Nuclear Magnetic Resonance Studies of Antibody–Hapten Interactions Using a Chloride Ion Probe*

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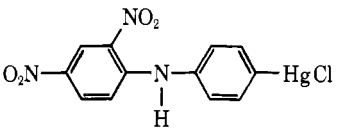
ABSTRACT: The interaction of a mercury-labeled hapten with antidinitrophenyl antibody has been investigated by ^{35}Cl nuclear magnetic resonance (nmr) spectroscopy. The hapten, 2,4-dinitro-4′-(chloromercuri)diphenylamine, binds strongly to antidinitrophenyl antibody in 1 M NaCl. The binding of chloride ions by the mercury atom of the hapten–antibody complex and the rapid exchange of the bound chlorine with chloride ions in the solvent produce a broadening of the ^{35}Cl nmr resonance at protein concentrations as low as 10^{-6} M. The nmr spectra provide information concerning three facets of the hapten–antibody interaction: (a) accessibility of the mercury atom in the hapten–antibody complex to chloride ion in the solvent; (b) binding affinity and stoichiometry of the hapten–antibody complex; and (c) rotational mobility of the bound hapten. It seems likely that the active sites of a variety of proteins can be similarly studied by ^{35}Cl nmr spectroscopy using substrates which contain a covalently bonded mercury atom.

The use of halide ions as chemical probes for nuclear magnetic resonance (nmr) studies of biological macromolecules in solution has been recently demonstrated by Stengle and Baldeschwieler (1966). A metal atom such as mercury labels specific sites in a macromolecule; the binding of chloride ions, for example, by a mercury–protein complex, and the rapid exchange of the bound chlorine with chloride ions in the solvent produce observable changes in the ^{35}Cl nmr line width at protein concentrations as low as 10^{-6} M. Information concerning the structure of the labeled site can then be inferred from the ^{35}Cl nmr spectrum.

In the work of Stengle and Baldeschwieler (1966), mercury atoms were bound to proteins at sulfhydryl sites. The method is also applicable to proteins without sulfhydryl residues; for example, detailed information concerning protein active sites can be obtained by using *substrate molecules which contain a covalently bonded mercury atom.* A broadening of the ^{35}Cl nmr line width will be observed if the mercury atom in the protein–substrate complex is accessible to chloride ions in the solvent. The magnitude of the broadening depends on the rotational mobility of the bound chloride. Thus, the degree of flexibility of a substrate molecule bound at an active site and the extent of its exposure to solvent can be determined by ^{35}Cl nmr spectroscopy.

An nmr study of the interaction of a mercury-labeled hapten (I, DNP-mercurial)[1] with antidinitrophenyl antibody using a chloride ion probe is reported in this



I

paper. Antidinitrophenyl antibody was chosen because it has been characterized by a variety of methods (Velick *et al.*, 1960; Metzger *et al.*, 1963; Eisen, 1964). Its use in evaluating new spectroscopic methods was demonstrated by Stryer and Griffith (1965) in their electron spin resonance study of the mobility of the antibody combining site. In this nmr study, it is shown that the mercury atom of the bound hapten is exposed to the solvent. Titrations of the antibody with DNP-mercurial are readily obtained from the ^{35}Cl nmr line widths at antibody concentrations as low as 10^{-6} M. The displacement of the DNP-mercurial hapten by another hapten, ε-*N*-dinitrophenyl-L-lysine, is also readily followed from the ^{35}Cl nmr spectrum. The ^{35}Cl nmr line widths provide an additional estimate of the rigidity of the antibody combining site.

Experimental Section

^{35}Cl nmr spectra were obtained with a Varian Associ-

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[1] Abbreviations used: DNP-lysine, ε-*N*-2,4-dinitrophenyl-L-lysine; DNP-mercurial, 2,4-dinitro-4′-(chloromercuri)diphenylamine; ν, line width in cycles per second; τ, rotational relaxation time.

ates V-4300 nmr spectrometer operating with a 4.3 Mcycle/sec fixed-frequency radiofrequency unit. The spectrometer was equipped with a Varian Associates 12-in. electromagnet system and standard accessories. The spectrometer system also included a Princeton Applied Research JB-4 lock-in-amplifier, a Hewlett-Packard 200 audiooscillator, and a Dyna Co. audio-amplifier to provide various modulation and detection options. Line widths were measured as the full width at one-half peak height of absorption mode signals. All line-width values reported are the average, and the uncertainties are the standard deviation of at least ten traces of the ^{35}Cl nmr spectrum under each set of conditions. The spectra were calibrated using sidebands generated by magnetic field modulation. The modulation frequency was measured to ± 0.1 cycle/sec with a Hewlett-Packard Model 521-C counter. The liquid samples were contained in 13-mm o.d. glass tubing in a standard Varian 15-mm nmr probe insert. Spectra were obtained with the liquid samples at $27 \pm 1°$.

The nmr titration of the interaction of DNP-mercurial with antibody was carried out by adding 20-μl aliquots of 6.5×10^{-4} M hapten in acetonitrile to 4 ml of 5.8×10^{-6} M antibody in 1 M NaCl at 27°. It was necessary first to dissolve the DNP-mercurial in acetonitrile because of its low solubility in water. At the end point, the concentration of acetonitrile was 2% by volume. After the end point was reached, two 50-μl aliquots of 8.2×10^{-4} M ϵ-*N*-2,4-dinitrophenyl-L-lysine in H_2O were added to the preceding solution.

The fluorescence quenching method of Velick *et al.* (1960) was used to determine the stoichiometry of binding and to obtain an upper limit on the dissociation constant of the hapten–antibody interaction. Fluorescence titrations were carried out on a spectrofluorimeter previously described (Stryer, 1965). For the fluorescence quenching measurements, aliquots of 2,4-dinitro-4′-(chloromercuri)diphenylamine in acetonitrile or of ϵ-*N*-2,4-dinitrophenyl-L-lysine in H_2O were added to 3 ml of 9.7×10^{-8} M antidinitrophenyl antibody in 1 M NaCl at 20°. At the end of the titration with the dinitrophenyl mercurial, the concentration of acetonitrile was 5% by volume. The antibody fluorescence was excited at 280 mμ and the emission was observed at 340 mμ.

2,4-Dinitro-4′-(chloromercuri)diphenylamine (I, DNP-mercurial) 2,4-dinitrofluorobenzene (132 mg, 0.71 mmole) in 2 ml of acetone was added to a refluxing solution of *p*-chloromercurianiline (Dimroth, 1906), (218 mg, 0.67 mmole) in 110 ml of absolute ethanol which contained 1 ml of triethylamine. The yellow solution was concentrated to 70 ml after it was refluxed for 30 min. A red-orange crystalline powder (163 mg) was obtained on cooling. A second crop of 30 mg was obtained by concentrating the filtrate to 45 ml. The product decomposed above 250°. The yield was 71%.

Anal. Calcd for $C_{12}H_8ClHgN_3O_4$: C, 29.16; H, 1.63; Cl, 7.17; N, 8.50. Found: C, 29.41; H, 1.77; Cl, 6.92; N, 8.42.

ϵ-N-2,4-Dinitrophenyl-L-lysine (DNP-lysine) was purchased from Mann.

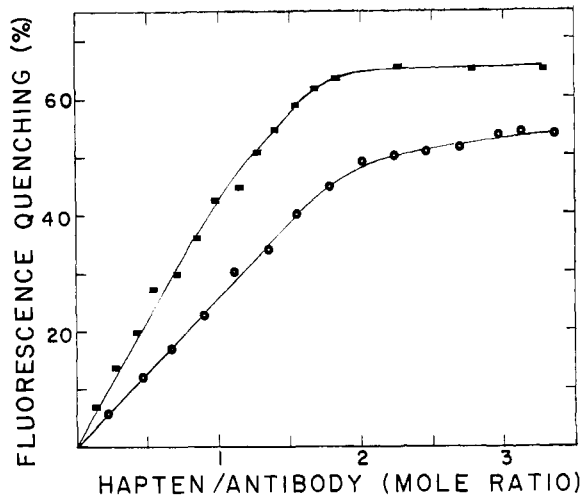

FIGURE 1: Fluorescence quenching titration of anti-dinitrophenyl antibody in 1 M NaCl by DNP-lysine (squares) and DNP-mercurial (circles).

Antidinitrophenyl antibody was the gift of Dr. Herman Eisen and Mr. Ernest Simms of the Washington University School of Medicine. The antibody was purified from a rabbit serum pool obtained 14 months after initial immunization with dinitrophenylated hemocyanin. A booster dose of this antigen was given 2 weeks prior to bleeding. Antibody concentrations were based on a specific absorption coefficient of 1.46 cm^2/mg and a molecular weight of 160,000.

Results

Fluorescence quenching titrations of DNP-lysine and DNP-mercurial with antidinitrophenyl antibody in 1 M NaCl are shown in Figure 1. The mercurianiline moiety evidently does not prevent binding of the mercury-labeled hapten with the antibody. From these fluorescence quenching titrations, it is clear that the DNP-mercurial binds to the antibody with an association constant greater than 10^7, and exhibits the expected stoichiometry of two haptens per antibody. The fluorescence of nonspecific γ-globulin is not quenched by 5×10^{-6} M DNP-mercurial. The DNP-mercurial does not appear to bind in a nonspecific manner at the concentrations used in this study. The fluorescence quenching titration (Figure 1) of a standard hapten (Velick *et al.*, 1960), DNP-lysine, indicates that the high ionic strength of the 1 M NaCl solutions used in this nmr study does not interfere with binding.

The ^{35}Cl nmr spectrum of a 1 M aqueous NaCl solution is shown in Figure 2A. The ^{35}Cl line width under these conditions is 16.0 ± 0.7 cycles/sec. The ^{35}Cl line width obtained for a 5.8×10^{-6} M solution of DNP-antibody in 1 M NaCl is 18.0 ± 1.0 cycles/sec. The addition of this concentration of antibody has only a slight broadening effect on the ^{35}Cl nmr line. This broadening effect is just outside the uncer-

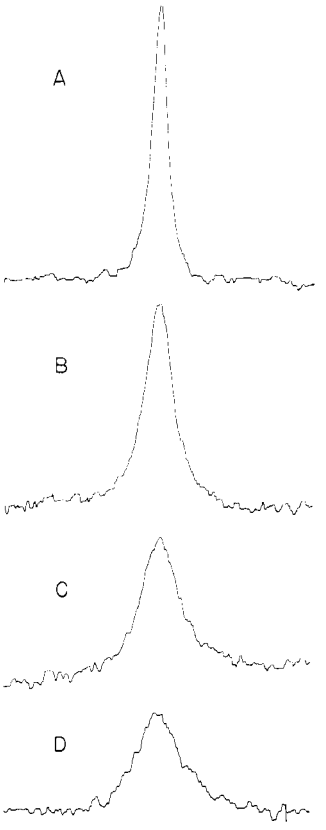

FIGURE 2: ^{35}Cl nmr spectrum of (A) 1 M NaCl and of 1 M NaCl containing 5.8×10^{-6} M antidinitrophenyl antibody and DMP-mercurial at DNP-mercurial: antibody mole ratios of (B) 0.56, (C) 1.12, and (D) 1.68.

tainty of the line-width measurements. Small effects of this sort presumably can arise from the weak binding and exchange of Cl^- with a variety of side chains of the antibody molecule.

The Addition of DNP-mercurial to the Antibody in 1 M NaCl Leads to a Broadening of the Line (Figure 2B–D). A line width of 61.6 ± 5.5 cycles/sec is observed at a hapten:antibody mole ratio of 1.68 (Figure 2D). The antibody concentration is 5.8×10^{-6} M. A titration curve showing the observed ^{35}Cl nmr line width as a function of DNP-mercurial: antibody ratio is given in Figure 3. Addition of DNP-mercurial beyond a mole ratio of about 2 yields no further line broadening. This end point agrees with the stoichiometry established by other methods (Velick *et al.*, 1960). There is no detectable change in line width when these amounts of DNP-mercurial are added to a 1 M NaCl solution that does not contain antibody.

The competition of two different haptens for binding to the antibody also influences the ^{35}Cl nmr spectra. Addition of DNP-lysine to a 1 M NaCl solution containing DNP-mercurial and antibody leads to a marked decrease in the ^{35}Cl nmr line width (Figure 4). The

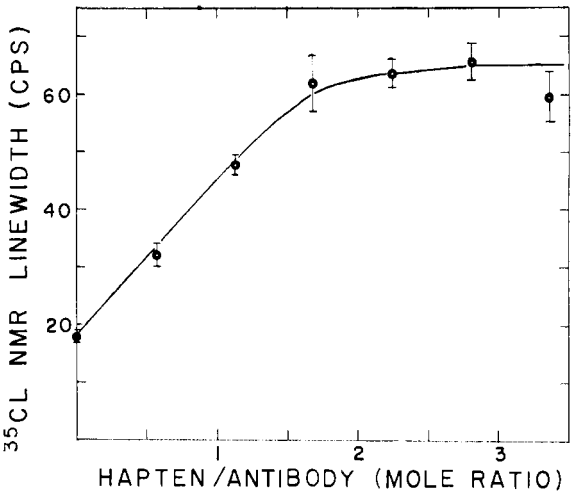

FIGURE 3: ^{35}Cl nmr titration of antidinitrophenyl antibody showing observed ^{35}Cl nmr line widths as a function of the DNP-mercurial:antibody mole ratio.

line width decreases from 59.2 ± 4.2 to 27.2 ± 2.0 cycles/sec when DNP-lysine is added to a 1 M NaCl solution to give the mole ratios DNP-lysine:DNP-mercurial:antibody = 1.78:3.36:1 as shown in Figure 4A,B. Further addition of DNP-lysine to a total of 3.54 equiv yields a line width of 25.6 ± 1.2 cycles/sec. *These spectra indicate that most of the DNP-mercurial is displaced by DNP-lysine from the antibody combining site when equimolar amounts of the haptens are present.*

It is feasible to apply the halide probe technique at antibody concentrations as low as 6×10^{-7} M in 1 M NaCl, as illustrated in Figure 5. In 1 M NaCl, the line width is 15.4 ± 0.6 cycles/sec, which is unchanged when 6×10^{-7} M antibody is present (Figure 5A). Addition of DNP-mercurial gives line widths of 19.6 ± 0.6 and 20.6 ± 1.0 cycles/sec for DNP-mercurial: antibody mole ratios of 2.5 and 5.0, respectively (Figure

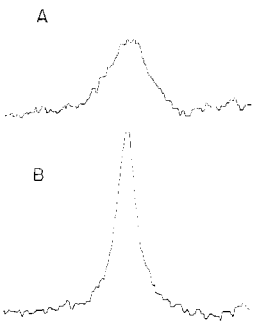

FIGURE 4: ^{35}Cl nmr spectrum of (A) 1 M NaCl containing DNP-mercurial and 5.8×10^{-6} M antidinitrophenyl antibody at a DNP-mercurial:antibody mole ratio 3.36 and (B) with the addition of DNP-lysine, to give a DNP-lysine:DNP-mercurial:antibody mole ratio of 1.78:3.36:1.0.

5B,C). Addition of an excess of DNP-lysine takes the line width back to 16.0 ± 0.5 cycles/sec (Figure 5D).

Discussion

The above results indicate that the ^{35}Cl nmr line width is sensitive to the details of the antibody–hapten interaction. For ^{35}Cl, fluctuations in the orientation and magnitude of the electric field gradient, q, at the side of the nucleus with quadrupole moment, Q, provide the dominant nuclear relaxation mechanism. Thus for a ^{35}Cl nucleus at a single site, the nmr line width is given simply by

$$\Delta\nu = (2\pi/5)[e^2qQ]^2\tau \qquad (1)$$

where the asymmetry factor is neglected, $\Delta\nu$ is the full line width at half-height in cycles per second, (e^2qQ) is the quadrupole coupling constant in cycles per second, and τ is the rotational relaxation time in seconds.

In an aqueous solution of NaCl, mercury-labeled hapten, and antibody, the chlorine atoms are in fact located at three kinds of sites, each of which is characterized by different values of (e^2qQ) and τ: (a) chloride symmetrically solvated by water; (b) chloride complexed to mercury-labeled hapten not bound to antibody; and (c) chloride complexed to mercury-labeled hapten which is bound to antibody. If exchange of chloride between the sites is more rapid than the reciprocal of the line width of the broadest line expressed in reciprocal seconds, a composite signal is observed. The line width of the composite signal is

$$\Delta\nu = (\Delta\nu_a)p_a + (\Delta\nu_b)p_b + (\Delta\nu_c)p_c \qquad (2)$$

where $\Delta\nu_a$, $\Delta\nu_b$, and $\Delta\nu_c$ are the line widths for sites a, b, and c above, and p_a, p_b, and p_c are the probabilities that chloride ion is at sites a, b, and c. When the chloride concentration, $[Cl^-]$, is much greater than that of the DNP-mercurial, [Hg], the change in the ^{35}Cl line width on addition of mercurial is

$$\Delta\nu - \Delta\nu_a = \frac{[Hg]}{[Cl^-]}(f_b\Delta\nu_b + f_c\Delta\nu_c) \qquad (3)$$

where f_b is the fraction of mercury-labeled hapten not bound to the antibody and f_c is the fraction bound to the antibody. It is assumed in eq 3 that the mercury–chlorine complex on the antibody must be accessible for exchange with chloride ions of the solvent. A mercury atom buried in the interior of a protein will make no detectable contribution to the ^{35}Cl line width. The ^{35}Cl nmr spectra (Figures 2 and 4) thus provide information concerning three facets of the hapten–antibody interactions: (a) accessibility of the mercury atom in the hapten–antibody complex to solvent; (b) binding affinity and stoichiometry; and (c) rotational mobility of chloride complexed to the bound hapten.

The large increase in the line width observed on addition of DNP-mercurial to antibody in 1 M NaCl clearly demonstrates that *the mercury atom of the bound hapten is exposed to the chloride ions of the solvent.* The observed broadening requires a chloride exchange rate greater than about 10^7 sec^{-1}, since if $f_c = 1$, a value of $\Delta\nu_c$ equal to 3×10^6 is obtained from eq 3, for $[Hg] = 1.16 \times 10^{-5}$ M, $[Cl^-] = 1$ M, and $(\Delta\nu - \Delta\nu_a) = 44$ cycles/sec (Figure 3). The finding that the mercury atom is exposed to solvent is not unexpected since the antibody specificity is directed toward the dinitrophenyl moiety of the hapten rather than the mercurianiline portion.

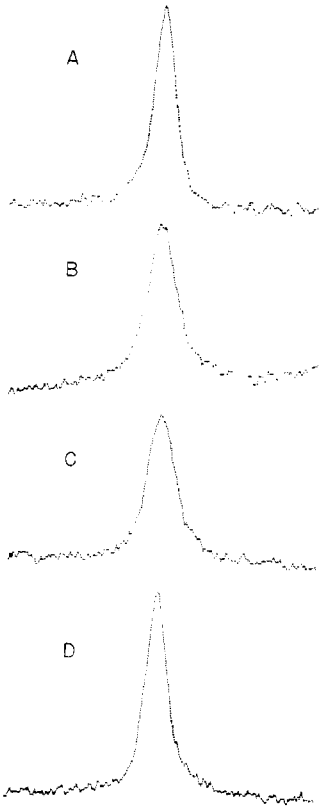

FIGURE 5: ^{35}Cl nmr spectrum of (A) 1 M NaCl and of 1 M NaCl containing DNP-mercurial and 6.0×10^{-7} anti-dinitrophenyl antibody at a DNP-mercurial:antibody ratio of (B) 2.5 and (C) 5.0 and (D) with the addition of an excess of DNP-lysine.

The ^{35}Cl nmr titration shown in Figure 3 indicates that about two haptens are bound per antibody molecule. The success of this type of titration depends on the fact that $\Delta\nu_c$ is some two orders of magnitude larger than $\Delta\nu_b$. Chloride ions complexed to the DNP-mercurial bound to the antibody have a much larger effect value of τ than chloride ions complexed to the simple unbound DNP-mercurial. These differences in τ are consistent with those observed for mercury–hemoglobin complexes and simple organomercury complexes (Stengle and Baldeschwieler, 1966). Since the titration curves essentially follow the stoichiometric line, it is apparent that the dissociation constant of the

hapten–antibody complex is less than 10^{-5} M. The fluorescence titration shown in Figure 1 is consistent with these conclusions, in agreement with well-established values for this hapten–antibody complex in lower ionic strength solutions (Velick *et al.*, 1960). The use of the chloride ion probe technique in determining the relative binding affinities of two haptens is illustrated in Figure 4. Displacement of DNP-mercurial by DNP-lysine is evident in the narrowing of the ^{35}Cl nmr line width. Furthermore, the magnitudes of the spectral changes reveal that the binding affinity of DNP-lysine for antibody is fivefold or more greater than that of the DNP-mercurial.

The ^{35}Cl nmr line width provides a measure of the rotational mobility of the active site. From eq 1, the rotational relaxation time can be determined from the line width if the quadrupole coupling constant is known. Assuming (e^2qQ) is 4×10^7 cycles/sec for the mercury–chlorine complex (Stengle and Baldeschwieler, 1966), and using a value of 3×10^6 cycles/sec for the $\Delta\nu$ of bound DNP-mercurial, τ is calculated to be 1.5×10^{-9} sec. In eq 1, τ refers to the relaxation time for the change in angle between the electric field gradient at the chlorine nucleus and the external magnetic field. This angle is affected by motions within the hapten and between the hapten and antibody, as well as by rotations of the entire antibody molecule. Hence the rotational relaxation time is a composite of these factors. Other methods for determining τ, such as fluorescence polarization and electron spin resonance, give different weights to these several motions. Thus, a comparison of values of τ determined by ^{35}Cl nmr spectroscopy with those ascertained by other methods must take these differences into account. In the electron spin resonance study (Stryer and Griffith, 1965) of antidinitrophenyl antibody, a rotational relaxation time of 3.6×10^{-8} sec was obtained. The shorter τ obtained in this nmr study is probably due to rotations about the C–Hg bond in the DNP-mercurial. This type of motion is not present in the spin-labeled hapten used in the electron spin resonance study. It is of interest to compare the rotational relaxation time obtained in the present nmr study with the τ previously measured for hemoglobin (Stengle and Baldeschwieler, 1966) by this technique. A value of 0.5×10^{-9} was obtained for hemoglobin, with the mercury atom at the β93 cysteine residue. The τ measured here for the hapten–antibody complex is some threefold greater.

This nmr study of the interaction of a mercury-labeled hapten with antibody shows that the halide ion probe technique (Stengle and Baldeschwieler, 1966) can be used to obtain significant information concerning protein–substrate interactions. The accessibility of the metal atom to the solvent is evident if there is any effect on the halide ion nmr line width. It is, of course, possible to substitute the metal label at a variety of sites on the substrate and thus map the accessibility of various parts of the bound substrate. The ^{35}Cl nmr titrations show that information on the stoichiometry of protein–substrate binding, and on relative binding affinities for different substrates is readily available by this technique. It is of particular importance to point out that this method is suitable for the study of weak binding. Since in general $\Delta\nu_o \gg \Delta\nu_b$, the presence of a large fraction of unbound substrate does not interfere seriously with the nmr line width measurements. For example, with the antibody–hapten system described in this paper it should be possible to detect the presence of 1 bound hapten/100 unbound hapten molecules. Finally, the halide ion nmr line width depends on a composite of all the motional correlation times that contribute to the rotational relaxation time of the halogen nucleus. A change in any of the components of τ produces an effect on the nmr line width. Therefore, a change in the gross structure or conformation of a protein, such as subunit dissociation or helix–coil transition, affects the halide ion nmr line width. A local change in the flexibility of the active site owing to structural changes in the protein or the presence of inhibitors (A. G. Marshall, to be published) is also apparent from the line widths. Thus, it should be possible to substitute the metal label at a variety of sites on the substrate and map the flexibility of various parts of the bound substrate.

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