

FIGURE 2: 270-MHz ^{1}H NMR spectrum of Fab' (1.5 mM) of (a) U61 and (b) the difference spectrum (scale ×2) after addition of 1:1 inulotriose, pH* 7.08 and T = 303 K. Scale is parts per million from DSS.

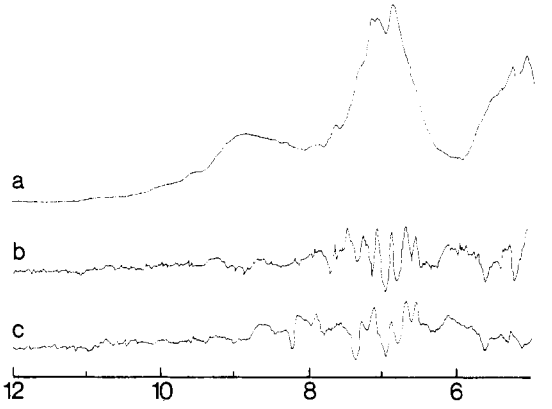

FIGURE 3: ^{1}H NMR aromatic region at 270 MHz of Fab (1.5 mM) at 303 K and pH* 7.08 of (a) U61, (b) U61 difference spectrum (×2) with inulotriose, and (c) E109 difference spectrum with inulotriose. Scale is parts per million from DSS.

(Figure 1b,c). Each of the histidines in U61 has an equivalent residue in E109, as judged by chemical shift and pK_a. The pK_as were found to be 7.4, 4.8, and 6.7. A pH titration of the 1:1 complex of U61 and inulotriose did not cause any change in the residues with pK_a = 7.4 and 4.8. However, the residue with pK_a = 6.7 appears to have its titration range slightly extended (by about 0.1 ppm).

Sequence data are available for these three Fab's. Table I gives the known, or inferred, histidine locations for both heavy and light chains. The constant region histidines are inferred from other BALB/c IgA myeloma proteins possessing the same allotypes (Svasti & Milstein, 1972; Francis et al., 1974). On the basis of this tabulation, and ignoring position 212_H, there are six histidines in the Fab' of J539 and U61 and seven in E109. Since there are two histidines with identical pK_as, chemical shift, and titration range in all three fragments, they are probably limited to those which are common, i.e., to His-145_H, His-189_L, or His-198_L (which are all in the constant region). Other ^{1}H NMR studies on λ light chains (Shimizu et al., 1980; Arata et al., 1978) have shown that His-189_L has a pK_a of 7.4 while His-198_L has a pK_a of 4.8 for non-Mcg-like λ chains and 5.2 for Mcg-like light chains. The third histidine, common to both U61 and E109 but not J539, must be limited to His-53_H or His-58_H (Table I), both of which are in the second hypervariable region. This third histidine is therefore potentially a residue which may be affected by binding of hapten.

Difference Spectra. The difference spectrum obtained after addition of inulotriose to U61 Fab' is shown in Figure 2. Apparently few resonances are perturbed by hapten, as has now been found with several other antibodies (Dower et al., 1978; Morris et al., 1980). Figure 2 also shows that there are comparable areas for the aromatic and aliphatic peaks. (The total area corresponds to approximately 20 protons.) In Figure 3, the aromatic region difference spectra for inulotriose binding to E109 and U61 are compared. There are numerous simi-

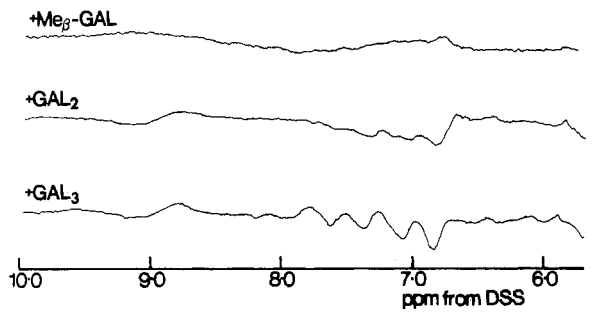

FIGURE 4: ^{1}H NMR aromatic region at 270 MHz of difference spectra between J539 and mono-, di-, or trisaccharide hapten. pH* 7.4; T = 303 K. The hapten concentration was sufficient to saturate the combining site.

larities. Resonances at 5.51, 6.24, 6.57, 6.73, 6.94, 7.72, and 8.98 ppm in the U61 difference spectrum are all reproduced in E109 (when identical spectrometer conditions are used) in position if not quite in intensity. There are dissimilarities, however, notably in the region from 7.1 to 7.6 ppm as well as the additional sharp resonance at 8.25 ppm which may, from its chemical shift and narrow line width, be a histidine C(2) proton.

Figure 4 shows the aromatic region difference spectra for the series of mono-, di-, and trigalactosyl haptens after binding to J539 Fab. The dissociation constants are 999, 87, and 7 μM, respectively (Jolley et al., 1974), and therefore excess of the monosaccharide is necessary to produce saturation of the antibody combining sites. As with E109 and U91 above and with Dnp binding antibody fragments (Dower et al., 1978), the total resonance area affected and appearing in the difference spectrum is a small percentage of the total. For the monosaccharide, very little appears to be perturbed on the protein, in contrast to the result with either the di- or trisaccharides (Figure 4). These last two difference spectra show large qualitative similarities. The broad amide feature at 8.8–9.1 ppm is common to both. Four peaks, appearing at 6.85, 7.07, 7.36, and 7.62 ppm in the Gal_3 difference spectrum, are paralleled by an extensive negative triangular peak covering the same region with indications of peaks at the first three of the above four peak positions and with a comparable area.

Binding of Nitrophenyl Derivatives to J539. It is known that J539 precipitates with *p*-azophenyl β-D-galactosyl BGG and that *p*-nitrophenyl β-D-galactopyranoside acts as an inhibitor. From recent binding studies of aromatic glycosides to J539 (Das et al., 1979b), it was concluded that the second sugar binding site can interact as strongly with a phenyl group as with a galactose residue and substitution on the phenyl ring enhances binding even more.

The region of the ^{1}H NMR spectrum of *o*-nitrophenyl β-D-galactopyranoside to low field of HOD is shown in Figure 5a. Assignments of the four aromatic ring protons were made by J decoupling. The chemical shift of the anomeric proton of the galactose residue is at 5.2 ppm compared with 4.3 ppm for the methyl galactoside. Addition of J539 Fab′ to give a small percentage of bound hapten results in differential broadening and shifting of the five, clearly distinguishable, low-field resonances at 313 K (Figure 5).

Cooling the sample results in a sharpening of the resonances. This behavior is characteristic of a system undergoing chemical exchange on the NMR time scale (Dwek, 1973). Analysis of the broadening and chemical shift then allows the exchange rate and fully bound shift to be obtained. When a small fraction of the hapten (P_M) is bound, the equations describing chemical exchange behavior for the observed relaxation rate

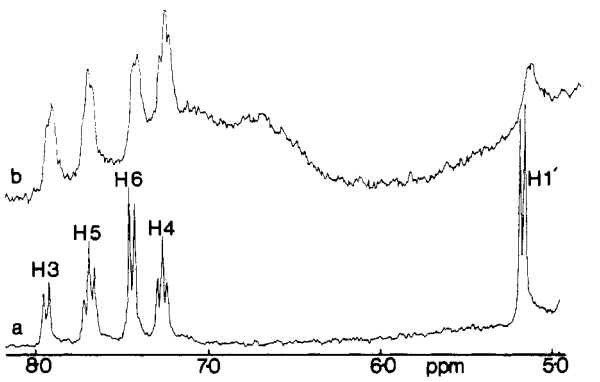

FIGURE 5: 270-MHz ^{1}H NMR spectrum of downfield region of *o*-nitrophenol β-D-galactoside (2 mM) (a) alone and (b) after addition of Fab′ from J539 (0.148 mM); T = 313 K. H(3)–H(6) are the aromatic resonances of the sugar, and H(1)′ is the anomeric proton resonance.

Table II: Exchange Broadenings and Shifts on ^{1}H Resonances of *o*-Nitrophenyl-β-D-galactose on Binding to J539 Fab′ at 313 K[a]

resonance	exchange broadening (±0.1 Hz)	obsd shift (±1.5 Hz)	fully bound shift (ppm)
C(1)	11.7	1.5	1.2
H(3)	2.2	1.9	0.41
H(4)	0.9	0.5	0.26
H(5)	2.1	-1.5	-0.41
H(6)	4.1	2.4	0.6

[a] The fraction of hapten bound was 0.037.

$1/T_{2,obsd}$ and chemical shift $\Delta\omega_{obsd}$ of the hapten are given by (Swift & Connick, 1962; Dwek, 1973)

$$\frac{1}{T_{2,obsd}} = \frac{1}{T_{2,free}} + \frac{P_M}{\tau_M}\left(\frac{\frac{1}{T_{2M}}\left(\frac{1}{T_{2M}} + \frac{1}{\tau_M}\right) + \Delta\omega_M^2}{\left(\frac{1}{T_{2M}} + \frac{1}{\tau_M}\right)^2 + \Delta\omega_M^2}\right) \quad (1)$$

$$\Delta\omega_{obsd} = \frac{P_M\Delta\omega_M}{(1 + \tau_M/T_{2M})^2 + \tau_M^2\Delta\omega_M^2} \quad (2)$$

where $1/T_{2,free}$ is the relaxation rate of the free hapten and $1/T_{2M}$ that when it is bound to the antibody. $\Delta\omega_M$ is the chemical shift when bound to the antibody, and $1/\tau_M$ is the chemical exchange rate of the hapten between the antibody and free solution. The observed line width $\Delta\nu$ in each case is related to the relaxation rate by

$$\frac{1}{T_2} = \pi\Delta\nu \quad (3)$$

From eq 2, lower limits for $\Delta\omega_M(\Delta\omega_{obsd}/P_M)$ and $1/\tau_M$-$(2\Delta\omega_{obsd}/P_M)$ can be obtained (Morris et al., 1980). When the value for the C(1) proton in Table II is used, then $1/\tau_M > 2479$ s^{-1} and $\Delta\omega_M > 1240$ s^{-1}. The expected value of $1/T_{2M}$ for a hapten bound to a protein of size 50000 daltons is ~60 s^{-1} (Morris et al., 1980). This effectively means that terms in $1/T_{2M}$ in eq 1 and 2 can be ignored [since $1/\tau_M \gg 1/T_{2M}$ and $\Delta\omega_M^2 \gg 1/(T_{2M}\tau_M)$]. Equations 1 and 2 can thus be used to solve for $1/\tau_M$ and $\Delta\omega_M$. This was done for the C(1)H resonance and gave a value of $1/\tau_M = 2545$ s^{-1}, but the values of the shifts for H(3)–H(6) are too small to allow their independent use in eq 1 and 2. We have calculated their fully bound shifts from eq 1, using the observed line broadenings,

Table II, and the value of $1/\tau_M$ calculated from C(1)H resonance.

When a similar titration of J539 (1 mM) into a solution of *m*-nitrophenyl acetate (0.59 mM) is carried out, the upfield shifts of 8, 11, 17, and 8 Hz, are observed for the aromatic ring protons H(2), H(4), H(5), and H(6), respectively, and for the acetate methyl group, a shift of 7 Hz is observed. The binding constant of this hapten to J539 is extremely weak, $\gg$10 mM. Under the conditions of this experiment, a very small fraction (1%) of the hapten is therefore bound. This implies that the scaled up fully bound shift changes are large and upfield, consistent with the presence of aromatic residues in this subsite.

Discussion

The application of 270-MHz proton NMR to the three sugar binding antibodies extends the range of combining sites which have now been studied by this technique. As with the dinitrophenyl binding and the phosphorylcholine binding antibodies, the unexpected but successful use of difference spectra in proteins of this size provides a structural fingerprint of the combining site. Such difference spectra generally contrast with those obtained from the binding of ligands to enzymes. For instance in the case of lysozyme, the binding of *N*-acetylglucosamine results in perturbations of many resonances in the NMR spectrum consistent with minor confromational changes all over the molecule (Dwek, 1977). The small number of protons perturbed in the NMR spectra of antibody fragments on addition of hapten suggests that most conformational changes are confined to the binding site. The generation of these difference spectra makes the study of antibody fragments (of molecular weight 50 000) by NMR feasible. Hapten difference spectra provide a rapid way of comparing or screening different antibody combining sites. Thus, for example, the similarity of the difference spectra with inulotriose of E109 and U61 points to very similar combining sites. If the concept of framework invariance of antibody structure proves well founded, then structures of combining sites can be predicted (Padlan et al., 1976; Potter, 1977; Kabat, 1978). NMR can then also provide a means of testing these combining sites and of differentiating between possible hapten modes of binding. An example of this is J539. In a postulated binding mode for the trisaccharide hapten (Feldmann et al., 1981), one of the subsites is nonaromatic and is adjacent to an aromatic subsite which is consistent with the results presented here. The NMR results, however, suggest that no titrating His residue is involved in the binding site. The suggested involvement of His-52_H (Feldmann et al., 1981) in the site may therefore not involve it as an intimate contact residue of the hapten or else it is a nontitratable histidine.

Of the histidine resonances visible in the ^{1}H NMR spectra of J539, U61 and E109, two behave identically. On the basis of the work of Shimizu et al. (1980) and Arata et al. (1978), we have assigned these to residues His-189_L and His-198_L in the constant domain of the Fab′. Their pK_as of 4.8 and 7.4 are markedly different from the value of $\sim$6.7 for a histidine residue in small peptides and therefore indicate quite distinct local environments in the antibody fragments. As such, they are good markers for the particular tertiary structure required to produce the observed pK_a, chemical shift, and chemical shift titration range of the ^{1}H resonances. The occurrence of these two histidines with identical behavior in three separate antibodies is expected from the equivalence of the primary sequences in the constant domain. However, it serves to emphasize the independence of the constant region structure from that of the variable region, where sequence differences do exist. For all three antibodies, hapten binding must produce no large-scale-induced conformational change in the constant domain, since these histidine markers are quite unaffected. Even in the variable region, the conformational change is probably again localized to the hapten contact residues, as judged by the small intensity appearing in the proton difference spectra. This is supported by the behavior of the third histidine resonance observed for U61 and E109, which we have assigned to either His-53_H or -58_H in the second heavy chain hypervariable region. This histidine residue is therefore relatively close to the combining site but is almost unperturbed in the presence of the hapten inulotriose, so that again it may not be an actual contact residue.

By way of a control, it is interesting to compare the behavior of the histidine residues in the proteins studied here with those of the chicken and rabbit triosephosphate isomerases (Browne et al., 1976). The chicken enzyme has eight histidine residues and the rabbit enzyme four (which are conserved). Three of the four conserved histidines do not titrate while the fourth, His-100, which is near the active-site region, is perturbed on addition of certain ligands. In the chicken enzyme, the addition of these ligands also affects the shift of a histidine residue, His-195 [on the other side of the molecule with respect to His-100 but more importantly well removed (20 Å) from the active site]. In a combined X-ray and NMR analysis, Brown et al. (1976) interpret these results in terms of a general tightening of the structure on addition of ligands, resulting in conformational changes involving movements of $\sim$0.05 nm. Their explanations for the nontitrating histidine residues involve either specific interactions between the nitrogen atom of the histidine and nearby peptide NH or that the ring is surrounded by nonpolar contacts and inaccessible to solvent. Such explanations could also hold for those histidines which do not titrate in U61, E109, and J539.

While it seems clear that no large hapten-induced conformational change occurs in the proteins studied here, this does not exclude the possibility of residues in or very near to the combining site changing their orientations slightly on hapten binding. It may well be that such changes are in fact those monitored in the T-jump experiments on hapten binding, rather than any arising from residues remote from the binding site (Vuk-Pavlovic et al., 1978; Zidovetzki et al., 1980).

Perhaps the most surprising result from the three sugar-binding myelomas studied here is the importance of resonances from aromatic residues in the NMR difference spectra. The presence of aromatic residues in the combining site may well be part of the explanation for the tight binding of phenyl glycosides and aromatic compounds. However, not all of the subsites have to be aromatic. For instance, the binding of the monogalactoside to J539 results in practically no aromatic residues being perturbed as shown by the difference spectra, in contrast to that for the digalactoside.

In the case of J539, Jolley et al. (1974) concluded that there was sequential occupation of the sugar subsites, such that the monosaccharide binds only in the first subsite and the disaccharide binds only in the first two subsites. Their argument is based on obtaining strictly linear Scatchard plots for the binding of shorter saccharides. The binding of nitrophenyl β-D-galactopyranosides would support the idea of an aromatic second subsite. Further, the large upfield shift of the anomeric proton and the shifts on the two aromatic ring protons closest to it, H(3) and H(6), are consistent with aromatic residues on the protein in the region of the first intersubunit glycoside linkage and the second subsite. This result raises the question of whether or not J539 can bind a nitrophenyl group alone with

any degree of specificity. The experiments reported here show that when J539 is added to *m*-nitrophenyl acetate there is good evidence for weak binding from the differential shifts on the ring and methyl proton resonance. Three distinct interactions can be expected to contribute to the thermodynamics of association of neutral oligosaccharides with antibody combining sites; these are hydrophobic, H bonding, and van der Waals (Ross & Subramanian, 1981). We suggest the importance of aromatic residues in the combining sites for D sugars arises in part from the sugars adopting a $^4C_1$ D conformation in which there is a planar hydrophobic face, which can interact with the planar rings of the aromatic residues forming favorable hydrophobic interactions. Support for this concept, that sugars can form hydrophobic interactions with aromatic amino acids, can be found from the X-ray crystallographic studies on the Fc fragment of immunoglobulin (Diesenhofer, 1981) and from work on the lysozyme–sugar complex (Perkins et al., 1981). Sugars in aqueous solution will be extensively H bonded with the solvent. The simple transference of these H bonds with solvent to an array within a combining site, which is itself extensively hydrated, would not be expected to contribute significantly to the energetics of association. However, an important contribution may be envisaged if the H bonds of the complex were formed in an environment of low dielectric constant such as may well arise in a combining site comprising predominantly nonpolar amino acids. The third factor, van der Waals forces, is difficult to estimate for saccharides at the present time. The loss of conformational entropy associated with the binding of tetrasaccharide would make the ΔS of association unfavorable (Janin & Chothia, 1978), although this would be offset by the structured water of hydration previously associated with both protein and ligand which would be returned to bulk solvent (Bhattacharjee et al., 1981).

A basic difference between sugar and nonsugar binding proteins is the concept of distinct subsites in the former. The shape and topology of these subsites will determine the specificity for a particular sequence of sugars. An individual subsite which may be aromatic might not distinguish between sugars and other hydrophobic (e.g., aromatic) ligands which have the correct shape. The specificity of sugar binding proteins such as those studied here will therefore arise from the presence of several subsites (which is shown by the increasing strength of binding of the tetra- and trisaccharides compared with that of the monosaccharide). This is also found to be true in lysozyme, where, for instance, trisaccharides bind 3 orders of magnitude more strongly than the monosaccharides. These subsites therefore favor carbohydrate antigens which are frequently polymeric and discriminate against smaller, hydrophobic ligands, which bind to an individual subsite but with relatively low affinity.

The dependence on correct shape is well illustrated by E109 and U61 which bind both grass levan, containing (2–6)-linked fructose units, and inulin, containing only (2–1) links (Streefkerk et al., 1979). These authors demonstrated that in their extended conformations, disaccharide units of levan are capable of mimicking disaccharide units of inulin. Possibly as a result of the requirement for large hydrophobic channels in such antibodies to accommodate the extensive haptens, the inulin binding groups of myeloma proteins all have exceedingly short third heavy hypervariable loop regions, thereby extending the cleft seen in other antibodies into a wide rectangular hole (Potter et al., 1977).

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Inhibition of Aromatic L-Amino Acid Decarboxylase by Coenzyme–Amino Acid Adducts†

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ABSTRACT: The coenzyme–amino acid adducts *N*-(5′-phosphopyridoxyl)-L-3,4-dihydroxyphenylalanine and *N*-(5′-phosphopyridoxyl)-L-*m*-NH_2-tyrosine inhibit hog kidney aromatic L-amino acid decarboxylase (Dopa decarboxylase). Kinetic studies on the nature of the inhibition caused by these adducts appeared to distinguish two distinct decarboxylase activities in purified enzyme preparations. It was determined that the appearance of two activities in purified enzyme preparations is an artifact of the system resulting from the following properties of Dopa decarboxylase: (1) the enzyme has a high affinity for pyridoxal 5′-phosphate, (2) Dopa decarboxylase can follow a decarboxylation-dependent transamination pathway forming apoenzyme as one of the products of this pathway, and (3) the phosphorylated adducts investigated readily bind to apo-Dopa decarboxylase but do not readily displace pyridoxal phosphate from holoenzyme. Incubation of holo-Dopa decarboxylase with *N*-(5′-deoxypyridoxyl)-DL-Dopa, in the absence of added coenzyme, causes a rapid inactivation of enzyme ($t_{1/2}$ = 5 min) which is associated with a decrease in the coenzyme content of the enzyme. However, incubation of holoenzyme with the phosphorylated adduct, PPxy-L-*m*-NH_2-Tyr, causes a much slower inactivation of enzyme ($t_{1/2}$ = 30 min), while a short incubation (10 min or less) with either of the phosphorylated adducts increases the activity of holoenzyme. Calculations indicate that the extent of reactivation of apoenzyme, formed via the decarboxylation-dependent transamination pathway, by excess exogenous coenzyme cannot be accounted for solely by reconstitution of holoenzyme. It is proposed that Dopa decarboxylase has either a second active site which has a low affinity for pyridoxal phosphate or a site(s) which when occupied by pyridoxal phosphate leads to an increase in the activity of the enzyme.

Coenzyme–amino acid adducts are inhibitors of many pyridoxal phosphate (PLP)[1] dependent enzymes (Ayling & Snell, 1968; Bayon et al., 1977; Borri-Voltattorni et al., 1975; Heller et al., 1975; Orlacchio et al., 1980; Raso & Stollar, 1975; Tunnicliff et al., 1977). Since these adducts combine both substrate and coenzyme into a single molecule, they might be expected to be potent enzyme inhibitors (Byers, 1978). In addition, these adducts can be useful in exploring mechanistic aspects of PLP-dependent enzymes. In a previous publication (Rudd et al., 1979), we reported that *N*-(5′-phosphopyridoxyl)-L-Dopa (PPxy-L-Dopa) and *N*-(5′-deoxypyridoxyl)-DL-Dopa were inhibitors of mouse liver Dopa decarboxylase (aromatic L-amino acid decarboxylase, EC 4.1.1.28). We report in this paper a detailed characterization of the interactions between coenzyme–amino acid adducts and purified hog kidney Dopa decarboxylase.

Dopa decarboxylase has been purified from porcine kidney (Christenson et al., 1970; Lancaster & Sourkes, 1972; Borri-Voltattorni et al., 1979). It has a very high affinity for its coenzyme and can exist as holoenzyme in the absence of ex-

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[1] Abbreviations used: PLP, pyridoxal 5′-phosphate; PMP, pyridoxamine 5′-phosphate; Dopa, 3,4-dihydroxyphenylalanine; Tyr, tyrosine; PPxy, 5′-phosphopyridoxyl; BSA, bovine serum albumin; DEAE, diethylaminoethyl; EDTA, ethylenediaminetetraacetic acid.

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