Carbon-13 Nuclear Magnetic Resonance Study of the Binding of Carbon-13-Enriched Tetra-L-alanine Haptens to Fab′ Fragments of Anti-poly(L-alanine) Antibodies†

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ABSTRACT: We have performed ^{13}C nuclear magnetic resonance (NMR) studies of the binding of tetra-L-alanine haptens, each selectively enriched with 90% ^{13}C in a single methyl group, to Fab′ fragments of purified sheep anti-poly(L-alanine) antibodies. Sheep were immunized with a poly(L-alanine)–human serum albumin conjugate, and purified immunoglobulin G antibodies were isolated by affinity chromatography. Two separate pools of purified, specific $F(ab')_2$ fragments were prepared, and each pool was reduced and alkylated to give approximately 200 mg of purified Fab′ fragments. Pool 1 Fab′ fragments bound tetra-L-[^{3}H]alanine with an average association constant (K_a) of 1.1×10^4 M^{-1} at 5 °C; pool 2 had a K_a value of 2.6×10^4 M^{-1} at 5 °C. The enthalpy of binding (ΔH) of pool 1 and pool 2 Fab′ fragments was −5.6 and −7.9 kcal M^{-1}, respectively; the entropy of binding (ΔS) was −1.8 and −8.2 cal mol^{-1} deg^{-1}, respectively. Sips constants for the first and second pools were 0.90 and 0.80, respectively. Both preparations of antibody fragments were found to be very heterogeneous when analyzed by isoelectric focusing. ^{13}C NMR difference spectra were obtained over a range of hapten–Fab′ fragment ratios in order to observe alterations induced in the [^{13}C]methyl resonances of the tetrapeptide by interaction with high- and low-affinity fragments. The higher affinity proteins induce essentially uniform downfield shifts of ~2.8 parts per million in all four methyl resonances of the bound peptide, and the lower affinity antibodies either affected fewer methyl groups or, in most cases, did not induce any shifts in the methyl resonances of the bound peptide. These downfield shifts appear to be due to van der Waals interactions between the methyl groups of the bound peptide and the binding sites of the higher affinity antibodies. The uniformity of the downfield shifts of all four methyl resonances of the bound peptide is consistent with relatively homogeneous hydrocarbon-like environments in the hapten's side-chain binding region of the higher affinity fragments. The direct correlation between the number of methyl groups of the bound hapten that exhibit this shift and the association constants of the antibody fragments suggests that the major difference between the higher and lower affinity Fab′ fragments is the presence or absence of side-chain van der Waals interactions with the hapten. The NMR line-width data indicate that the methyl groups of the peptide are free to rotate in the antibody–hapten complexes whereas the entire backbone of the peptide appears to be firmly bound to both high- and low-affinity fragments. The chemical shift data of the N-terminal methyl group of the peptide are consistent with the amino group being protonated when bound to the Fab′ fragments, which suggests that there may be electrostatic binding between the hapten at this position and the antibody binding sites. The chemical shift of the methyl resonance of the C-terminal residue suggests that the carboxyl group of tetra-L-alanine is protonated when bound to higher affinity antibodies and ionized when bound to lower affinity fragments. The tetrapeptide was found to be in the NMR "slow-exchange" limit with the Fab′ fragments, and calculations indicated that the forward rate constant(s) of peptide binding is (are) relatively slow. The kinetic results suggest that there may be substantial orientational requirements for binding of the hapten to the antibodies.

Elucidation of the molecular basis of antigen–antibody reactions has long been a goal of immunochemical research. Studies of antibody binding specificity include determining the size of the antibody combining site, the chemical determinants of the antigen or hapten that make contact with antibodies, and the nature of the forces that stabilize the antigen–antibody complex. Present concepts of antibody binding sites are derived mostly from information obtained by conventional immunological techniques, such as equilibrium dialysis, hapten inhibition of precipitation, or complement fixation, and the binding of a series of structurally related haptens to antibodies (cf. Kabat, 1976). Although these approaches have demonstrated the remarkable ability of antibodies to discriminate between related structures and have established an upper limit for the size of antigenic determinants, they do not provide direct information concerning the molecular interactions between haptens and antibodies.

Data on the size and shape of immunoglobulin binding sites are emerging from X-ray crystallographic studies of homogeneous immunoglobulins obtained from patients or animals bearing myelomas. Many myeloma proteins have been found to bind small molecules such as phosphorylcholine or dinitrophenyl derivatives, and X-ray crystallographic data have been reported for several of these proteins as well as their ligand complexes (cf. Davies et al., 1975). Questions still remain as to the intrinsic binding specificity of myeloma

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proteins and the relationship of these studies to the binding specificity of specific antibodies. Furthermore, X-ray crystallographic studies do not usually provide kinetic or thermodynamic data on ligand–protein interactions, and any structural conclusions drawn from each study must be confirmed by experiments carried out under physiological conditions in solution.

Nuclear magnetic resonance (NMR) spectroscopy can provide both structural and dynamic data on the interactions between ligands and macromolecules in solution (cf. Dwek, 1973). Using ^{1}H NMR techniques, Dwek and co-workers (Dower et al., 1977) have monitored the binding of dinitrophenyl haptens to the dinitrophenyl-binding mouse myeloma protein MOPC 315. There have also been ^{19}F NMR studies of the binding of ^{19}F-substituted aromatic ligands to myeloma proteins (Hardy & Richards, 1978) and studies of phosphorylcholine binding to myeloma proteins with ^{13}C and ^{31}P NMR (Goetze & Richards, 1977). There have been few attempts to apply NMR techniques to investigate the binding specificity of conventional antibodies, principally because of the difficulties associated with the heterogeneity of these proteins.

We have used ^{13}C NMR to investigate the binding specificity of heterogeneous Fab′ fragments from sheep anti-poly(L-alanine) antibodies by observing the interaction of these fragments with tetra-L-alanine haptens that are selectively enriched with 90% ^{13}C in the methyl groups. This system was chosen because of the extensive immunochemical studies by Schechter (1970, 1971) of rabbit anti-poly(L-alanine) antibodies which implicated the methyl groups of alanine peptides as important molecular determinants in the binding of these haptens to the antibodies. Our results show that ^{13}C NMR techniques can provide a powerful, direct method for determining the nature of the molecular interactions between a hapten and specific heterogeneous antibodies.

Materials and Methods

L-[3-^{3}H]Alanine (16.1 Ci/mmol) was obtained from New England Nuclear. Alanine peptides were purchased from Aldrich Chemicals and Sigma Chemicals. Myoglobin, human serum albumin (HSA), and bovine serum albumin (BSA) were obtained from Sigma Chemicals.

Preparation of Antigens. Poly(L-alanine) was conjugated to HSA (poly(L-Ala)–HSA), BSA (poly(L-Ala)–BSA), and human myoglobin by the method of Schechter et al. (1966). The total amount of L-alanine coupled to the protein was determined by amino acid analysis of the original protein and the conjugate. The number of polyalanine chains and the average chain length were calculated from the lysine content before and after modification of the protein (Anfinsen et al., 1962). The HSA and BSA conjugates each contained approximately 30 chains of polyalanine per mol of protein, and the average number of alanine residues per chain was 6.5.

Immunization Procedure. Adult female sheep were immunized with 0.1–10-mg quantities of poly(L-Ala)–HSA. For the primary immunization, the antigen was administered in complete Freund's adjuvant containing 10 mg/mL *M. tuberculosis* R_{37}HA. Each sheep received a total of 2 mL of the antigen–adjuvant mixture, half of which was administered intramuscularly and the remainder in a series of intradermal injections along the back and 0.25 mL of *H. pertussis* vaccine in a separate intramuscular site. Additional injections of antigen, either the original immunogen or the poly(L-Ala)–BSA, were given intramuscularly in incomplete Freund's adjuvant.

Preparation of Polyalanine Immunoadsorbent. Diaminopentane was coupled to Sepharose 4B by the procedure of Cuatrecasas (1970). The diaminopentane–Sepharose 4B was reacted with *N*-carboxy-L-alanine anhydride according to the procedure of Wilchek (1973). The product was stored at 5 °C in an aqueous solution containing 0.25% phenol.

Purification of Anti-poly(L-alanine) Antibodies. Two pools of antisera were collected from 2 groups of 5 sheep over a period of 6–12 months. The first pool consisted of ~10 L of sera, the second pool 15 L of sera. Each pool, containing 2 mM EDTA,[1] was passed through an immunoadsorbent column (10 × 8 cm) at 5 °C. The column was then washed with 2 mM EDTA in phosphate-buffered saline (PBS) (PBS–EDTA) until the absorbance of the effluent at 280 nm was less than 0.04. The specific anti-poly(L-alanine) antibodies were eluted from that column by 5 mM tetra-L-alanine in PBS–EDTA and concentrated to 20 mg/mL in an Amicon ultrafiltration system containing a PM 10 membrane. The concentration of the antibody solution was calculated by using the value $A_{280}^{0.1\%} = 1.4$. The antibodies were then separated into immunoglobulin M (IgM) and immunoglobulin G (IgG) fractions by chromatography on a Sephadex G-200 column at 5 °C in PBS–EDTA buffer. IgG was the major component of the hapten eluate, and the small quantity of nonimmunoglobulin impurities, mostly albumin, was removed from the antibody preparation by the gel chromatography procedure. Immunoelectrophoretic analysis showed IgG_2 as the major subclass.

Preparation of F(ab′)$_2$ and Fab′ Fragments from Purified Anti-poly(L-alanine) Antibody. The IgG antibodies were digested with pepsin to give F(ab′)$_2$ fragments (Turner et al., 1970). The reduction of F(ab′)$_2$ to Fab′ fragments was performed by a modification of the procedure of Nisonoff et al. (1970) (Geller, 1976). Fab′ fragments were separated from the unreduced F(ab′)$_2$ on a Sephadex G-100 column; the overall recovery was about 80%.

Isoelectric Focusing. Isoelectric focusing in polyacrylamide gels was performed by the method of Righetti & Drysdale (1971) in a commercial apparatus (MRA Corp., Clearwater, FL).

Hapten Binding Assay. The binding of the two pools of purified anti-poly(L-alanine) Fab′ fragments to tetra-L-alanine was examined by equilibrium dialysis at 5, 22, and 37 °C by using the apparatus and method described by Colowick & Womack (1969). Tritiated tetra-L-alanine was used as the radioactive hapten, and the experiments were performed in PBS–EDTA. The data were subjected to Sips (Sips, 1948; Karush, 1956) and Scatchard (1949) analyses. The concentrations of Fab′ used in these experiments were 20–40 mg/mL in PBS–EDTA buffer, pH 7.6. Initial concentration of the labeled hapten was about 0.03 mg/mL.

Synthesis of Tetra-L-[^{3}H]alanine. Tritiated tetra-L-alanine, containing L-[3-^{3}H]alanine in the N-terminal residue, was synthesized by a modification of the procedure of Schechter & Berger (1966) (Geller, 1976). The product was stored in 70% ethanol and examined for purity by using paper chromatography.

^{13}C-Enriched Tetra-L-alanine Peptides. L-Alanine, enriched with 90% ^{13}C in the methyl carbon, was obtained from Merck Sharp & Dohme of Canada. Synthesis of the tetra-L-alanine

[1] Abbreviations used: C-1 peptide, 90% ^{13}C-enriched methyl group in the N-terminal alanine residue of tetra-L-alanine; C-2 peptide, 90% ^{13}C-enriched methyl group in the alanine residue adjacent to the N-terminal residue in tetra-L-alanine; C-3 peptide, 90% ^{13}C-enriched methyl group in the alanine residue adjacent to the C-terminal residue in the tetra-L-alanine; C-4 peptide, 90% ^{13}C-enriched methyl group in the C-terminal residue of tetra-L-alanine; EDTA, ethylenediaminetetraacetic acid.

peptides containing selectively enriched ^{13}C methyl carbons was carried out at Research Triangle Institute, Research Triangle Park, NC, courtesy of Dr. Monroe E. Wall and John A. Kepler. The procedure followed that described by Schechter & Berger (1966). Four tetrapeptides, each containing a single methyl group enriched to 90% ^{13}C, were synthesized. The tetrapeptide containing a ^{13}C-enriched methyl group in the N-terminal alanine residue was designated as C-1,[1] and peptides enriched in successive alanine residues were designated C-2, C-3, and C-4. The peptides were pure according to elemental analysis, optical rotations, paper chromatography, and electrophoretic analysis. The ^{13}C NMR spectra of the peptides were consistent with their labeling patterns.

^{13}C NMR Experiments. ^{13}C nuclear magnetic resonance experiments were performed on a JEOL PFT-100 spectrometer operating at 25.2 MHz which was interfaced with a Nicolet 1080 computer and a Bruker WH-360 spectrometer operating at 90.5 MHz which was interfaced with a Nicolet 1180 computer. All spectra were obtained by using Fourier transform techniques. Sample tubes were 10 mm in diameter (Wilmad) and contained vortex plugs. Spectra were broad band proton noise decoupled, and an internal deuterium field frequency lock was used by locking on 20% deuterium oxide that was added to the sample solutions (total volume 1.2 mL). Dioxane was used as an internal ^{13}C standard. Chemical shifts are reported with respect to tetramethylsilane with dioxane located 67.4 ppm downfield. Spectra obtained of protein samples that were recorded on the Bruker instrument employed a pulse width of 16 μs, which corresponded to a 60° flip angle, and quadrature detection for signal enhancement. A sweep width of 20000 Hz was typically used, and an acquisition of 0.41 s was employed. All spectra were subject to a convolution–deconvolution procedure (Campbell et al., 1973) to remove low-frequency base-line oscillations. An exponential time constant was also used in transforming the data which introduced a 15-Hz line broadening. NMR difference spectra were obtained by subtracting the transformed protein spectrum from the spectrum of the protein plus ^{13}C-enriched peptide. Typically, 8000–32000 scans were recorded for such spectra, depending on the concentration of peptide present. All samples were recorded at 5 °C under nonspinning conditions.

Results

Preparation of Antibodies. Poly(L-alanine) conjugates are weak immunogens, and trials of different immunization protocols indicated that the most effective procedure was to administer 0.5–1 mg of poly(L-Ala)–HSA in complete Freund's adjuvant initially and to boost with an equal amount of poly(L-Ala)–HSA or poly(L-Ala)–BSA intramuscularly in incomplete adjuvant. In our initial attempts to purify anti-poly(L-alanine) antibodies, we used immunoadsorbents containing poly(L-alanine) coupled to a protein that was not used for immunization; e.g., poly(L-Ala)–HSA was used for immunization, and poly(L-Ala)–BSA was coupled to Sepharose for use as an immunoadsorbent. The material eluted from these columns contained a considerable amount of antialbumin antibodies and nonimmunoglobulin contaminants. When poly(L-alanine) diaminopentane Sepharose was used as the immunoadsorbent matrix, the eluate provided over 95% pure, specific antibodies when eluted with tetra-L-alanine. The average yield of antibody was about 0.3 mg/mL of serum.

In our initial ^{13}C NMR experiments, in which concentrations of 20–30 mg/mL antibody were used, we noted that the tetra-L-alanine haptens were slowly hydrolyzed by small amounts of peptidase activity that contaminated both the specific IgG antibodies and the nonspecific sheep IgG. The peptidase activity remained associated with the antibodies through hapten elution, gel filtration, and ion-exchange chromatographic procedures. This activity could not be inhibited by a variety of protease and peptidase inhibitors or by heat inactivation, but it could be separated from the antibodies by gel filtration on Sephadex G-200 in the presence of 2 M thiocyanate or by preparation of $F(ab')_2$ fragments. The latter procedure was chosen since Fab' fragments were used for the NMR experiments. Two pools of antisera, 10–15 L each, were collected from different groups of sheep.

Table I: Temperature Dependence of the Average Association Constants, K_a, obtained by Equilibrium Dialysis and the Thermodynamic Parameters for the Binding of Tetra-L-[^{3}H]alanine by Sheep Anti-poly(L-alanine) Fab' Fragments

pool 1 Fab' fragments[a]			pool 2 Fab' fragments[b]		
T (°C)	K_a (M^{-1})	ΔG (kcal/mol)	T (°C)	K_a (M^{-1})	ΔG (kcal/mol)
5	1.10×10^4	−5.14	5	2.64×10^4	−5.65
22	6.07×10^3	−5.11	22	1.26×10^4	−5.52
37	3.83×10^3	−5.08	37	6.45×10^3	−5.40

[a] For pool 1, $a = 0.90 \pm 0.04$, where a is Sips heterogeneity coefficient, $\Delta H = -5.6 \pm 0.2$ kcal/mol, and $\Delta S = -1.8 \pm 0.2$ cal/mol^{-1} deg^{-1}. [b] For pool 2, $a = 0.80 \pm 0.04$, $\Delta H = -7.9 \pm 0.3$ kcal/mol, and $\Delta S = -8.2 \pm 0.8$ cal mol^{-1} deg^{-1}.

Affinity of Specific Fab' Fragments. The average association constants, K_a, for both pools of fragments are given in Table I. Data were obtained at 5, 22, and 37 °C by equilibrium dialysis with tetra-L-[^{3}H]alanine, and the Scatchard plots are shown in Figure 1A. The corresponding Sips plots of the binding data are shown in Figure 1B, and the Sips heterogeneity coefficient, a, for each pool is given in Table I. The thermodynamic data, ΔH, ΔS, and ΔF, for the tetrapeptide binding to the two Fab' pools are also given in Table I. Both pools of anti-poly(L-alanine) Fab' fragments were found to be extremely heterogeneous when analyzed by isoelectric focusing, and there was no indication of predominant clones of antibodies.

^{13}C-Enriched Tetra-L-alanine Peptides. Under proton decoupled conditions, a single resonance is observed for each peptide, as shown in Figure 2 for the C-2 peptide. The ^{13}C chemical shifts of the C-1, C-2, C-3, and C-4 ^{13}C-enriched tetra-L-alanine peptides are given in the legend of Figure 2. The chemical shift positions of the C-1 methyl resonance are 17.45 and 20.38 ppm for the protonated and unprotonated forms of the N-terminal amino group, respectively. The resonance positions of the C-4 methyl group are 18.15 and 17.00 ppm, respectively, for the ionized and protonated forms of the C-terminal carboxyl group. The pK_a values for the N-terminal amino group and C-terminal carboxyl groups at 5 °C are pH 8.7 and 3.8, respectively. These values were determined from changes in the NMR chemical shift positions of the C-1 and C-4 peptides as a function of pH. The chemical shifts of the C-2 and C-3 peptides are insensitive to changes in pH. (The complete pH dependence of the chemical shifts of these peptides will be reported elsewhere.)

Interaction of ^{13}C-Enriched Peptides with Fab' Fragments of Anti-poly(L-alanine) Antibodies. The proton-decoupled ^{13}C NMR spectrum of purified anti-poly(L-alanine) Fab' fragments shows the expected pattern of protein resonances. Aliphatic carbons of the proteins give rise to the resonance envelope between 0 and 60 ppm, and the aromatic and carboxyl carbons give rise to peaks located in the regions 120–130 and 160–170 ppm, respectively (Geller, 1976).

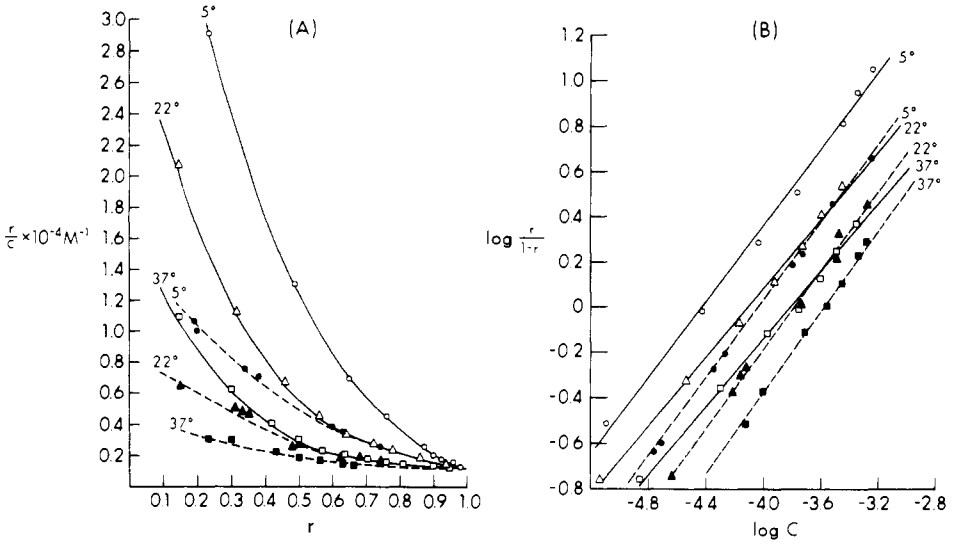

FIGURE 1: (A) Scatchard plots of the binding of tetra-L-[^{3}H]alanine to anti-poly(L-alanine) Fab′ fragments. In the plots, r is the ratio of moles of hapten bound per mole of Fab′ fragments and c is the concentration of free hapten. Binding data at 5, 22, and 37 °C for pool 1 fragments are given by ●, ▲, and ■, respectively. Binding data at 5, 22, and 37 °C for pool 2 fragments are given by ○, △, and □, respectively. (B) Sips plots of the binding of tetra-L-[^{3}H]alanine to anti-poly(L-alanine) Fab′ fragments. The ratio of moles of hapten bound per mole of Fab′ fragments is given by r, and c denotes the concentration of free hapten. Binding data at 5, 22, and 37 °C for pool 1 fragments are given by ●, ▲, and ■, respectively. Binding data at 5, 22, and 37 °C for pool 2 fragments are given by ○, △, and □, respectively. The solid lines through the points are theoretical curves obtained by a least-squares method by using linear regression analysis. The slopes of the plots give the Sips heterogeneity coefficient, a, and the intercepts give the average association constants, K_a.

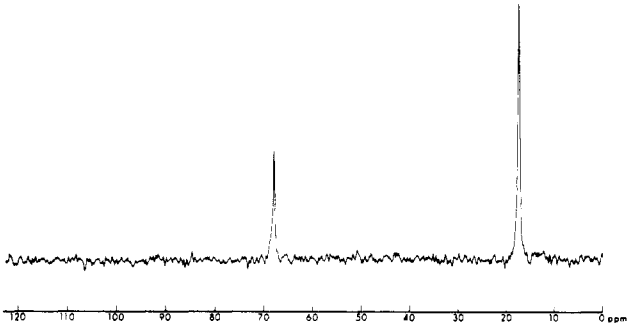

FIGURE 2: Proton-decoupled ^{13}C NMR spectrum at 90.5 MHz of 0.44 mM C-2 peptide (tetra-L-alanine enriched with 90% ^{13}C in the methyl group of the second residue) in pH 7.2 PBS buffer containing 20% D_2O at 5 °C. The upfield resonance at 17.34 ppm is due to the peptide; the downfield resonance at 67.4 ppm is due to internal dioxane. The chemical shift positions of the C-3 and C-4 peptides at pH 7.2 in 0.02 M phosphate and 0.15 M NaCl at 5 °C are 17.23 and 18.15 ppm, respectively. The chemical shift position of the C-1 at pH 6.8 in the same buffer at 5 °C is 17.45 ppm.

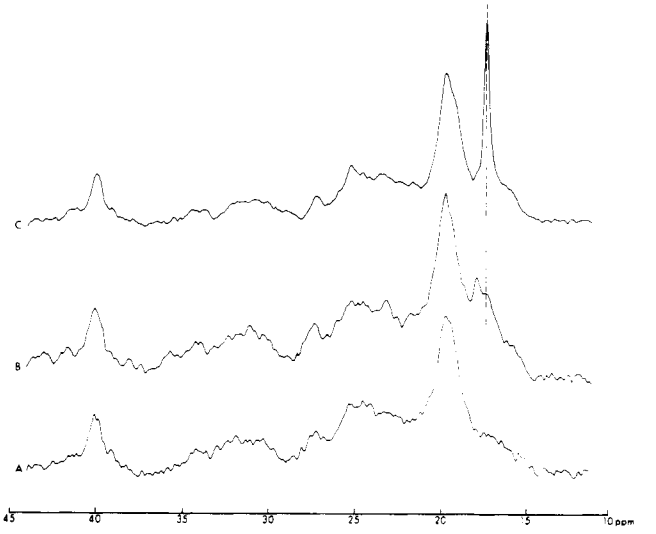

FIGURE 3: Proton-decoupled ^{13}C NMR spectra at 90.5 MHz of (A) the aliphatic region of 0.74 mM Fab′ fragments from pool 1, (B) the specific fragments in the presence of 0.65 mM C-2 peptide, and (C) nonspecific sheep Fab′ fragments in the presence of 0.65 mM C-2 peptide. The pH of the solutions was 7.2, and each contained 20% D_2O. The temperature was 5 °C. The dashed line is the chemical shift position of the free C-2 peptide.

Preliminary binding studies of the ^{13}C-enriched peptides with the specific Fab′ fragments in which the molar ratio of peptide to protein was varied from 4:1 to 12:1 indicated the peptide to be in the NMR "slow-exchange" time scale (Geller, 1976). In order to obtain spectra of the peptide haptens directly bound to the Fab′ fragments, a series of experiments were carried out in which the molar ratio of peptide to Fab′ fragments was varied from antibody excess to hapten excess.

The aliphatic region of the Fab′ fragments (0.74 mM, pool 1) from 10 to 45 ppm is shown in Figure 3A. A main aliphatic resonance band is observed at approximately 20 ppm. When 0.65 mM of the C-2 peptide is added to the protein solution at 5 °C, resulting in essentially all of the peptide bound, a new shoulder is seen on the upfield side of the main aliphatic protein band (Figure 3B). Under the same conditions but in the presence of nonspecific Fab′ fragments, the single resonance of the free C-2 peptide is observed at 17.34 ppm (Figure 3C) without detectable broadening (other than that induced by instrumental filtering). These results indicate that the changes observed in the spectrum of the C-2 peptide in Figure 3B are due to specific binding interactions between the peptide hapten and the anti-poly(L-alanine) Fab′ fragments.

In order to observe the resonance of the directly bound hapten more clearly, we obtained ^{13}C NMR difference spectra of each ^{13}C-enriched peptide bound to the antibody fragments. This procedure eliminates interfering resonances from the protein because of the high ^{13}C enrichment of the labeled peptides. The molar ratios of peptide to antibody fragments used in experiments were 0.52, 0.79, and 1.58. Since the antibody Fab′ fragments are heterogeneous with respect to their binding constants to tetra-L-alanine, ranging between $\sim 5 \times 10^3$ and 1×10^5 M^{-1}, the lowest ratio, 0.52, results in binding

Table II: Relative Amounts of "Shifted" and "Unshifted" ^{13}C Resonance Density of the ^{13}C-Enriched Tetra-L-alanine Peptides in the Presence of Anti-poly(L-alanine) Fab' Fragments

	C-1		C-2		C-3		C-4	
hapten–Fab' fragment ratio	% shifted resonance (20.0 ppm)	% unshifted[d] resonance (17.45 ppm)	% shifted resonance (20.2 ppm)	% unshifted resonance (17.34 ppm)	% shifted resonance (20.2 ppm)	% unshifted resonance (17.23 ppm)	% shifted resonance (19.8 ppm)	% unshifted resonance (18.15 ppm)
0.52[a]	100		100		100		100	
0.79[b]	50 ± 8	50 ± 8	60 ± 8	40 ± 6	60 ± 8	40 ± 6	50 ± 7	50 ± 7
1.58[c]	30 ± 6	70 ± 8	25 ± 5	75 ± 8	25 ± 5	75 ± 8		

[a] 0.44 mM peptide, 0.85 mM protein. [b] 0.66 mM peptide, 0.84 mM protein. [c] 1.30 mM peptide, 0.82 mM protein. [d] Unshifted resonance positions were obtained from solutions of the free peptides; the pH for the C-2, C-3, and C-4 peptide solutions was 7.2 while the pH of the C-1 peptide solution was 6.8.

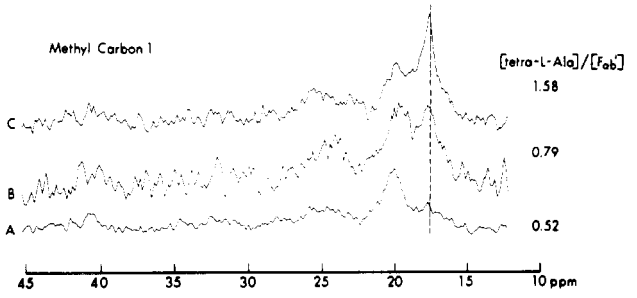

FIGURE 4: Proton-decoupled ^{13}C NMR difference spectra at 90.5 MHz of (A) 0.44 mM C-1 peptide in the presence of 0.85 mM Fab' fragments from pool 2, (B) 0.66 mM C-1 peptide in the presence of 0.84 mM Fab' fragments from pool 2, and (C) 1.30 mM C-1 peptide in the presence of 0.82 mM Fab' fragments from pool 2. The pH of the solutions was 6.8, and each contained 20% D_2O. The temperature was 5 °C. The dashed line represents the chemical shift position of the free C-1 peptide. Quadrature detection was used to enhance the signal-to-noise ratio in spectrum A.

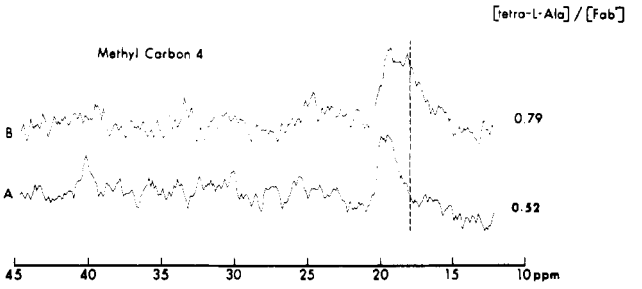

FIGURE 5: Proton-decoupled ^{13}C NMR difference spectra at 90.5 MHz of (A) 0.44 mM C-4 peptide in the presence of 0.85 mM Fab' fragments from pool 2 and (B) 0.66 mM C-4 peptide in the presence of 0.84 mM Fab' fragments from pool 2. The pH of the solutions was 7.2, and each contained 20% D_2O. The temperature was 5 °C. The dashed line is the chemical shift position of the free peptide. Quadrature detection was used to enhance the signal-to-noise ratio in spectrum A.

of the peptide to the highest affinity fragments. As the concentration of peptide is increased, interactions also take place with lower affinity populations. Difference spectra for the C-1 and C-4 peptides are shown in Figures 4 and 5; the results for the C-3 and C-4 peptides were similar to that of C-1. Only the pool 2 antibody fragments are shown since they are representative of the spectra obtained for both pools. All spectra were recorded at 5 °C to optimize the binding affinity of the protein.

At a ratio of 0.52 hapten to Fab' fragments (0.44 mM peptide, 0.85 mM protein), the C-1 peptide shows a broad resonance located at approximately 20 ppm (Figure 4A) that is approximately 90 Hz in width (half-peak height, corrected for instrumental broadening). This resonance is nearly 2.6 ppm downfield of the chemical shift position of the free C-1 peptide in solution at pH 6.8 (indicated by the dashed line). Further addition of the C-1 hapten to give a molar ratio of 0.79 (0.66 mM peptide, 0.84 mM protein) in which greater than 90% of the peptide is bound results in a second peak appearing, which is centered at the chemical shift position of the unshifted peptide (Figure 4B). The line width of the unshifted resonance is approximately the same as that of the downfield shifted resonance, and since both resonances are observable, the peptide is clearly in the NMR "slow-exchange" time scale while exchanging between these two types of protein environments. An estimate of the amount of shifted vs. unshifted resonance density was obtained by cutting out the respective peaks (some extrapolation was required since they are not clearly separated) and weighing them. The results (Table II) indicate that ~50% of the total resonance density is associated with the shifted resonance. When excess C-1 peptide is added to give a molar ratio of 1.58 (1.3 mM peptide, 0.82 mM protein) (Figure 4C), the resonance intensity of the unshifted peak is observed to increase, as expected from the contribution of free, unbound peptide, while considerable resonance density remains at the shifted position. Under these conditions, approximately 30% of the total resonance density is associated with the shifted band (Table II).

The results for the C-2 and C-3 peptides were similar to that for C-1. At the lowest ratio of hapten–Fab' fragments, 0.52, single downfield shifted resonances are observed for both peptides, and in each case the line width is approximately 90 Hz. These resonances represent 2.9 and 2.8 ppm downfield shifts from the C-2 and C-3 peptide's free chemical shift positions, respectively. Upon further addition of the haptens to a ratio of 0.79, a second peak appears in each spectrum at the unshifted resonance positions which appears as broad as the shifted resonances. The amount of shifted resonance density in these spectra is approximately 60% (Table II). Addition of excess hapten in both cases shows substantial increases in the intensity of the unshifted resonances, reflecting bound and free peptide at this position.

The results for the C-4 peptide are shown in Figure 5. At the lowest ratio of 0.52, the C-4 peptide in the presence of specific Fab' fragments gives rise to a broad resonance centered at approximately 19.8 ppm, with a 90-Hz line width (Figure 5A). In contrast to the results obtained with the other ^{13}C peptides, this resonance is located 1.6 ppm downfield from the free peptides' chemical shift. It is interesting to note, however, that the resonance is located 2.8 ppm downfield from the chemical shift position of the free C-4 peptide in the protonated carboxyl state. This observation will be discussed later. Further addition of the peptide to a ratio of 0.79 results in a considerable spread in the resonance of the C-4 peptide (Figure 5B). It is difficult to estimate the amount of shifted vs. unshifted resonance density present in the spectrum, but a reasonable estimate is roughly 50% for each. At a hapten/Fab' fragment ratio of 1.58, there is increased resonance intensity

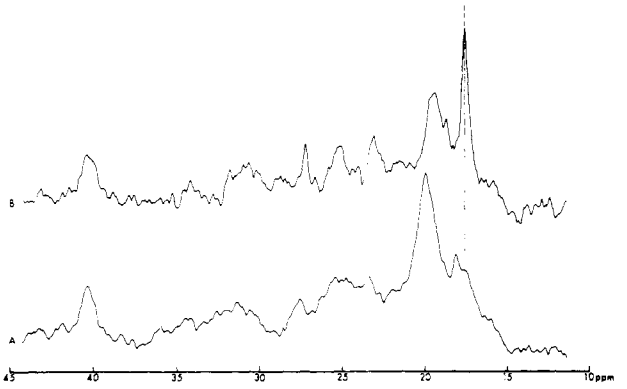

FIGURE 6: Proton-decoupled ^{13}C NMR spectra at 90.5 MHz of (A) 0.65 mM C-2 peptide in the presence of 0.74 mM Fab′ fragments from pool 1 and (B) the same solution containing 3.0 M potassium thiocyanate. The pH of the solutions was 7.2, and each contained 20% D_2O. The temperature was 5 °C. The dashed line is the chemical shift position of the free C-2 peptide.

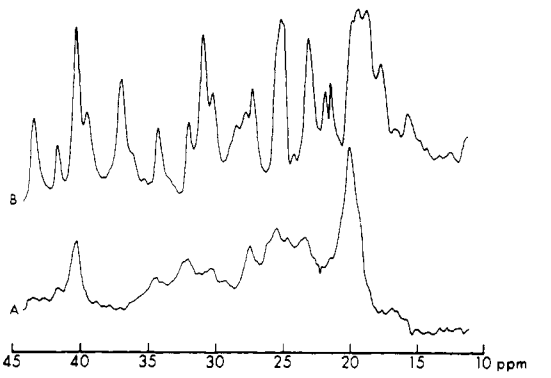

FIGURE 7: Proton-decoupled ^{13}C NMR spectra at 90.5 MHz of (A) 0.74 mM nonspecific sheep Fab′ fragments and (B) the same solution containing 8 M guanidine hydrochloride. The pH of the solutions was 7.2, and each contained 20% D_2O. The temperature was 5 °C.

at the unshifted position (not shown), as observed for the other three peptide derivatives.

Protein Denaturation Experiments. These experiments were performed to provide insight into the origin of the downfield shift of the methyl resonances of the peptide when bound to the higher affinity Fab′ fragments. Figure 6A shows the ^{13}C spectrum of the aliphatic region of the specific Fab′ fragments in the presence of bound C-2 peptide (the same as Figure 3B). When 3 M potassim thiocyanate is added to the solution, the protein is partially denatured (Figure 6B), and the peptide is no longer bound. Of particular interest is that the spectrum of the partially denatured protein appears to be an averaged one; that is, the denatured fraction of protein is in "fast exchange" with intact structures. Furthermore, the main aliphatic peak of the fragments in Figure 6A is observed to be shifted upfield by approximately 0.8 ppm. In addition, a single resonance is shifted far enough upfield to be observed individually at 18 ppm on the high-field shoulder of the main aliphatic band of the protein.

In the second experiment, the Fab′ fragments were totally denaturated in the presence of 8 M guanidine hydrochloride. The ^{13}C spectrum of the protein under these conditions is shown in Figure 7B. Figure 7A shows the spectrum of the intact protein for comparison. The aliphatic region of the denatured proteins consists of many relatively sharp resonances, essentially all of which can be assigned to specific aliphatic amino acid side chains (Horsley et al., 1970). Of particular interest is the resonance located at 17.4 ppm which represents the methyl resonances of alanine residues. The chemical shift position is characteristic of alanine methyl groups in free peptides (Keim et al., 1973). This resonance appears to be the small single resonance observed in Figure 6B on the high-field shoulder of the main aliphatic band of the partially denatured protein, judging from its intensity. Therefore, most of the alanine methyl resonances of the intact protein seem to be located in the main aliphatic resonance band centered at ~20 ppm, and upon complete denaturation in 8 M guanidine hydrochloride, these resonances shift upfield to the chemical shift position characteristic of free alanine residues in aqueous solution.

Discussion

Properties of Specific Fab′ Fragments. The average association constants for pool 1 and pool 2 sheep anti-poly(L-alanine) Fab′ fragments at 5 °C were 1.1×10^4 M^{-1} and 2.6×10^4 M^{-1}, respectively. The K_a for tri-L-alanine is about an order of magnitude less than that of the tetrapeptide, and di-L-alanine has a K_a more than two orders of magnitude smaller than the tetrapeptide (Geller, 1976). These results are in agreement with those reported by Schechter (1971) using precipitin–inhibition techniques for the relative affinities of rabbit anti-poly(L-alanine) antibodies eluted from an immunoadsorbent column with tetra-L-alanine. The relative binding affinities of the alanine oligopeptides for sheep anti-poly(L-alanine) Fab′ fragments are consistent with the view that the binding sites of a large proportion of the proteins are sufficiently large to bind all four residues of the tetrapeptide. However, Schechter (1970) has shown that although tetra-L-alanine binds to rabbit antibodies better than tri-L-alanine, the tri-L-alanine amide binds nearly as well as the tetrapeptide. Since there was insufficient evidence to assume a fourth subsite in the binding sites of the antibodies, Schechter concluded that the rabbit antibodies could accommodate between 3 and 4 alanine residues.

The Sips constants of 0.90 and 0.80 obtained for the two antibody preparations are relatively high compared to those usually obtained by immunization of outbred animals with heterogeneous immunogens. These values can be compared with *a* values of 0.36–0.6 reported by Schechter (1971) for rabbit anti-poly(D-alanine) antibodies. Despite the relatively high Sips constants, the heterogeneity of the sheep antibodies was demonstrated by isoelectric focusing, which indicated no predominant clones present, and by the NMR results.[2]

A number of considerations led to use of the Fab′ fragments in the NMR experiments rather than intact IgG molecules or $F(ab')_2$ fragments. First, pepsin digestion used to prepare

[2] Several factors contribute to the heterogeneity of the antibodies. Principal among these were the nature of the immunogen used, the low immunogenicity of the poly-L-alanine determinant, and the requirement for large quantities of antibodies for the NMR experiments which necessitated immunization of several sheep over a relatively long period of time. The poly(L-Ala)–HSA conjugate used as the immunogen contained approximately 30 substituted chains per molecule, with an average of 6–7 alanine residues per chain. The heterogeneity of these chains with respect to the different amino acid sequences around them, their steric accessibility, and the differences in their chain lengths contribute to the heterogeneity of the antibodies. We were unable to synthesize conjugates containing more homogeneous carriers such as gramicidin because of difficulties related to the solubility of the reactants and products. Further, since we required about 200–300 mg of Fab′ fragments to perform the NMR experiments and the highest antibody titers obtained in the sheep were ~0.3 mg/mL, we had to pool sera collected from many sheep over a long period of time. The procedure introduced two factors that contributed to the heterogeneity of the specific antibodies: (1) variations in the immune response between outbred animals and (2) changes in the immune response of single animals over a period of time.

the Fab′ fragments eliminated the peptidase activity which hydrolyzed the ^{13}C-enriched peptide haptens during the NMR experiments. Second, the high concentration of protein required to completely bind the peptidases for the NMR experiments could only be achieved with the more soluble Fab′ fragments. Finally, analysis of the NMR line-width data of the bound ^{13}C-enriched tetrapeptide required an accurate determination of the rotational correlation time, τ_R, of the protein. Diamagnetic solvent proton relaxation dispersion studies performed by the method of Hallenga & Koenig (1976) indicated that intact IgG molecules have complex, multiple rotational correlation times which have their origin in the segmental motions of individual domains of the molecules (W. Ganz, S. Geller, C. F. Brewer, and R. D. Brown, III, unpublished results). Use of Fab′ fragments greatly simplified determination of τ_R.

Chemical Shift Effects upon Peptide Binding to the Fab′ Fragments. Upon binding to higher affinity Fab′ fragments of purified anti-poly(L-alanine) antibodies, the resonances of the methyl carbons of tetra-L-alanine are shifted downfield ~2.8 ppm (the apparent smaller shift of the C-4 peptide will be discussed later). An explanation for this observation is suggested by a comparison of the spectra of the native and denatured Fab′ fragments.

In the presence of 8 M guanidine hydrochloride (Figure 7B), a main aliphatic resonance band of the intact fragments centered at ~20 ppm (Figure 7A) is observed to break up and shift into several upfield resonances. The methyl groups of alanine residues are located at 17.4 ppm, and those of valine are located at 18.4 and 19.2 ppm. These upfield shifts appear to be due to exposure of these residues to aqueous solvent in the course of denaturation. The shifts that the alanine methyl groups exhibit in the native protein are similar in magnitude to the downfield shifts experienced by the methyl groups of tetra-L-alanine when bound to higher affinity specific Fab′ fragments. Since these shifts may provide a clue as to the nature of the combining sites of the specific antibodies, it is worth discussing their possible origins and the molecular environments that can give rise to such effects.

Chemical shifts induced by circulating ring-current effects in unsaturated molecules such as aromatic groups are well-known (Stothers, 1972). Because of the strict dependency of these shifts on orientation and distance, it is difficult to conceive of the rather uniform downfield shifts observed in this study as arising from aromatic side chains of the proteins. Electric field effects arising from charged groups in the proteins could also induce resonance shifts in surrounding nuclei, but in view of the essentially uniform downfield shifts observed, it is difficult to conceive of this as the responsible mechanism. The lack of hydrogen bonding potential of methyl groups removes this type of interaction from consideration. ^{13}C shifts are known to be sensitive to steric effects, but they usually result in upfield shifts (Grant & Cheney, 1967). The possibility also exists that the conformation of the peptide changes upon binding, which could affect the chemical shifts of the methyl resonances. The uniformity of the shifts of the hapten also weighs against this possibility.

Several studies have demonstrated that in the presence of van der Waals interactions, the ^{13}C resonances of methyl groups in small aliphatic molecules shift downfield from 2 to 4 ppm (Cans et al., 1976; Tiffon & Doucet, 1976; Jackowski & Raynes, 1977). The importance of van der Waals interactions in stabilizing molecular interactions and protein structure is well-known.[3] This mechanism of inducing shifts is consistent with the results of the denaturation experiments with the Fab′ fragments and the correlation between the shifts of the bound hapten and increased affinity of the proteins.

These results suggest that the downfield shifts observed in the peptide are due to van der Waals interactions between the methyl groups of the hapten and residues of the protein. This is the first apparent observation of van der Waals attractive interactions in a biological system by using ^{13}C NMR spectroscopy although van der Waals effects in proteins have been reported in several ^{19}F NMR studies (cf. Hull & Sykes, 1976), and Sternlicht & Wilson (1967) have observed small downfield shifts (0.25 ppm) for methyl proton resonances of aliphatic residues in lysozyme that appear to be induced by van der Waals interactions.

The similar magnitude of the downfield shifts observed for all four methyl groups of the peptide suggests that the magnitude of the interactions is essentially the same for all four side chains of the hapten (Tiffon & Doucet, 1976). The individual sites for methyl groups must be similar on different high-affinity antibody fragments, and all four "subsites" must be relatively homogeneous in their chemical character and most likely "aliphatic-like" in nature (Krigbaum & Kormoriya, 1979).

Charge State of the Bound Peptide Hapten. When the C-1 peptide is bound to the higher affinity Fab′ fragments (Figure 4A), a shifted resonance is observed at ~20 ppm. This is ~2.6 ppm downfield of the position for the protonated form of the C-1 amino group, which is present in solution under the experimental conditions, and ~0.4 ppm upfield from unprotonated C-1. Since the C-1, C-2, and C-3 haptens' methyl groups all experience downfield shifts of the same magnitude when bound to high-affinity fragments and since the C-2 and C-3 methyl groups are not affected by the charge state of the peptide, we believe that the N-terminal amino group of the hapten is protonated while bound to the higher affinity fragments and that the resonance observed in Figure 4A results from a downfield shift of this form of the bound peptide. The fact that a resonance is observed at 17.4 ppm when C-1 is bound to lower affinity fragments (Figure 4B) indicates that the protonated amino form of the hapten also binds to these antibodies.

When bound to higher affinity Fab′ fragments, the C-4 peptide's resonance exhibits an ~1.6-ppm downfield shift from the position of the ionized form of C-4 and an ~2.8-ppm downfield shift from the protonated form. The magnitude of the latter shift is similar to those observed for the other three methyl groups of the peptide, which suggests that the peptide's carboxyl group is protonated when bound to higher affinity fragments. At higher hapten–antibody ratios, the C-4 peptide binds to lower affinity antibody fragments, and a resonance appears close to the chemical shift position of the ionized carboxylate form of the hapten (Figure 5B). This suggets that the peptide is binding to the weaker binding fragments with the C-terminal group ionized.

Kinetics of Binding of the Peptide. The NMR results

[3] The importance of van der Waals interactions in proteins has been pointed out by Chothia (1975) who studied the X-ray crystallographic data of 15 proteins and determined that the packing density of atoms in a protein is essentially the same as found in crystals of amino acids. Krigbaum & Komoriya (1979) have concluded from an analysis of the structure of globular proteins that van der Waals interactions are a primary determinant in the folding of proteins, principally through the interactions of side chains of similar polarity. These observations suggest that the methyl groups of the tetrapeptide, when bound to the highest affinity fragments, are optimally positioned adjacent to atoms of the Fab′ binding sites for maximum packing density.

clearly show that at a hapten–Fab′ ratio of 0.79, where >90% of the hapten is bound, two resonances are clearly present in the spectra of the C-1, C-2, and C-3 peptides. This indicates that the exchange rate of the peptide between these two chemically shifted sites in the fragments is slow relative to the difference in frequency separating the two resonances. Since this frequency difference is ~2.8 ppm, or ~250 Hz, the off rate of the hapten must be less than $2\pi\Delta\nu$ or $<1600\ s^{-1}$. The C-4 peptide in Figure 5B shows a broad resonance that appears to be composed of a shifted resonance and unshifted resonance that overlap with each other. The spectra obtained for the C-4 peptide in marked hapten excess with pool 1 Fab′ fragments (not shown) (Geller, 1976) exhibit two separate resonances. Since the frequency difference for the shifted and unshifted peaks of C-4 is less than that for the other methyl groups of the bound peptide, this provides an upper limit for k_{-1}. Thus, $\Delta\nu$ is ~140 Hz for C-4, and $k_{-1} < 900\ s^{-1}$ for the peptide binding to pool 1 fragments.

^{13}C NMR experiments were also performed at 24 kG with an excess of hapten relative to pool 1 antibody fragments (Geller, 1976). Line broadening of ~4 Hz of the C-1, C-2, C-3, and C-4 resonances was observed at a 5:1 ratio of hapten to Fab′ fragments. On the basis of temperature-dependent measurements, the broadening was attributed to slow exchange and partial averaging of free and bound resonances. This provides an upper limit for $k_{-1} < \pi$ (5 × 4 Hz), or $<63\ s^{-1}$.

An estimate of the forward rate constant, k_1, for tetra-L-alanine binding to the Fab′ fragments can also be made from the relationship $k_1 = K_a k_{-1}$. For pool 1 fragments, the average association constant is $1.0 \times 10^4\ M^{-1}$, and k_{-1} is $<63\ s^{-1}$; thus, $k_1 < 7 \times 10^5\ M^{-1}\ s^{-1}$. Pecht (1975) reports k_1 values in the range of 10^5–$10^6\ M^{-1}\ s^{-1}$ for binding D-alanine haptens containing a dansylated alkyl side chain to rabbit anti-poly(D-alanine) antibodies as determined by fluorescence relaxation measurements. These forward rate constants are much less than diffusion-controlled values of 10^9–$10^{10}\ M^{-1}\ s^{-1}$ and also slower than typical k_1 values of 10^7–$10^8\ M^{-1}\ s^{-1}$ reported for aromatic haptens binding to antibodies (cf. Pecht, 1975). The slower association rates of tetra-L-alanine binding to the Fab′ fragments may reflect greater conformational and orientational requirements for binding of oligomeric ligands. This conclusion is supported by the kinetic studies of Pecht (1975) on binding of oligomeric haptens to antibodies and myeloma proteins. Relatively slow forward rate constants may also reflect a two-step encounter mechanism (Haselkorn et al., 1974).

Mobility of Bound Haptens. The effective rotational correlation time, τ_g, experienced by a freely rotating methyl group of an alanine residue is one-third that of an immobilized methyl group (Doddrell et al., 1972). When the assumption is made that the carbon backbone of the bound peptide is immobilized and tumbles with the overall rotational correlation time, τ_R, of the protein, then the resonance line width of the rotating methyl groups would be ~90 Hz (ignoring small corrections for anisotropy effects), while that for a nonrotating methyl group would be ~270 Hz (see Appendix). Thus, it should be possible to distinguish between these two cases. (If the carbon backbone of the bound peptide is not immobilized but segmentally mobile, the observed line widths will be correspondingly narrower.)

The results show that at the lowest concentration of hapten bound to the specific Fab′ fragments, the shifted resonances for all four methyl groups have line widths of ~90 Hz (corrected for instrumental broadening). It appears that the methyl groups of the peptide are free to rotate when bound in relatively hydrophobic regions of higher affinity fragments. This is not surprising since the methyl groups of alanine residues in ribonuclease, where the packing density of atoms is presumed to be similar, for example, appear to be free to rotate (Glushko et al., 1972). These results also suggest that the backbone of the peptide is firmly bound to the fragments at all four residues.

Further addition of hapten, which results in binding to lower affinity fragments, gives rise to a resonance centered at the unshifted position for each methyl resonance, and this new resonance appears to possess about the same line width as the corresponding shifted resonance. These results suggest that the difference in binding constants between higher and lower affinity fragments in the heterogeneous pool does not result from a difference in the "size" of their combining sites because all of the Fab′ fragments appear to equally immobilize the entire backbone of the bound peptide.[4] Smaller binding sites would not be expected to bind all of the residues of the hapten. This would result in increased segmental mobility and narrower line widths for those unbound residues.

Forces Involved in Binding the Peptide to the Antibody Fragments. The association constants for pool 2 specific Fab′ fragments vary over an order of magnitude, roughly between 5×10^3 and $1 \times 10^5\ M^{-1}$, with the average value calculated to be $2.6 \times 10^4\ M^{-1}$. The downfield shifts observed when the hapten binds to the higher affinity fragments appear to correlate with an increase in binding energy of approximately an order of magnitude for the peptide, which is ~1.3 kcal/mol of binding energy. Tanford (1962) has measured the free energy of transfer of the side chains of amino acids from water to ethanol and determined a value of −0.7 kcal/mol for the methyl group of alanine. This appears to be a reasonable estimate of the favorable free energy available for the methyl group to bind to a protein. The four methyl groups of tetra-L-alanine could thus contribute ~2.8 kcal/mol of binding energy. This represents nearly a tenfold greater increase in binding than found from the NMR results, which suggests that additional unfavorable binding interactions must also occur. We suggest that part of this is due to protonation of the carboxyl group of the peptide upon binding to the higher affinity fragments (cf. Tanford, 1962).

The NMR data indicate that the N-terminal amino group of the peptide is protonated when bound to both higher and lower affinity fragments. This suggests the possibility of an electrostatic interaction between the amino group of the hapten and a counterion of the antibodies, which could provide up to 5 kcal/mol of binding energy. Other possible interactions that could occur between the peptide and the Fab′ fragments are hydrogen bonds involving amide groups of the hapten and van der Waals interactions between the protein and the protons of the peptide.

The thermodynamic data in Table I indicate that the free energy of binding of the hapten is due to the enthalpy contribution. It appears that most of the binding energy is associated with probable electrostatic interactions of the protonated amino group of the hapten with the antibodies while differences in binding energy between higher and lower affinity fragments appear to be due to the ability of the former to bind all four methyl groups of the peptide via van der Waals interactions. Results for pool 1 suggest that there are intermediate affinity populations that bind to the C-1 and C-2

[4] The results of the present study suggest that the relative affinity of antibodies for an oligomeric hapten such as tetra-L-alanine may not depend as much on the "size" of the combining sites but rather on maximizing the number of complementary interactions between residues of the hapten and the binding sites.

methyl groups of the peptide but not to the C-3 and C-4 methyl groups (Geller, 1976).

Previous Studies. Schechter (1970, 1971) has previously investigated the interaction of L-alanine peptides and their derivatives with anti-poly(L-alanine) antibodies from rabbits by precipitin–inhibition measurements. The principal conclusions of this study were that the antibody combining sites were made up of subsites, that the methyl group of the N-terminal residue fits into a deep subsite, and that the succeeding subsites become shallower and allow less contact with other methyl residues of the peptide.

In comparing Schechter's results and conclusions with ours, it should be noted that antibodies from different species, rabbits and sheep, were used, but despite this difference, we feel that certain comparisons can be made. The conclusions that the combining sites of higher and lower affinity antibodies differ in size and that the binding sites consist of subsites in which the first subsite binds the N-terminal alanine methyl group more tightly than the second subsite binds the methyl group of the second residue are not consistent with our data. First, the equivalent line widths of all four methyl groups do not support the idea that the C-1 methyl group is bound more tightly than other methyl groups. Since the entire backbone of the peptide appears to be immobilized when bound to either high- or low-affinity antibodies, the binding sites of lower and higher affinity molecules may be of comparable sizes. Second, the near-equivalency of the downfield chemical shifts of all four methyl resonances of the ^{13}C peptides when bound to higher affinity fragments (assuming protonation of C-4) is also not consistent with differences in the strength of interaction of each methyl group with the antibodies. The data are more consistent with similar interactions taking place at each residue.

Our results are consistent with Schechter's findings on the importance of the charged amino group of the peptide in binding and with his observation that a charged carboxylate anion destabilizes binding. As noted above, the ^{13}C NMR chemical shift data for the C-4 peptide binding to higher affinity Fab′ fragments are consistent with the carboxylate group's undergoing protonation to the free acid form. This increase in the apparent pK_a of the carboxyl group suggests that this end of the peptide is binding in a relatively nonpolar region and that the overall process is energetically unfavorable for complex formation. Interestingly, the carboxylate group appears to remain ionized when the peptide is bound to lower affinity fragments, which suggests that this region of the binding site is more polar than the corresponding binding region in higher affinity antibodies.

Schechter's observation that modification of the N-terminal alanine residue of the hapten lowered the association constant of the hapten–antibody interaction to a much greater extent than modification of adjacent alanine residues, and many similar observations made with carbohydrate determinants have been interpreted as indications that the terminal "immunodominant" residue contributes more binding energy than succeeding residues (cf. Kabat, 1976). This may be true in some instances, but at least two other factors deserve consideration. We observed that a larger proportion of the anti-poly(L-alanine) antibodies interacted with the C-1 and C-2 methyl groups than with the C-3 and C-4 methyl groups in pool 1 (Geller, 1976). Replacement of the C-1 methyl groups by a proton would, therefore, affect the binding of hapten to more antibodies than replacement of the C-4 methyl group, although the binding energy contributed by the interaction of the C-1 and C-4 methyl groups with the antibody might be equal. Another consideration is that modification of the sterochemistry, polarity, or solvation of the terminal residue might destabilize the interaction of a considerable portion of the hapten with the antibody by dislocating the hapten from its position of closest congruency with the antibody sites or by altering the conformation of the hapten (Kabat & Wu, 1973).

The results of the present study show that ^{13}C NMR techniques can provide valuable insights into the nature of the molecular interactions that occur between a hapten and specific heterogeneous antibodies. We are currently expanding these studies with the use of other selectively labeled alanine peptide haptens as well as the production of monoclonal anti-poly(L-alanine) antibodies by cell fusion techniques.

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Appendix

Mobility of Bound Hapten. Information on the mobility of the methyl groups of the bound hapten can be obtained from measurements of the ^{13}C transverse relaxation time (T_{2M}) of the bound peptide. The line width of a bound resonance is related to T_{2M}, in the absence of chemical shift effects due to exchange averaging and broadening of the peak by exchange kinetics (i.e., $T_{2M} < \tau_m$, as is the present case), by the relationship $\pi\nu_{1/2} = T_{2M}^{-1}$, where $\nu_{1/2}$ is the resonance line width at half-peak height. For a 90-Hz resonance line width, T_{2M} = 3.5 ms. The relationship between T_{2M} and the rotational correlation time of the ^{13}C nuclei containing directly bound protons is given by the equation $T_{2M}^{-1} = (n/5)\hbar^2\gamma_C^2\gamma_H^2 r^{-6}\tau_R$ (Doddrell et al., 1972), where n is the number of directly bound protons, $\hbar$ is Planck's constant divided by 2π, γ_C and γ_H are the carbon and proton gyromagnetic constants, respectively, r is the distance separating the directly bound proton(s) from the carbon atom, and τ_R is the rotational correlation time of the carbon atom. An experimental value of τ_R of 66 ns for the Fab′ fragments at 5 °C was obtained from diamagnetic proton solvent magnetic relaxation dispersion measurements performed by Dr. R. D. Brown, III, Dr. S. H. Koenig, and Mr. W. Ganz (cf. Hallenga & Koenig, 1976). This value is in good agreement with values of 33 ns obtained for the Fab′ fragments at 25 °C (Yguerabide et al., 1970) and 38 ns obtained by electron spin resonance techniques (Stryer & Griffith, 1965) when viscosity changes in the solution are taken into account at the two temperatures.

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L-*myo*-Inositol-1-phosphate Synthase from Bovine Testis: Purification to Homogeneity and Partial Characterization†

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ABSTRACT: L-*myo*-Inositol-1-phosphate synthase has been purified to homogeneity from bovine testis by $(NH_4)_2SO_4$ precipitation on Celite followed by reverse $(NH_4)_2SO_4$ gradient elution, DEAE chromatography, gel filtration, and hydroxylapatite chromatography. The enzyme is then pure by the criteria of elution profile from the hydroxylapatite, electrophoresis, and sedimentation properties. We find no overall (gluconeogenic) reversibility of the enzyme using 6 mM DL-*myo*-inositol-1-P. The first three steps of the reaction are reversible as determined by uptake of isotope from a D_2O incubation medium into the 6 position of D-glucose-6-P. Thus, substrate binding, dehydrogenation, and proton removal prior to the aldol cyclization are all reversible steps. The enzyme is <5% NAD^+ independent and is not inhibited by substrate or product (5 mM D-glucose-6-P or 0.8 mM DL-*myo*-inositol-1-P). The enzyme is twofold stimulated by either 50 mM NH_4^+ or 50 mM K^+; the activation by these ions is not additive. Sodium ions inhibit the enzyme by 78% at 153 mM. The effect of sodium and potassium is not on the K_m of D-glucose-6-P but on V_{max}. We propose that K^+ activates the enzyme by stabilizing a carbanion intermediate. Ethanol stimulates the enzyme 2-fold and 2.5-fold with added K^+. The effect of ethanol appears to be via lowering of the D-glucose-6-P K_m. In the presence of ethanol the effect of salt on V_{max} disappears.

L-*myo*-Inositol-1-phosphate synthase (M1P[1] synthase) (EC 5.5.1.4) and L-*myo*-inositol-1-phosphatase (EC 3.1.3.25), provide the only known biological pathway for the de novo formation of *myo*-inositol. The substrate for the synthase is

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[1] Abbreviations used: DTT, dithiothreitol; GC, gas chromatography; GC–MS, gas chromatography–mass spectrometry; 5-ketoglucose-6-P, D-*xylo*-hexos-5-ulose 6-phosphate; M1P, L-*myo*-inositol 1-phosphate; synthase, L-*myo*-inositol-1-phosphate synthase; Me_3Si, trimethylsilyl.

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