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Magnetic Resonance Studies of the Binding Site Interactions between ^{19}F-Labeled Nitrophenyl Haptens and Specific Mouse Myeloma Immunoglobulin MOPC-315†

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ABSTRACT: The interactions between MOPC-315, a mouse myeloma protein with specificity for nitrophenyl haptens, and ^{19}F-substituted haptens have been investigated using nuclear magnetic resonance (NMR) spectroscopy. The haptens studied are mono- or dinitrophenyl derivatives of γ-aminobutyric acid, lysine, or glycine which have trifluoromethyl groups attached to the phenyl rings. Upon binding to immunoglobulin, the ^{19}F nucleus experiences a downfield shift whose magnitude depends on the position of the trifluoromethyl group on the phenyl ring but is independent of other structural changes in the hapten such as the number of nitro groups attached to the phenyl ring. Further, the chemical shift of bound hapten is not influenced by the amount of the constant region attached to the binding site; we accordingly conclude that the presence of the distal, constant regions of the immunoglobulin molecule does not influence binding site interactions.

As a part of a program aimed at gaining a molecular understanding of the specific recognition and binding of antigen by antibody (Goetze and Richards, 1977), we have used magnetic resonance techniques to study the interactions of the MOPC-315 myeloma protein, and its binding fragments, with specific haptens containing ^{19}F as a probe for magnetic resonance observation. In particular, trifluoromethyl groups have proved to be sensitive probes of their environment when used, for example, to study the allosteric changes which occur on ligation of hemoglobin (Huestis and Raftery, 1972) and to probe the binding site of chymotrypsin (Smallcombe et al., 1972a,1972b; Gammon et al., 1972; Maddox et al., 1975).

Mouse plasmacytoma MOPC-315 grows in Balb/c mice (Eisen et al., 1968) and produces an α class immunoglobin with a λ_2 light chain (Dugan et al., 1973). The antibody has a high affinity for ligands which incorporate a di- or trinitrophenyl group (Eisen et al., 1968; Johnston et al., 1974), and its binding specificity and affinity (Haimovich and Eisen, 1971; Johnston et al., 1974), amino acid sequence (Dugan et al., 1973; Francis et al., 1974), binding kinetics (Haselkorn et al., 1974), and fragmentation by proteolytic enzymes (Inbar et al., 1971, 1972; Hochman et al., 1973) have been previously studied. The nature of the binding packet of MOPC-315 has also recently received attention (Dwek et al., 1977; Padlan et al., 1977).

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Materials and Methods

Maintenance of MOPC-315 Plasmacytoma. Balb/c mice injected with MOPC-315 plasmacytoma were obtained from Herman Eisen (Washington University School of Medicine, St. Louis, Mo.) and Salk Institute for Biological Studies (San Diego, Calif.). The tumor was transplanted by a subcutaneous injection into Balb/c female mice (Potter, 1972). Mice were obtained from a variety of vendors and, as long as the mice were healthy, there was no noticeable difference in tumor growth.

Purification of MOPC-315 Immunoglobulin. MOPC-315 protein was initially acquired from the serum obtained by bleeding the tumor-laden Balb/c mice by the throat 20–21 days after tumor injection. The average mouse produced 0.5 mL of serum from which 8–11 mg/mL of purified protein could be obtained.

Better yields were obtained from mineral oil primed CDF (Balb/c × DBA/2) hybrid females injected intraperitoneally with screened MOPC-315 tumor cells as described by Potter (1972). These mice produced 3–5 mL of ascites per tapping and could be tapped twice a week for 2 to 3 weeks. From the ascites, 3–5 mg/mL of purified MOPC-315 immunoglobulin could be obtained.

MOPC-315 antibody content of the ascites or serum was occasionally tested by immunodiffusion on Ouchterlony plates against DNP-BGG.[1]

The MOPC-315 immunoglobulin was purified by affinity chromatography as described by Inbar et al. (1971) with only slight modification. The ascites was filtered through glass wool and diluted with one-tenth its volume of 2 M Tris-HCl, pH 8.6. This buffered ascites was reduced with 0.01 M dithiothreitol for 1 h at room temperature followed by alkylation in 0.03 M iodoacetamide for 2 h in an ice bath. The reduced and alkylated protein was then loaded directly onto a Sepharose column conjugated with DNP-lysine and eluted with 0.5 M DNP-glycine.

MOPC-315 7S monomer used for binding studies was further purified on a DE-52 column. The 7S monomer purified by affinity chromatography was eluted from a DE-52 column by using a 0.03 to 0.3 M potassium phosphate gradient (pH 8.0) at 4 °C. This gave a single asymmetric peak. Only fractions at the top of the peak were used. Protein purified in this manner was clearer at high concentrations, but no qualitative differences were noticed in NMR experiments.

Preparation of Fab′ and Fv Fragments of MOPC-315. Fab′ fragments were made by a 1% pepsin digest of the purified MOPC-315 antibody in 0.05 M sodium acetate buffer, pH 4.7, for 6 h at 37 °C (Inbar et al., 1971). The fragments were purified by affinity chromatography as described above. Purification was checked by 7.5% sodium dodecyl sulfate gel electrophoresis which indicated greater than 90% Fab′, in agreement with Inbar et al. (1971).

Fv fragments were made by 1% pepsin digest of the Fab′ fragment in 0.1 M sodium acetate buffer, pH 3.6, for 4 h at 37 °C (Inbar et al., 1972; Hochman et al., 1973). After purification on the DNP-lysine Sepharose column, the Fv fragments were further purified by gel filtration chromatography on a Sephadex G-75 column. Purification was checked by 7.5% sodium dodecyl sulfate gel electrophoresis and showed that 90% of the protein migrated as a single band. The other 10% migrated with a molecular weight similar to a normal light chain. The purified Fv fragments were dialyzed against distilled water and lyophilized.

Preparation of NMR Samples. Purified MOPC-315 immunoglobulin, 7S monomer, was dialyzed against PBS* buffer (0.15 M NaCl, 0.01 M sodium phosphate, 0.001 M EDTA, and 0.02% NaN_3 at pH 7.4) and concentrated to the desired concentration under pressure by ultrafiltration. Normal NMR samples had protein concentrations of 30–120 mg/mL determined from the protein's absorption at 278 nm (Abs_{278}^{1cm} = 1.35 mg/mL, mol wt 153 000) (Underdown et al., 1971). The fluorinated haptens were added to this protein solution in one of two ways: a small aliquot was added from a standard solution, or the protein solution was added to a centrifuge tube in which a premeasured aliquot of the fluorinated nitrophenyl solution had been lyophilized.

NMR samples of MOPC-315 Fab′ fragments were prepared in the same manner (Abs_{278}^{1cm} = 1.35 mg/mL, mol wt 50 000 gave the best agreement with the integrated areas of the bound and free peaks seen in ^{19}F NMR spectra with 2NTP-GABA and DNTP-GABA).

NMR samples of Fv fragments of MOPC-315 were reconstituted from the lyophilized protein. A preweighed sample of the Fv protein was added to a centrifuge tube containing 2.5 mL of PBS* buffer. The protein was dissolved in the buffer by a combination of stirring and centrifugation (all but a small amount dissolved). The supernatant was removed and the fluorinated hapten was added as described above. Concentrations of the Fv protein were determined either by assuming all dissolved or from the absorption at 278 nm (Abs_{278}^{1cm} = 1.50 mg/mL, mol wt 25 000; Hochman et al., 1973). This reconstituted protein showed about 70–80% of the calculated binding activity as determined by ratio of bound to free peaks in ^{19}F NMR spectra. NMR spectra were usually determined on solutions which contained 10–30 mg/mL of Fv protein.

Fluorine NMR spectra were observed on 2.0–3.0 mL of sample in a 12-mm tube containing a 5-mm tube of D_2O for locking. The spectra were determined on a Varian XL-100-15 operating at 94.1 MHz for ^{19}F NMR.

γ-N-(2,6-Dinitro-4-trifluoromethylphenyl)aminobutyric Acid (DNTP-GABA). DNTP-GABA (see Figure 1) was prepared by modification of the procedure described by Porter (1950). 3,5-Dinitro-4-chlorobenzotrifluoride (Columbia Organics) was purified by dissolving in ether, washing with 0.1 M Na_2CO_3 (pH 10.5), and then removing the ether to give phenol-free 3,5-dinitro-4-chlorobenzene. γ-Aminobutyric acid (1.0 g, 9.7 mmol) and $NaHCO_3$ (2.2 g) were dissolved in 28 mL of water and titrated to pH 7.5. To this solution was added an ethanolic solution (54 mL) of 3,5-dinitro-4-chlorobenzotrifluoride (3.48 g, 12.6 mmol). This mixture was allowed to react for 20 h at room temperature with vigorous stirring. The ethanol was removed under reduced pressure, the pH was raised to 10.5 to dissolve the DNTP-GABA produced, the solution was washed with 3 × 25 mL of ether, and the product was precipitated by acidification with HCl. This filtered and dried product weighed 2.0 g, 61% yield. A portion of product was further purified by recrystallization in benzene, mp 139–140 °C. Infrared spectra of DNTP-GABA (KBr pellet) had absorptions at 3300 cm^{-1} (N—H), 1700 cm^{-1} (C=O), and 1630 cm^{-1} (C=C); NMR spectrum (acetone-d_6) had a

[1] Abbreviations used: DNP-BGG, dinitrophenylated bovine γ-globulin synthesized as described by Eisen (1968) or purchased from Sigma; DNTP-GABA, *γ-N-(2,6-dinitro-4-trifluoromethylphenyl)*aminobutyric acid; NTCB, 2-nitro-4-trifluoromethylchlorobenzene; 2NTP-GABA, γ-*N*-(2-nitro-4-trifluoromethylphenyl)aminobutyric acid; NTP-GABA, γ-*N*-(4-nitro-2-trifluoromethylphenyl)aminobutyric acid; DNTP-Gly, γ-*N*-(2,6-dinitro-4-trifluoromethylphenyl)aminobutyric acid; NTP-Lys, ε-*N*-(4-nitro-2-trifluoromethylphenyl)-L-lysine; EDTA, ethylenediaminetetraacetic acid; PBS, 0.15 M NaCl, 0.01 M sodium phosphate, pH 7.4; PBS*, PBS plus 0.02% NaN_3 and 1 mmol of EDTA; DNTP-Lys, *ε-N-(2,6-dinitro-4-trifluoromethylphenyl)*lysine; 2NTP-Lys, ε-*N*-(2-nitro-4-trifluoromethylphenyl)lysine; Cbz, benzyloxycarbonyl.

singlet at δ 8.52 ppm (2 H), a quartet δ 3.50 ppm (2 H), two overlapping multiplets centered at δ 2.1 (2 H) and 2.5 ppm (2 H) and a broad N—H signal under the phenyl peak; ^{19}F NMR (PBS*) had a single peak at 13.75 ppm downfield from an external standard of trifluoroacetic acid (2.5 mmol of TFA in a D_2O, H_2O mixture); visible spectra (PBS*) max 428 nm with a molar absorptivity of 6.36×10^3 cm^{-1} M^{-1}. Anal. Calcd for $C_{11}H_{10}F_3N_3O_6$: C, 39.18; H, 2.99; F, 16.90; N, 12.46. Found: C, 39.21; H, 3.04; F, 16.87; N, 12.44.

2-Nitro-4-trifluorochlorobenzene (*NTCB*). NTCB was prepared from 4-amino-3-nitrobenzotrifluoride using a modification of the procedure outlined by Gunstone and Tucker (1963). The yield was 75% after petroleum extraction. This product was used in the synthesis of 2NTP-GABA without further purification.

γ-N-(2-Nitro-4-trifluoromethylphenyl)aminobutyric Acid (*2NTP-GABA*). NTCB[2] (2.9 g, 13 mmol) was dissolved in 54 mL of ethanol and added to a stirred solution of 1.0 g (9.7 mmol) of γ-aminobutyric acid and 2.2 g of $NaHCO_3$ in 28 mL of water. This mixture was refluxed for 20 h and the product isolated by the procedure used for DNTP-GABA. The yield was 2.1 g (74%). Part of the product was purified by recrystallization in benzene to give yellow crystals, mp 102–103 °C. Structure of 2NTP-GABA was verified by IR spectroscopy (KBr pellet) which had absorptions at 3300 cm^{-1} (N—H), 1700 cm^{-1} (C=O), and 1620 cm^{-1} (C=C); the NMR spectrum (acetone-d_6) had a split singlet at δ 8.40 ppm (1 H), a doublet of doublets at δ 7.75 ppm (1 H), a doublet centered at δ 7.27 ppm, (1 H), a quartet centered at δ 3.57 ppm (2 H); two overlapping multiplets at δ 2.5 (2 H) and δ 2.1 ppm (2 H), and the N—H protein giving a broad peak at δ 8.5 ppm; the ^{19}F NMR spectrum (PBS*) had a single peak at 13.97 ppm downfield from an external standard trifluoroacetic acid solution; visible spectrum PBS* λ_{max} 429 nm with a molar absorptivity of 6.06×10^3 cm^{-1} M^{-1}. Anal. Calcd for $C_{11}H_{11}F_3N_2O_4$: C, 45.21; H, 3.79; F, 19.50; N, 9.59. Found: C, 45.12; H, 3.83; F, 19.55; N, 9.58.

γ-N-(4-Nitro-2-trifluoromethylphenyl)aminobutyric Acid (*NTP-GABA*). 5-Nitro-2-fluorobenzotrifluoride (3.6 g, 17.2 mmol) from Pierce Chemical was dissolved in 54 mL of ethanol and added to a stirred solution of 1.0 g (9.7 mmol) of γ-aminobutyric acid and 2.2 g of $NaHCO_3$ in 28 mL of water adjusted to pH 9.0. After reacting for 60 h at ambient temperature, NTP-GABA was isolated as described for DNTP-GABA. Crude yield of product was 0.94 g, 36% (increased yields and faster reaction times would probably be obtained by heating the reaction mixture to reflux). Some NTP-GABA was recrystallized in benzene and had a mp of 181–182 °C, and its structure was verified by: IR spectroscopy (KBr pellet) which had peaks at 3350 cm^{-1} (N—H), 1680 cm^{-1} (C=O), and 1570 cm^{-1} (C=C); NMR spectrum (acetone-d_6) had a multiplet centered at δ 8.3 ppm (2 H), a doublet at δ 7.10 ppm (1 H), a quartet at δ 3.55 ppm (2 H), two overlapping multiplets at δ 2.43 (2 H) and 2.06 ppm (2 H), and a single broad N—H peak at δ 6.44 ppm (1 H); ^{19}F NMR spectrum (PBS*) had only a single absorption 12.03 ppm downfield from an external standard of a trifluoroacetic acid solution; visible spectra (PBS*) had a λ_{max} at 384 nm and a molar absorptivity of 1.90×10^4 cm^{-1} M^{-1}. Anal. Calcd for $C_{11}H_{11}F_3N_2O_4$: C, 45.21; H, 3.79; F, 19.50; N, 9.59. Found: C, 45.29; H, 3.79; F, 19.64; N, 9.54.

γ-N-(2,6-Dinitro-4-trifluoromethylphenyl)aminoacetic Acid (*DNTP-Gly*). Washed 3,5-dinitro-4-chlorobenzotrifluoride (5.0 g, 18 mmol) in 54 mL of ethanol was added to a stirred solution of 2.0 g (27 mmol) of aminoacetic (Gly) acid and 2.2 g of $NaHCO_3$ in 27 mL of water (pH 8.5). This mixture was heated on a steam bath to form a homogeneous solution and the product was isolated as mentioned for DNTP-GABA after 3 h to give 4.7 g of product, 84% yield. Recrystallization in benzene gave yellow crystals that melted at 192–196 °C. Structure was verified by: IR spectroscopy (KBr pellet) showed peaks at 3300 cm^{-1} (N—H), 1730 cm^{-1} (C=O), and 1600 cm^{-1} (C=C); NMR spectrum (acetone-d_6) had absorptions at δ 8.5 ppm (2 H) a singlet, a multiplet at δ 4.0 ppm (2 H), and a broad peak at δ 9.3 ppm (1 H); ^{19}F NMR spectrum (PBS*) had a single absorption 13.71 ppm downfield from an external trifluoroacetic acid standard; and a visible absorption λ_{max} 426 nm with a molar absorptivity of 6.42×10^3 cm^{-1} M^{-1}. Anal. Calcd for $C_9H_6F_3N_3O_6$: C, 34.63; H, 1.94; N, 13.46. Found: C, 35.17; H, 2.05; N, 13.19.

Solutions of DNTP-Gly in PBS* formed a precipitate on sitting for long periods of time.

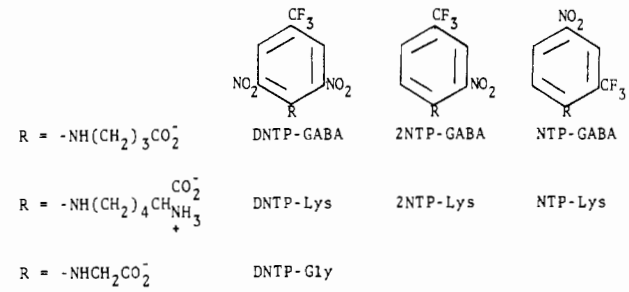

FIGURE 1: Structure and abbreviations of fluorinated nitrophenyl haptens.

ϵ-N-(4-Nitro-2-trifluoromethyl)-L-lysine (*NTP-Lys*). NTP-Lys was prepared by reacting 2-fluoro-5-nitrobenzotrifluoride with α-*N*-carbobenzoxy-L-lysine (Sigma) by a slight modification of the procedure used by Goetzl and Metzger (1970a) to make ϵ-*N*-*p*-nitrophenyllysine. Yield of yellow solid was 70%. NTP-lysine was purified by multiple precipitations at its isoelectric point. A final attempt was made to crystallize NTP-Lys by adding just enough HCl to cause it to dissolve when heated on a steam bath, but it precipitated out of solution without forming crystals. NTP-Lys decomposed at 198–200 °C. Structure was verified by: IR spectroscopy (KBr pellet) showed peaks at 3500 cm^{-1} (N—H), 1625 cm^{-1} (C=O), and 1600 cm^{-1} (C=C); NMR spectrum (1:1 TFA, D_2O) had an overlapping singlet and doublet centered at δ 8.2 ppm (2 H), a doublet at δ 6.8 ppm (1 H), two unresolved triplets at δ 4.3 ppm (1 H) and 3.4 ppm (2 H), and a multiplet centered at δ 2.0 ppm (6 H); and a single fluorine absorption (PBS*) was observed at 11.96 ppm downfield from an external trifluoroacetic acid standard. Anal. Calcd for $C_{13}H_{16}F_3N_3O_4$: C, 46.57; H, 4.81; N, 12.53. Found: C, 45.44; H, 4.86; N, 11.79; and 0.7% Cl. No further attempt was made to purify NTP-Lys.

ϵ-N-(2,6-Dinitro-4-trifluoromethylphenyl)-L-lysine (*DNTP-Lys*). DNTP-Lys was made by reacting 2,6-dinitro-4-trifluoromethylchlorobenzene with α-*N*-Cbz-L-lysine by the procedure of Goetzl and Metzger (1970b). The product was crystallized in water by adding just enough HCl to cause DNTP-Lys to dissolve when heated on a steam bath. Yield was 33% of yellow crystals that decomposed at 204–205 °C. An IR spectrum (KBr pellet) had absorptions at 3400 cm^{-1} (N—H), 1650 cm^{-1} (C=O), and 1590 cm^{-1} (C=C); NMR spectrum (1:1 TFA, D_2O) had a single phenyl absorption at δ 8.5 ppm (2 H), two unresolved triplets at δ 4.1 ppm (1 H) and 3.1 ppm

[2] 4-Fluoro-3-nitrobenzotrifluoride can be used in place of NTCB and is now commercially available from Columbia Organics.

(2 H), and a broad multiplet centered at δ 1.9 ppm (6 H); and ^{19}F NMR spectrum (PBS*) showed a single absorption at 13.75 ppm downfield from trifluoroacetic acid. Anal. Calcd for $C_{13}H_{15}F_3N_4O_6$: C, 41.06; H, 3.98; N, 14.78. Found: C, 39.14; H, 3.80; N. 12.72; Cl, 3.1. No further efforts were made to purify DNTP-Lys. DNTP-Lys was very difficult to work with, because its solubility in PBS* at pH 7.4 was only about 2×10^{-4} M and it decomposed when stored in higher pH solutions.

ϵ-N-(2-Nitro-4-trifluoromethylphenyl)-6-lysine (2NTP-Lys). 2NTP-Lys was prepared by reacting NTCB with ϵ-*N*-Cbz-L-lysine. The product was crystallized by adding just enough HCl to dissolve 2NTP-Lys in hot water giving a 14% yield of yellow crystals which decomposed at 201–203 °C. An IR spectrum (KBr pellet) had peaks at 3350 cm^{-1} (N—H), 1640 cm^{-1} (C=O), and 570 cm^{-1} (C=C); NMR spectra (1:1 TFA, D_2O) showed a singlet at δ 8.3 ppm (1 H), a doublet, δ 7.6 ppm (1 H), a doublet at δ 7.0 ppm (1 H), and broad multiplets at δ 4.3 ppm (1 H), 3.5 ppm (2 H), and 2.0 ppm (6 H); and there was a ^{19}F NMR absorption (PBS*) at 13.99 ppm with a small impurity (<5%) at 13.83 ppm downfield from an external trifluoroacetic acid standard. Anal. Calcd for $C_{13}H_{16}F_3N_3O_4$: C, 46.57; H, 4.81; N, 12.53. Found: C, 46.07; H, 4.84; N, 11.93; Cl, 2.42. No further attempts were made to purify 2NTP-Lys.

Determination of Binding Affinities. Binding affinities of the fluorinated nitrophenyls were determined by NMR spectroscopy and fluorescence quenching.

Fluorescence Quenching. Initial determinations of the binding affinity of DNTP-GABA and 2NTP-GABA to MOPC-315 7S monomer were hampered by high affinity, nonspecific quenching. This nonspecific quenching was minimized by using protein further purified on a DE-52 column. The remaining nonspecific quenching and machine fluctuations were corrected by using a solution of Fc peptides (material not absorbed on the affinity column after pepsin digest to make Fab′ fragments).

Protein samples in PBS* buffer were adjusted to a concentration of 0.05–0.5 mg/mL and centrifuged to remove any dust or precipitate[3] and the concentration rechecked by absorption at 278 nm. The fluorescence spectra were run on 3 mL of this protein solution using a Perkin-Elmer fluorescence spectrophotometer MPF-4. The excitation wavelength was 290 nm and the fluorescence was observed at 332 nm. The protein was titrated with 10-μL aliquots of hapten solution and the blank fluorescence was obtained by a simultaneous titration of a sample containing only the buffer. Nonspecific binding and machine fluctuations were determined by a concurrent titration of the Fc peptides of equal absorption at 278 nm. The fluorescence data were analyzed and the dissociation constant determined from the slope and intercept of the Sips plot (Eisen, 1968).

NMR Binding Determinations. The binding affinities of the fluorinated haptens were also determined by NMR spectroscopy. The ^{19}F NMR spectra of NTP-GABA are in fast exchange on the NMR time scale. The observed chemical shift, δ, of this fluorinated label is dependent on the ratio of the concentration of the label bound, [AbHap], to the total hapten concentration [Hap_0] and on the total chemical shift, Δ, eq 1.

$$\delta = \frac{[\text{AbHap}]}{[\text{Hap}_0]}\Delta \tag{1}$$

[3] Originally solutions were filtered though a 0.45-μm Millipore filter, but the coatings on the filter added to protein and buffer absorption at 278 nm.

From the ^{19}F NMR spectra of a number of different antibody hapten ratios, it is possible to determine the dissociation constant as described by Gammon et al. (1972). Using this method the dissociation constant and total chemical shift, Δ, for NTP-GABA and NTP-Lys were determined.

Once the dissociation constant and Δ are determined for NTP-GABA the binding affinity of the other labels can be determined by inhibition of NTP-GABA binding. The procedure for determination of the binding of a competitive substrate by NMR spectroscopy is outlined by Smallcombe et al. (1972a) for chymotrypsin. The procedure used by them is slightly more complicated than needed here because they included enzyme dimerization. Their equations can be rewritten without dimerization in terms of antibody, [Ab], hapten [Hap], and competitive hapten [Hap′] concentration to give:

$$[\text{Ab}_0] = [\text{Ab}] + [\text{AbHap}] + [\text{AbHap}'] \tag{2}$$

$$[\text{Hap}_0] = [\text{Hap}] + [\text{AbHap}] \tag{3}$$

$$[\text{Hap}_0'] = [\text{Hap}'] + [\text{AbHap}'] \tag{4}$$

$$K_d = \frac{[\text{Ab}][\text{Hap}]}{[\text{AbHap}]} \tag{5}$$

$$K_d' = \frac{[\text{Ab}][\text{Hap}']}{[\text{AbHap}']} \tag{6}$$

$$K_d' = \frac{([\text{Ab}_0] - [\text{AbHap}] - [\text{AbHap}'])([\text{Hap}_0'] - [\text{AbHap}'])}{[\text{AbHap}']} \tag{7}$$

$$[\text{AbHap}'] = \frac{K_d[\text{AbHap}] - [\text{Ab}_0][\text{Hap}_0] + [\text{AbHap}][\text{Hap}_0] + [\text{Ab}_0][\text{AbHap}] - [\text{AbHap}]^2}{[\text{AbHap}] - [\text{Hap}_0]} \tag{8}$$

from eq 1

$$[\text{AbHap}] = \frac{\delta[\text{Hap}_0]}{\Delta} \tag{9}$$

Results

Binding Affinities. Table I lists the binding affinities for various fluorinated haptens as determined both by magnetic resonance and by fluorescence quenching techniques. In the case for protein concentrations of less than 0.5 mg/mL the binding constants were calculated from the slope and intercept of Sips plots. In the case of magnetic resonance techniques, protein concentration was about 40 mg/mL. Early work seemed to substantiate the claim (Johnston et al., 1974) that binding affinities for MOPC-315 proteins are dependent on protein concentration. However, studies using DE-52 purified protein and Fc peptides to correct for nonspecific quenching gave binding affinities which were essentially in agreement with those determined by the magnetic resonance techniques which gave values only to one significant figure. This inaccuracy is not inherent in the NMR method, but, in these cases, to obtain good signal to noise sensitivity in a reasonable observation time, one must use protein concentrations considerably greater than the hapten–antibody dissociation constant. The fact that $[\text{Ab}_0] \gg K_d$ leads to the inaccuracy.

The binding affinities of DNTP-Lys and 2-NTP-Lys were not determined because of difficulties of obtaining pure samples and the low solubility at pH 7.4 of DNTP-Lys; however, the binding affinities of these two ligands are probably roughly similar to those for their γ-aminobutyric acid analogues. For example, Haimovich Eisen (1971) found the binding affinity

TABLE I: Binding Affinity[a] of Fluorinated Haptens for MOPC-315 Immunoglobulin Determined by Fluorescence Quenching and NMR Spectroscopy.

	K_a by fluorescence quenching	Q_{max}[b]	Sips[c] coeff.	K_a of NMR spectroscopy
DNTP-GABA	3.4×10^6	67	0.99	1×10^6
2NTP-GABA	7.8×10^5	66	1.01	$>3 \times 10^5$
NTP-GABA	1.3×10^5	67	1.06	2×10^5
DNTP-Gly	2.4×10^4	69	1.02	3×10^4
NTP-Lys				1×10^5

[a] Binding affinities measured at 25.0 ± 0.1 °C for fluorescence quenching and 25.5 ± 1 for NMR spectroscopy. [b] Sips index of heterogeneity from fluorescence measurements. [c] Fraction of protein fluorescence quenched when saturated by hapten.

TABLE II: Total Chemical Shift, Δ, of Fluorinated Haptens Bound to MOPC-315 Immunoglobulin and Its Proteolytic Fragments.[a]

	7S immunoglobulin	Fab′ fragment	Fv fragment
DNTP-GABA	1.79 ± 0.05	1.79 ± 0.05	1.81 ± 0.05
DNTP-Lys	1.79 ± 0.05	1.79 ± 0.05	1.77 ± 0.05
DNTP-Gly		>1.53	>1.51
NTP-GABA	0.62	0.60 ± 0.05	0.66 ± 0.05
NTP-Lys	0.72 ± 0.05	0.62 ± 0.05	
2NTP-GABA	1.85 ± 0.05	1.83 ± 0.05	1.79 ± 0.05
2NTP-Lys			1.76 ± 0.05

[a] Note: All shifts are downfield and measured in ppm.

of MOPC-315 for DNP-GABA (7.0×10^7 M^{-1}) to be higher by a factor of 3.5 than for DNP-Lys (2.0×10^7 M^{-1}) at 4 °C. Johnston et al. (1974) measured the binding of DNP-Lys at both 4 and 25 °C; binding was stronger by an order of magnitude at 4 °C relative to 25 °C. Though the affinities of MOPC-315 for DNP-GABA and TNP-GABA (the analogues of the haptens we have studied in which a nitro group replaces a trifluoromethyl group) were not determined, we can estimate the effect of exchanging a trifluoromethyl group for nitro group if we assume that the DNP-GABA analogue still binds more strongly at 25 °C than the DNP-Lys analogue by a factor of about 3.5. Using this type of comparison together with the experimental results in Table I, we estimate that substitution of a *p*-nitro substituent by a *p*-trifluoromethyl group causes ΔG for binding to increase by about 1 kcal/mol (i.e., p-CF_3 binds less strongly). Substitution of an *o*-nitro group by an *o*-trifluoromethyl group causes ΔG for binding to increase by about 2 kcal/mol. Various published determinations of the affinity for DNP-Lys differ by about 0.5 kcal/mol (Johnston et al., 1974, measured the binding affinities by both fluorescence quenching and equilibrium dialysis and also list several other literature values). While such comparisons are not likely to be quantitatively precise, they suggest two general conclusions: (i) substitution of a nitro group by a trifluoromethyl group does not cause a major change in binding energy; (ii) binding is somewhat more sensitive to such substitutions at the ortho compared with the para position of the phenyl ring.

Though we have not determined the affinities for binding of the various fluorinated haptens to the Fab′ and Fv fragments of MOPC-315, they are probably similar to those for binding to the intact immunoglobulin molecule. Hochman et al. (1973) found the affinity for binding of DNP-Lys to the intact MOPC-315 7S protein and to the Fab′ fragment to be equal (2.4×10^6 M^{-1}) while that to the Fv fragment was only slightly stronger (2.8×10^6 M^{-1}).

Chemical Shift. The fluorine nuclei of all the haptens studied in this work show a downfield shift when the haptens bind to MOPC-315. Table II tabulates these shifts and shows that their magnitudes depend primarily on the structural relationship between the side chain of the hapten and the trifluoromethyl group and are essentially independent of the other substituents on the aromatic ring. In contrast to these observations for binding to MOPC-315, these same haptens show strikingly different behavior when they interact with other anti-DNP myeloma proteins. For example, in this work we have found that DNP-GABA when bound to MOPC-315 shows a downfield shift of 1.8 ppm whereas, in other work, binding to XRPC-25 causes an upfield shift of 1.0 ppm. Thus both the direction and magnitude of the shifts observed are crucially dependent on the protein to which a given hapten binds.

Discussion

Binding Affinities. The data of Table I show that neither the nature of the substituents nor their relative position about the phenyl ring greatly affect the binding constants. Thus 2NTP-GABA, NTP-CABA, and DNTP-GABA have K_{ass} values within about an order of magnitude of each other. In contrast the side chain has a larger effect (DNTP-GABA binds 140 times more strongly to MOPC-315 than does DNTP-Gly). Previous work (Hochman et al., 1973) indicates that the Fv fragment of MOPC-315 has a slightly stronger affinity for DNP-Lys than does either the Fab′ fragment or the 7S monomer.

Our results for the binding affinities of various haptens can be compared with structural models proposed by Padlan et al. (1977) and Dwek et al. (1977) which place the DNP ring into a hydrophobic aromatic box. In this location, the major interactions between the hapten and the protein can be viewed as ring stacking or charge transfer involving the nitrophenyl ring of the hapten and Trp-93 of the light chain of the immunoglobulin together with hydrogen bonding between the *p*-nitro group and Tyr-34 (L) and between an *o*-nitro group and Asn-36 (L). As both a nitro and trifluoromethyl group are strongly electron withdrawing, substitution of one for the other might well not significantly alter charge transfer interactions between hapten and protein.

Inter- and intramolecular hydrogen bonds have been reported involving nitro groups (Baitman et al., 1964). In contrast only weak intramolecular hydrogen bonding in dilute solutions is usually observed for trifluoromethyl groups (Doddrell et al., 1969; Kiehlmann et al., 1973; Baker and Shulgin, 1965). Thus, substitution of a nitro group by a trifluoromethyl group should weaken somewhat that part of the binding affinity which results from hydrogen bonding interactions.

Other characteristics of a nitro group and trifluoromethyl group are somewhat similar. For example, the dipole moments of both should be in the same direction so that both should participate similarly in dipole–dipole and ion–dipole interactions which, as in the case of hydrogen bonding, will probably not be of the same strength. The van der Waals radii of a trifluoromethyl (2.7 Å) and a nitro group (2.6 Å) are essentially the same. (These values are calculated from bond distances determined by x-ray diffraction of a trifluoromethylphenyl compound (Cohen-Addad, 1973) and of nitrobenzene (Trotter, 1959) together with van der Waals radii listed by Pauling (1960)). Of course, the trifluoromethyl group, being spherical, will occupy more space than the planar nitro group.

Thus, qualitatively, one might observe as we do, that a tri-

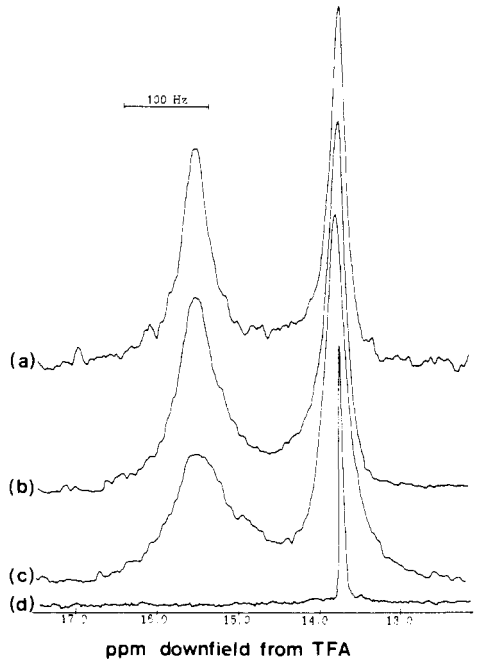

FIGURE 2: ^{19}F NMR spectra of DNTP-GABA: (a) 4.4×10^{-4} M with 3.3×10^{-4} M Fv MOPC-315; (b) 7.6×10^{-4} M with 3.5×10^{-4} M Fab' MOPC-315; (c) 5.8×10^{-4} M with 3.0×10^{-4} M (in binding site) 7S MOPC-315; (d) 10^{-3} M in buffer with no protein (0.5 kHz of 2.5 kHz spectra width, 0.3 c-s acquisition time, 2-s pulse delay, 9–23 K transients, 0.1-s sensitivity enhancement, 8K Fourier transform, 90° rf pulse, D_2O lock). Probe temperatures were 25 °C. Chemical shifts are in ppm downfield relative to an external trifluoroacetic acid standard (2.5 mmol in D_2O, H_2O).

fluoromethyl and a nitro group interact similarly with MOPC-315. The weaker binding of NTP-GABA (with the CF_3 group in the ortho position) relative to 2NTP-GABA (with the $-CF_3$ group in the para position) suggests a stronger hydrogen bond to the ortho than to the para substituent (which should favor a nitro over a trifluoromethyl group). Alternately, this subsite in the protein for the *o*-nitro group may be narrow and oblong which could force the phenyl ring to be rotated 180° so that the *o*-CF_3 group in fact occupies the subsite normally occupied by a third nitro group at C-6 of TNP-haptens.

The binding data for 2,6-dinitro, 4-nitro, 2,4-dinitro, and 2,4,6-trinitro compounds (Haselkorn et al., 1974) indicate strong binding interactions between the protein and nitro groups in the 2 and 4 positions; a nitro group in the 6-position interacts much more weakly. The binding affinities of the NTP compounds when compared with 4-nitrophenyl compounds show a strong interaction between the *o*-CF_3 group and the protein which causes us to favor a binding mode in which this *o*-CF_3 group does, in fact, occupy the position normally occupied by a 2-NO_2 substituent.

Environment of Bound Ligand. The environment of the bound hapten can be probed by observing the chemical shift of the ^{19}F nucleus of the trifluoromethyl group of the hapten (Table II). Though very sensitive to environmental effects, the total chemical shift Δ (for the hapten in the hapten–antibody complex relative to the hapten free in solution) should be independent of such other factors as binding affinity and dynamics (Gammon et al., 1972). (This shift, Δ, should be distinguished from the chemical shift, δ, actually observed under a given set of experimental conditions; in the case of fast exchange, δ depends crucially on such factors as binding affinity).

Downfield shifts of ^{19}F nuclei on binding to protein have previously been observed, for example, in chymotrypsin–inhibitor complexes (Gammon et al., 1972). Many factors can be responsible for such shifts but one which is likely to be particularly germane to antibodies, such as MOPC-315 with specificity for aromatic haptens, is the downfield shift experienced by nuclei brought near the plane of an aromatic ring in the protein. These shifts arise from the effects of the diamagnetic ring currents in the delocalized π-electron systems of aromatic rings. Since the magnetic currents resulting from these fields do not distort the electronic wave functions of the probe nucleus, the magnitude of the shift observed for a given ring current will be independent of the kind of nucleus (Millet and Raftery, 1972). The shifts to be expected for nuclei at various distances and directions from benzene rings have been calculated (Emsley et al., 1965; Johnson and Bovey, 1958). For a 1.7-ppm downfield shift to be caused entirely by diamagnetic ring current, deshielding would require that the fluorine nuclei be held very close to an aromatic system. The likely importance of such ring current contributions to the observed chemical shift finds support in the observation that nitrophenyldiazonium affinity labels react specifically with tyrosine-34 of the light chain of MOPC-315; this amino acid is in the first hypervariable region (Goetzl and Metzger, 1970b). Further evidence comes from circular dichroism studies of the binding of ϵ-*N*-2,4,6-trinitrophenylaminocaproate of MOPC-315; the circular dichroism spectrum of the complex showed strong interactions between this hapten and a nearby tryptophan residue(s) of the antibody (Freed et al., 1976; Orin et al., 1976). They calculated that an interaction of the hapten with only one tryptophan residue (with parallel or tilted orientations) would require a separation of 3.5 Å. Interaction with four tryptophans increased their calculated distance to 7 Å. More precise deductions about such interactions have recently been drawn (Dwek et al., 1977; Padlan et al., 1977) which place the nitrophenyl ring of the hapten in a pocket lined by aromatic residues and in intimate contact with Trp-93 (L).

Other effects which can contribute to a change in chemical shift for a nucleus in a hapten free in solution compared with one bound to antibody include van der Waals interactions, electric fields, and specific bonding with neighboring groups, such as hydrogen bonding (Millet and Raftery, 1972). These are more difficult to evaluate quantitatively, but moving a ^{19}F nucleus into an environment with a higher polarizability (Evans, 1960) or the formation of a new hydrogen bond (Doddrell et al., 1962; Kiehlmann et al., 1973) both usually cause downfield shifts. Given the specificity of MOPC-315 for nitrophenyl haptens, the possibility of hydrogen bond donating residues in the binding pocket seems high. For example, such hydrogen bonds have been suggested between Tyr-34 (L) and a para substituent and between Asn-36 (L) and an ortho substituent in the hapten (Dwek et al., 1977; Padlan et al., 1977).

Two further points about the observed chemical shift deserve emphasis: (i) the shift depends only on the location of the ^{19}F nucleus on the phenyl ring of the hapten; it does not depend on the nature or location of other substituents about the phenyl ring and is, in this sense, relatively insensitive to the changes in affinity caused by such alterations in the structure of the hapten; (ii) the chemical shift is not influenced by the amount of the constant region of the antibody which is present (Fv,Fab, or 7S immunoglobulin, Figure 2).

The essential constancy of the chemical shift of a *p*-CF_3 group in DNTP-GABA, DNTP-Lys, DNTP-Gly, 2NTP-GABA, and 2NTP-Lys suggests that the binding pocket of MOPC-315 for the phenyl ring is a relatively rigid, preformed pocket (lock and key model), which provides the main specific

attractive interactions between antibody and hapten. Thus changing the number of nitro groups from two to one, or changing the side chains from GABA to Lys or Gly does not appreciably alter the orientation of the hapten phenyl group relative to the binding pocket of the antibody. These changes can nevertheless alter the total attraction between hapten and antibody with resulting variations in the binding affinities.

The independence of chemical shift on the size of the immunoglobulin fragment (Fv, Fab′, 7S, IgA) suggests that the structure of the binding site when filled with hapten is essentially the same regardless of how much of the rest of the immunoglobulin molecule is attached. Further, these results do not indicate any observable differences between the two binding sites of a single immunoglobulin molecule. Thus, there are not two classes of binding sites. Our best evidence for this contention is the finding that, in those cases where the hapten is in slow exchange, the spectra show only two species of hapten, one bound and the other free in solution (Figure 2). The fact that we observe no differences between the two binding sites by NMR spectroscopy is consistent with binding data we determined by fluoroescence quenching. Table I lists the Sips coefficient for the binding of MOPC-315 to the fluorinated haptens. The Sips coefficient measures heterogeneity in the observed binding affinities. A value of one indicates that all binding sites have equal binding affinity and do not interact. Thus, antigen binding can be accurately described by a simple mass action equation such as eq 5, which assumes equivalent and noninteracting binding sites on the antibody molecule (Nisonoff and Pressman, 1958).

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