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NMR Study of the Complexes between a Synthetic Peptide Derived from the B Subunit of Cholera Toxin and Three Monoclonal Antibodies against It†

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ABSTRACT: The contact interactions between a synthetic peptide and three different anti-peptide monoclonal antibodies have been studied by nuclear magnetic resonance (NMR). The synthetic peptide is CTP3 (residues 50–64 of the B subunit of cholera toxin) suggested as a possible epitope for synthetic vaccine against cholera. The hybridoma cell lines TE33 and TE32 derived after immunization with CTP3 produce antibodies cross-reactive with the native toxin. The cell line TE34 produces anti-CTP3 antibodies that do not bind the toxin. Selective deuteriation of the antibodies has been used to simplify the proton NMR spectra and to assign resonances to specific types of amino acids. The difference spectra between the proton NMR spectrum of the peptide–Fab complex and that of Fab indicate that the combining site structures of TE32 and TE33 are very similar but differ considerably from the combining site structure of TE34. By magnetization transfer experiments with selectively deuteriated Fab fragment of the antibody, we have found that in TE32 and TE33 the histidine residue of the peptide is buried in a hydrophobic pocket of the antibody combining site, formed by a tryptophan and two tyrosine residues. The hydrophobic nature of the pocket is further demonstrated by the lack of any pH titration effect on the chemical shift of the C4H of the bound peptide histidine. In contrast, for TE34 we have found only one tyrosine residue in contact with the histidine of the peptide. No magnetization transfer was observed between the tryptophan residues of TE34 and the histidine residue of the peptide. The involvement of a tryptophan residue in the combining site of TE33 and TE32 is further demonstrated by the considerable quenching of antibody fluorescence observed upon the binding of the peptide. Such quenching is not observed in TE34. The observed differences between these two types of monoclonal antibodies may be relevant to the complementarity of their respective binding sites to the conformation of the CTP3 peptide in the native toxin.

Nuclear magnetic resonance (NMR) spectroscopy is a very useful tool in obtaining information on the tertiary structures of compounds of biochemical interest, such as peptides and proteins, as well as for monitoring structural changes induced by complex formation between biologically active molecules, e.g., enzymes and their substrates, or antibodies and antigens.

Indeed, NMR spectroscopy was previously employed for the study of antibody combining site structure in the pioneering work of Dwek, Richards, and their respective co-workers. Dwek's group demonstrated that the difference between the NMR spectrum of the Fv fragment of the antibody and the NMR spectrum of the Fv complex with dinitrophenyl (DNP) can be used as a fingerprint for the combining site structure (Dower & Dwek, 1979; Dower et al., 1977). Richards and his co-workers used phosphorus and fluorine NMR to study

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the binding kinetics of several haptens to antibodies (Goetze & Richards, 1978; Kooistra & Richards, 1978). Later McConnell's group used specifically deuteriated Fab fragments of the anti-DNP spin-label antibody ANO2 to determine the amino acid composition of its combining site, as well as the distances of several amino acids to the paramagnetic center (Anglister et al., 1984a,b, 1985; Frey et al., 1984). Recently, magnetization transfer experiments were used by Anglister et al. (1987) to identify contact interactions between the DNP moiety of the hapten and tryptophan residues of the anti-DNP spin-label antibody ANO2. In all these studies, the specificity of antibodies was toward small haptens. The technique has not yet been applied to peptides or protein antigens.

In the last few years, evidence has accumulated that relatively short linear sequences of amino acids, which seem to mimic sequential epitopes, can give rise to antibodies capable of cross-reacting with native intact protein, despite the fact that most immunogenic determinants on globular proteins are conformation dependent. As a result, rapid advances have been made, and synthetic antigens leading to effective immune response against bacterial toxins, viruses, and hormones have been found [reviewed by Arnon (1987) and Steward and Howard (1987)]. Although the use of synthetic peptides has become an important strategy for new vaccine development, very little is known about the structural requirements of these peptides in inducing the necessary production of antibodies cross-reactive with the native antigens.

The immunization of animals with a short synthetic peptide results in a very diversified antibody response since the peptide in solution exists usually in different conformations in fast equilibrium. Each of these conformations may elicit the production of specific antibodies. Antibodies specific for a given conformation may recognize different portions of the peptide. Even antibodies with the same specificity may differ in their amino acid sequences and affinities to the peptide as a result of somatic mutations or because of being coded by different germ line genes. An investigation of the different monoclonal antibodies elicited by a synthetic peptide and their modes of interaction with the peptide could illustrate this point.

In previous studies, the CTP3 peptide (residues 50–64 of the B subunit of cholera toxin) was shown to elicit the production of polyclonal antibodies highly cross-reactive with the native toxin (Jacob et al., 1983). These antibodies were able to inhibit cholera toxin induced cyclic AMP to an extent of 60–70% and to neutralize significantly the biological activity of the toxin (Jacob et al., 1983, 1984a). Moreover, anti-CTP3 antibodies were found to cross-react and neutralize several cholera toxin related diarrheagenic proteins of high clinical importance, such as heat-labile toxin isolated from either human or porcine sources (LT) (Jacob et al., 1984b, 1986). Understanding the different antigenic structures that CTP3 presents to the immune system may help us in the future to design an efficient synthetic vaccine against cholera.

In the present study, we describe the production of monoclonal antibodies to CTP3 and the applicability of NMR spectroscopy to the study of the specific interactions between this peptide and three different monoclonal anti-peptide antibody molecules.

Materials and Methods

Materials. Cholera toxin (CT) was purchased from Schwarz/Mann, Orangeburg NY. Purified *Escherichia coli* heat-labile toxins of human origin (H-LT) and porcine origin (P-LT) were the generous gift of Professor R. A. Finkelstein, Columbia MS. Tetanus toxoid (TT) was generously donated by Rafa Laboratories, Jerusalem, Israel. The following deuteriated amino acids were obtained from MSD isotopes: L-tryptophan-*2,4,5,6,7-d*$_5$ (96.9% D), L-*β*-(4-hydroxyphenyl)-alanine-*2,3,3-d*$_3$ (tyrosine) (96.9% D), L-phenyl-*d*$_5$-alanine-*3,3-d*$_2$ (98% D), and L-(4-hydroxyphenyl-*2,6-d*$_2$)alanine-*2-d*$_1$. L-Tryptophan-*4,6,7-d*$_3$ (60% H in positions 2 and 5) was a generous gift from Dr. Mei Whittaker, Stanford University.

Peptide Synthesis and Purification. The peptide CTP3 (amino acid residues 50–64 of the B subunit of CT, Figure 1A) and the control peptide K-2 (a 17 amino acid peptide derived from the human epidermal 56K type II keratin sequence) were prepared by Merrifield solid-phase synthesis (Erickson & Merrifield, 1976). CTP3 was purified by gel filtration on Sephadex G15 followed by CM52 cation-exchange chromatography. The peptide was loaded on the ion-exchange column in 10 mM acetic acid, washed with acetate buffer, pH 5, and eluted with 200 mM ammonium acetate, pH 7. The peptide was checked by high-performance liquid chromatography (HPLC) and found to be >97% pure, and its composition was verified by amino acid analysis. For immunization of mice, CTP3 was coupled to tetanus toxoid (TT) as described (Jacob et al., 1983).

Dinitrobenzene was coupled to CTP3 by reacting 1-fluoro-2,4-dinitrobenzene in 0.1 M bicarbonate, pH 9, with the protected peptide still coupled to the resin. The *tert*-butyloxycarbonyl protecting group of the N-terminus was removed before the reaction. The DNP-CTP3 was fully deprotected and cleaved from the resin by hydrofluoric acid and purified on Sephadex G25 and a CM52 cation-exchange column. The DNP-CTP3 was loaded in 50 mM acetic acid, and after the column was washed, it was eluted with 200 mM ammonium acetate buffer, pH 5. Purity was verified by HPLC.

Preparation of Monoclonal Antibodies. Eight-week-old Balb/C mice were used as cell donors. The cell line used for fusion was the NSO/1 myeloma cell line described by Galfre' and Milstein (1981). Mice were immunized into the foot pad with 50 μg of CTP3–TT (CTP3 coupled to tetanus toxoid) emulsified in complete Freund's adjuvant. Spleen lymphocytes from immunized donors were combined with NSO/1 myeloma cells in a ratio of 5:1 and fused with poly(ethylene glycol) according to the procedure of Galfre' and Milstein with minor modifications as described (Eshhar, 1985). Fused cells were plated in HAT medium, made with Dulbecco's modified Eagle's medium (DMEM) including 4.5 g/L glucose (GIBCO, Grand Island, NY), 20% fetal calf serum, 100 units/mL penicillin, 100 μg/mL streptomycin, and 0.25 μg/mL fungizone (all from GIBCO). Two weeks later, cell culture supernatants were screened for binding CTP3 and CT by solid-phase radioimmunoassay (RIA). From about 80 positive clones producing antibodies specific for CTP3, several representative clones were selected for further cloning and characterization. For the production of antibodies, the cells were adjusted to grow on RPMI 1640 medium supplemented with only 2% fetal calf serum.

The class identification of the immunoglobulins secreted by the different hybridomas was done by the Ouchterlony immunodiffusion test using anti-mouse classes and subclass antibodies from Miles-Yeda, Rehovot.

Solid-Phase Radioimmunoassay (RIA). The assay was performed on antigen-coated (0.5–1 μg per well) V-bottom flexible microtiter plates [precoated with glutaraldehyde (0.2%) according to Sutter (1982) whenever peptides were used as antigens]. The coating was done by overnight incubation with the antigen, followed by incubation with 3% bovine serum albumin in phosphate-buffered saline. The washed and dried

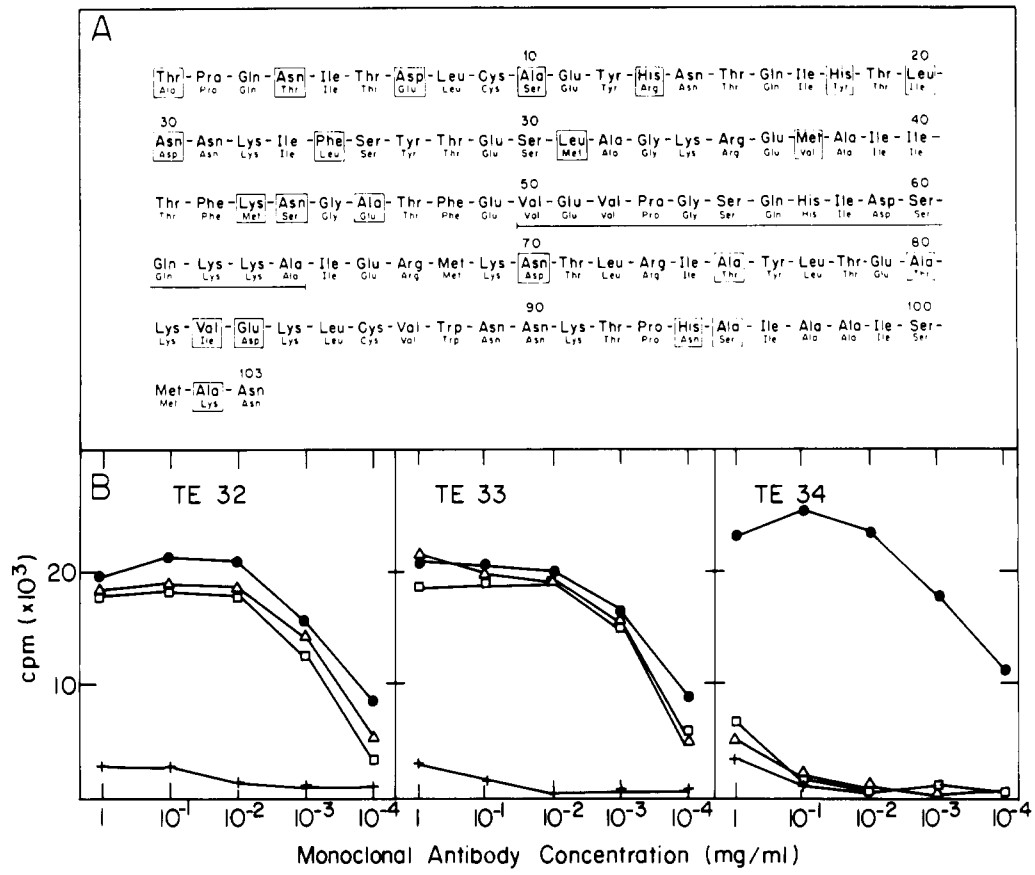

FIGURE 1: (A) Primary amino acid sequence of the B subunit of cholera toxin. CTP3 peptide (residues 50–64) is underlined. Residues in boxes are those which differ in the sequence from the heat-labile toxin of *E. coli*. (B) Binding of TE32, TE33, and TE34 monoclonal antibodies to CTP3 (●); nonrelevant control peptide (+), CT (△), and H-LT (□) as done by solid-phase radioimmunoassay (RIA).

wells were separated by cutting, and the radioactivity was counted in a γ counter.

Preparation of Samples for NMR Experiments. Deuteriated anti-CTP3 antibodies were obtained by growing the hybridoma cells on specifically deuteriated amino acids according to the RPMI 1640 recipe (GIBCO, Grand Island, NY, technical manual). Antibodies were purified by a protein A affinity column (Pharmacia), and Fab fragments were prepared by papain cleavage and then purified by a G75 column connected in series with a protein A column. The Fab fragments were concentrated to 1.5–2.0 mM and dialyzed extensively against deuterium oxide buffered with 0.01 M phosphate, pH 8.

NMR Experiments. The spectra were measured on GE 500-MHz and Bruker 400-MHz instruments. For the measurements of spectra and difference spectra, 200 scans were collected per sample. For the magnetization transfer experiments, the free induction decays (FID) were collected in an interleaved manner, changing the irradiation frequency after 44 scans (the first 4 were not recorded). The reference spectra for the magnetization transfer difference spectra were collected with irradiation frequency outside the envelope of the protein's absorption. Typically, 600 scans were collected per irradiation frequency. Spectra were measured at 47 °C with delays of 3 s on the 400-MHz instrument and 4 s on the 500-MHz instrument.

Fluorescence Measurements. The fluorescence was measured with Perkin-Elmer MPF-44E fluorometer. The excitation wavelength was 280 nm, and emission was measured at 364 nm. Concentrations of Fab were 5.6×10^{-7}, 4.45×10^{-7}, and 1.33×10^{-7} M for TE32, TE33, and TE34, respectively. No correction for absorption was required for the titration with CTP3 since it does not have a chromophoric group absorbing light at 280–370 nm. The inner filter effect of DNP-CTP3 was negligible at the concentrations used in the experiments.

Results

Of about 80 hybridomas producing antibodies that bind CTP3, 3 representatives, namely, TE32, TE33, and TE34, were chosen for further cloning and characterization. By the Ouchterlony immunodiffusion test, all three antibodies were found to be of the IgG1 class possessing κ light chains. Solid-phase RIA (Figure 1b) and Western blot analysis (data not shown) indicate that TE32 and TE33 are highly cross-reactive with cholera toxin and H-LT, a heat-labile toxin produced by enterotoxigenic strains of *E. coli* isolated from humans. The affinities of these antibodies to the toxins were found to be only slightly lower than their affinities to the homologous peptide CTP3. In contrast, TE34 did not show any significant cross-reactivity with CT or H-LT although its reactivity with the CTP3 peptide was as high or even somewhat higher than TE33.

Fluorescence quenching was used to determine the binding constants of antibodies (Eisen, 1964). We have found that saturation of the Fab of TE33 and TE32 with CTP3 result in a considerable quenching of the Fab fluorescence, 40% quenching in the case of TE33 and 23% in that of TE32. On the other hand, saturation of TE34 with CTP3 does not result in any detectable change in the antibody fluorescence. A 60% quenching of TE34 fluorescence is obtained by saturating the Fab with DNP-CTP3. The binding constants of the TE33 and TE32 antibodies were determined by following the tryptophan fluorescence quenching as a result of titrating the Fab with CTP3. In the case of TE34, DNP-CTP3 was added to the Fab. These results are displayed in Figure 2. The binding

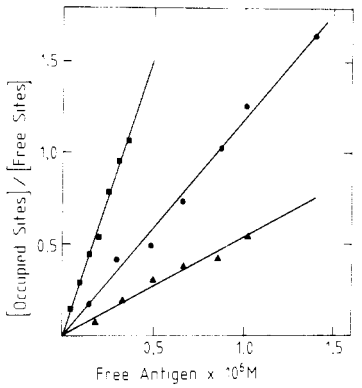

FIGURE 2: Binding of CTP3 to the Fab of TE32 (▲) and to the Fab of TE33 (●) and the binding of DNP-CTP3 to the Fab of TE34 (■). The ratio between occupied sites and free sites is obtained by fluorescence quenching measurements (Eisen, 1964).

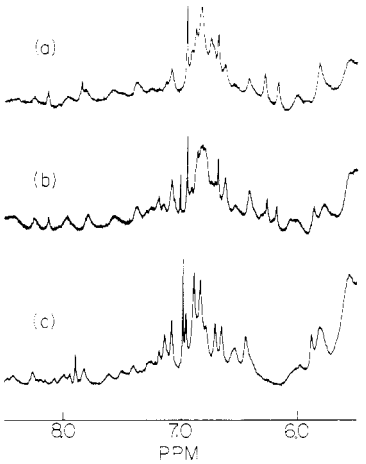

FIGURE 3: Comparison of the aromatic region of the proton NMR spectra of the Fab fragments of three different anti-CTP3 antibodies. Tryptophan and phenylalanine residues are perdeuteriated, while tyrosine residues are deuteriated in ring positions 2 and 6. (a) The spectrum of TE33 which cross-reacts with cholera toxin. (b) The spectrum of TE32 which like TE33 cross-reacts with cholera toxin. (c) The spectrum of TE34 which does not bind cholera toxin.

constants of CTP3 to TE33 and TE32 are 1.2×10^6 and 0.5×10^6 M^{-1}, respectively. The binding constant of DNP-CTP3 to TE34 is 3×10^6 M^{-1}. The binding constant of CTP3 to TE34 was determined by competition between CTP3 and DNP-CTP3 and is 2.5×10^6 M^{-1}. Measurements were carried out at room temperature (23 °C).

The proton NMR spectrum of Fab fragment is very poorly resolved. It contains contributions from more than 2000 nonexchangeable protons of which each single resonance is considerably broadened on the order of 10 Hz or more due to the slow rotational motion of the 50-kilodalton (kDa) Fab. Selective incorporation of deuteriated amino acids is therefore required in order to simplify the spectrum and improve resolution. The proton NMR spectra of the partially deuteriated three monoclonal anti-CTP3 antibodies TE32, TE33, and TE34 are given in Figure 3. All tryptophan and phenylalanine antibody residues are perdeuteriated, while tyrosine residues are deuteriated at aromatic ring positions 2 and 6. Only the spectral region containing contributions of the aromatic protons is shown. The proton NMR spectrum of the labeled Fab of TE33 shown in Figure 3a is well resolved, allowing us to identify resonances of individual tyrosine residues. The spectrum of TE32 Fab, given in Figure 3b, is somewhat similar to that of TE33. These two spectra differ greatly from the proton NMR spectrum of the labeled Fab of TE34 shown in Figure 3c.

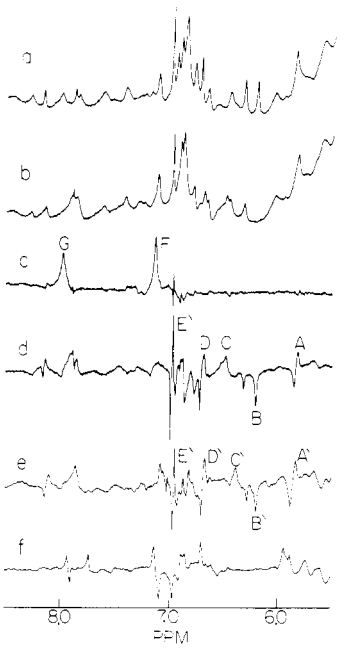

FIGURE 4: Difference spectra of partially deuteriated antibodies. Tryptophan and phenylalanine residues are perdeuteriated, while tyrosine is deuteriated in ring positions 2 and 6. (a) The NMR spectrum of TE33. (b) The NMR spectrum of TE33 complexed with the peptide CTP3. (c) A difference spectrum between the NMR spectrum of Fab with a large excess of CTP3 and that of the Fab of TE33 saturated with CTP3. Resonances G and F are those of the imidazole protons of the histidine residue of the free peptide. (d) The difference between the NMR spectrum of TE33 complexed with the peptide (b) and the NMR spectrum of the free TE33 Fab (a). (e) The difference spectrum between the NMR spectrum of the Fab complex with CTP3 and the NMR spectrum of the Fab for TE32. (f) The difference spectrum of TE34.

As previously pointed out by Dwek and his co-workers (Dower & Dwek, 1979), the difference between the NMR spectrum of the Fab–hapten complex and that of the noncomplexed Fab contains mostly, if not solely, contributions from those amino acids within the combining site. The binding of the hapten causes these amino acids to experience changes in chemical environment usually accompanied by changes in chemical shift. Figure 4 shows the difference spectra obtained for the Fab fragments of TE32, TE33, and TE34 (the individual proton NMR spectra of which have been given in Figure 3.) Figure 4a is the NMR spectrum of the labeled Fab fragment of the TE33 antibody. Figure 4b is that of the Fab–CTP3 complex. Figure 4c illustrates the contributions of free CTP3 to the spectrum of the Fab–CTP3 complex with a 6-fold excess of CTP3. Spectrum 4c is obtained by calculating the difference between the NMR spectrum of the Fab with an excess of CTP3 and that of the Fab saturated with CTP3. The only aromatic amino acid found in CTP3 is the position 8 histidine (corresponding to position 57 in cholera toxin). Figure 4d represents the difference between the spectrum of Figure 4b and that of Figure 4a and shows all resonances of the tyrosine residues which changed chemical shift upon binding of the peptide. The resonances of the Fab complex appear as positive peaks while those of the free Fab are negative in this presentation. Figure 4e is the difference spectrum obtained for TE32, and Figure 4f is that of TE34. There is much resemblance between the difference spectra of TE33 and TE32. Resonances A and D have the same chemical shifts as A′ and D′, respectively, while the chemical shifts of B′ and E′ differ only slightly from those of B and E, respectively. The chemical shift of C′ differs by more than 0.05 ppm from the chemical shift of C. It should be noted that

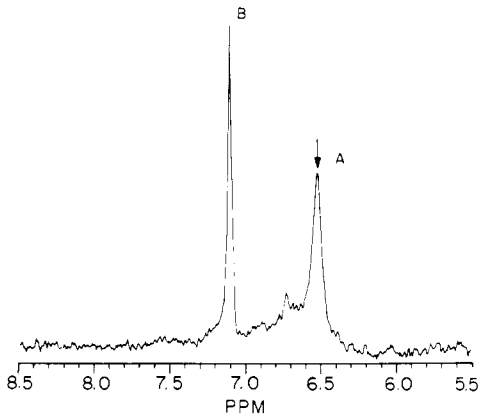

FIGURE 5: Magnetization transfer difference spectrum showing transfer due to chemical exchange between the peptide bound to TE33 and the free peptide. Resonance A assigned to the C4H ($C_{\delta 2}H$) histidine of the bound peptide was irradiated, and a transfer to resonance B is observed. Resonance B is assigned to the C4H histidine of the free peptide. All tyrosine, phenylalanine, and tryptophan residues of TE33 Fab are perdeuteriated. The concentration of CTP3 is 15 times greater than that of the Fab.

there is considerable more resemblance in the difference spectra of these two antibodies than in their respective proton NMR spectra. Comparison of the three different spectra shows clearly that there is very little resemblance between the difference spectrum of TE34 and that of the other two antibodies.

The two resonances of the histidine residue of the free CTP3 are clearly observed in the NMR spectrum of TE33, in which all tryptophan, tyrosine, and phenylalanine residues are perdeuteriated and the peptide is in large excess. Irradiation of the histidine C4H ($C_{\delta 2}H$) resonance of the free peptide at 7.13 ppm results in magnetization transfer to a broad resonance at 6.54 ppm. We have noticed that while all resonances of the Fab are narrowed when the temperature is raised from 37 to 47 °C, the resonance at 6.54 ppm becomes broader by 14 Hz. This indicates that the broad resonance is of a proton in slow chemical exchange between two forms. By raising the temperature, the exchange approaches medium rate relative to the difference in chemical shift between the two forms of the proton. Irradiation of the broad resonance results in a very strong magnetization transfer to the resonance of the free CTP3 even after an irradiation period as short as 10–30 ms as shown in Figure 5. We therefore conclude that this broad resonance represents the histidine C4H resonance of the bound peptide. The magnetization transfer is found to be linearly dependent upon the irradiation period up to at least 30 ms when the peptide is in 15-fold excess. From the dependence of the transfer as a function of the irradiation period, we conclude that the off rate is 30 s^{-1} at 47 °C and about 12 s^{-1} at 37 °C [discussions of the effect of the exchange rate on the line width of the exchanging species and the saturation transfer experiment are presented thoroughly by Jardetzky and Roberts (1981), Gutowsky and Saika (1953), McConnell (1958), and McConnell and Thompson (1959)]. The spin–lattice relaxation times (T_1) of the Fab and the peptide protons were found to be close to 1 s. Therefore, the measured chemical exchange is fast relative to T_1. We have noticed that while the chemical shift of the free peptide histidine C4H changes from 7.1 to 7.4 ppm at pH 5.7, the chemical shift of the broad resonances does not undergo any observable changes in the pH range 5.7–8.2.

The dependence of the magnetization transfer between two protons as a function of the irradiation period of one of them can be used to calculate the distance between the two protons

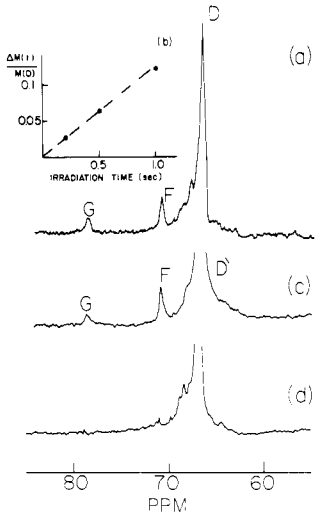

FIGURE 6: Magnetization transfer difference spectra showing transfer between a tyrosine residue of the antibodies to the histidine residue of the peptide. Phenylalanine and tryptophan residues of the antibodies are perdeuteriated with tyrosine deuteriatd in ring positions 2 and 6. The tyrosine resonance at approximately 6.7 ppm was irradiated. (a) Resonance D of TE33 was irradiated, and a transfer was observed to resonances F and G, both assigned to the histidine residue of the free peptide. (b) The dependence of the magnetization transfer to G presented in (a) as a function of the irradiation period. (c) Resonance D′ of TE32 was irradiated, and transfer to the resonances of the protons of the histidine residue of the free peptide, F and G, was observed. (d) Magnetization transfer difference spectrum for TE34. No transfer to protons of the histidine was observed.

(Noggle & Schirmer, 1971; Gordon & Wutrich, 1978). In order to determine the contact interactions between the tyrosine residues of the antibodies and the histidine residue of the peptide, all resonances appearing in the difference spectra shown in Figure 4 were irradiated. For this experiment, we used the Fab fragments (corresponding to Figure 3) in solution with 6-fold excess of CTP3. Figure 6a shows the magnetization transfer difference spectrum obtained when resonance D in Figure 4d is irradiated for 500 ms. A strong transfer to resonances G and F is observed. Resonance G has the same chemical shift as the imidazole C2H ($C_{\epsilon 1}H$) of the histidine of the free peptide while resonance F has the same chemical shift as the imidazole C4H resonance of the free peptide. This transfer is not observed when tyrosine is perdeuteriated or when there are equimolar amounts of CTP3 and Fab. This proves that magnetization transfer is occurring between a tyrosine residue of the antibody and the histidine protons of the bound peptide. As a result of chemical exchange between the bound and free peptide, which is fast relative to T_1, a magnetization transfer to the free peptide is observed. Figure 6b shows the dependence of the magnetization time on the irradiation period. The initial rate of change in the magnetization of the hapten is given by (Clore & Gronenborn, 1983)

$$dM_H/dt = -\sigma_{H,F}M_F(0)$$

where $M_F(0)$ is the equilibrium magnetization of a single Fab proton, M_H is the magnetization of the hapten (bound + free), t is the irradiation period, and $\sigma_{H,F}$ is the rate of cross-relaxation between the Fab proton and the hapten proton. From the saturation transfer experiment, we obtain the value of $dM_{H(free)}/dt$ which is smaller than dM_H/dt, and, therefore, we are able to calculate only an upper limit of the distance between the tyrosine protons and the histidine proton. By measuring the magnetization transfer between Fab protons separated by a fixed distance (tryptophan and tyrosine protons of adjacent carbons), we found that the dipolar correlation time for the Fab is 2.2×10^{-8} s. Using this value, the upper limit

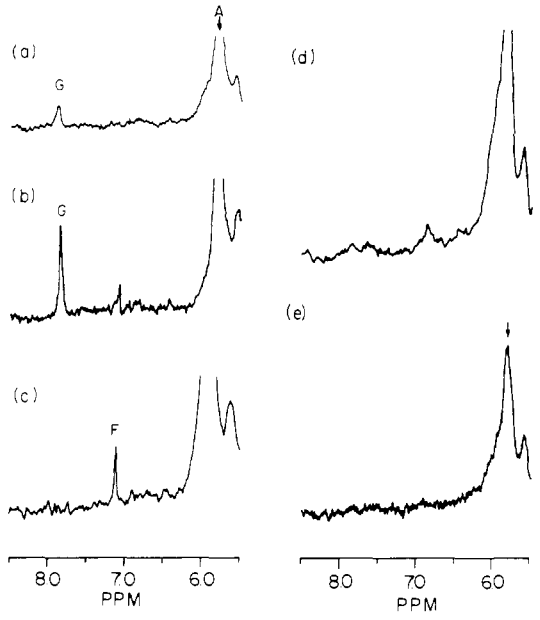

FIGURE 7: Magnetization transfer difference spectra showing transfer between tyrosine residues of three different monoclonal anti-CTP3 antibodies to the histidine protons of the peptide. Phenylalanine and tryptophan are perdeuteriated, with tyrosine residues deuteriated in ring positions 2 and 6. The tyrosine resonance at approximately 5.8 ppm was irradiated. (a) Resonance A of TE33 was irradiated, and a transfer was observed to resonance G, assigned to the imidazole C2 proton of the free peptide histidine. (b) Resonance A′ of TE32 was irradiated, and a transfer to resonance G is observed. (c) Irradiation of a TE34 tyrosine resonance at 5.8 ppm resulted in a magnetization transfer to resonance F, assigned to the imidazole C4 proton of the free peptide histidine. Two control experiments proving that the transfer observed is that between the tyrosine protons of the antibodies and the histidine protons of the free peptide: (d) magnetization transfer difference spectrum of TE33 saturated with CTP3; the resonance of a tyrosine residue at 5.8 ppm was irradiated, and no transfer to other resonances in the aromatic region was observed; (e) magnetization transfer difference spectrum of TE33 in which tryptophan, phenylalanine, and tyrosine residues are perdeuteriated. The concentration of CTP3 is 6 times the concentration of the Fab. The Fab was irradiated at 5.8 ppm, and no transfer to resonances of aromatic protons was observed. Irradiation time was 300 ms.

of the distance between the antibody's tyrosine proton and the peptide's histidine proton is found to be 4.5 Å. As shown in Figure 6c, a similar transfer is observed when resonance D′ of TE32 is irradiated for 300 ms. The intensity of the transfer is comparable in its magnitude to that observed in TE33. Irradiation of the resonance of TE34 appearing in the difference spectrum at 6.7 ppm does not cause any magnetization transfer to the histidine proton of CTP3.

A strong transfer between another tyrosine residue of TE33 and the C2H of the histidine of the free peptide is observed when a H3,5 tyrosine resonance at 5.8 ppm (resonance A in Figure 4d) is irradiated for 300 ms as shown in Figure 7a. This transfer is about 70% stronger than that shown in Figure 6. As shown in Figure 7b, a transfer 3.5 times stronger is observed in TE32 after irradiation of resonance A′ (Figure 4e) at 5.8 ppm. In TE34, there is a tyrosine residue that has a chemical shift of 5.95 ppm and appears in the difference spectrum between the NMR spectrum of the Fab complex with the peptide and the NMR spectrum of the Fab. As presented in Figure 7c, irradiation of the TE34 tyrosine resonance at 5.95 ppm, that appears in the difference spectrum in Figure 4f, results in a magnetization transfer to the free peptide C4H histidine resonance. The intensity of this transfer is similar to that observed in TE33. As illustrated in the examples given in Figure 7d,e, when the Fab tyrosine residues

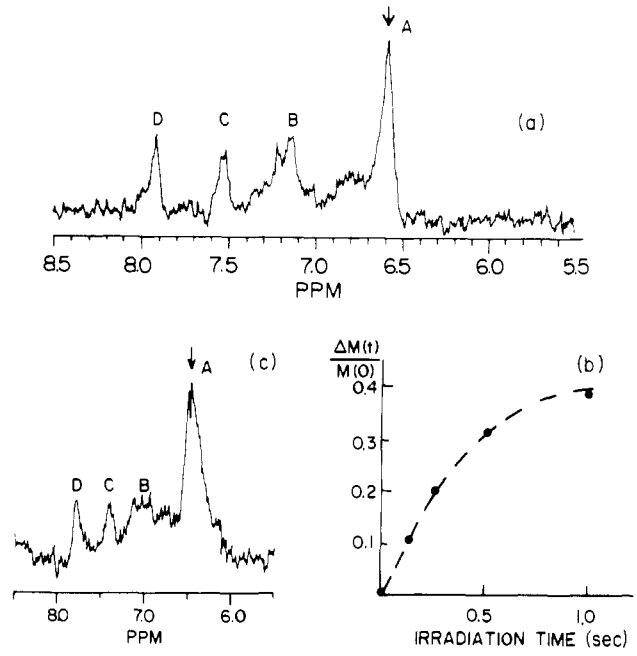

FIGURE 8: Magnetization transfer difference spectra showing transfer between the histidine residue of the peptide and tryptophan protons of two anti-CTP3 antibodies cross-reactive with cholera toxin. The Fab is saturated with CTP3. Tyrosine and phenylalanine residues of the antibody are deuteriated. The imidazole C4H resonance of the bound peptide histidine at 6.52 ppm (A) was irradiated, and transfer of magnetization to at least three tryptophan protons of the Fab was observed (B–D). (a) Magnetization transfer difference spectrum of TE33 irradiated for 500 ms measured on a 500-MHz instrument. (b) The dependence of transfer to resonance D on the duration of irradiation of the TE33 Fab complexed with CTP3. (c) The magnetization transfer difference spectrum measured for TE32 antibody on a 400-MHz instrument. Irradiation time was 400 ms.

are deuteriated or there are equimolar amounts of Fab and CTP3 present, the magnetization transfer shown in Figure 7a–c is not observed. Irradiation of the remaining resonances of TE34 does not cause any magnetization transfer to the protons of the histidine residue of the free peptide.

A difference spectrum between the NMR spectrum of the Fab complex of TE33 and CTP3 and the NMR spectrum of the Fab with its tyrosine and phenylalanine residues perdeuteriated, but its tryptophan not, indicates that at least one tryptophan residue contributes to the combining site. In the NMR spectrum of this labeled Fab saturated with CTP3, the C4H resonance of the bound peptide histidine is clearly observed and very well resolved. Figure 8a shows the magnetization transfer difference spectrum obtained when the histidine proton of the bound peptide is irradiated. A strong magnetization transfer to three resonances is clearly observed. This transfer is not observed when tryptophan is deuteriated, implying that transfer occurs between the peptide histidine and a tryptophan residue of the antibody. The difference spectrum between the NMR spectrum of the Fab–CTP3 complex with tyrosine and phenylalanine residues deuteriated and that of the complex when tyrosine, phenylalanine, and *tryptophan* residues of the Fab are deuteriated reveals that there are no resonances of Fab tryptophan protons overlapping with the C4H resonance of the bound peptide histidine. This rules out the possibility that the observed transfer occurs between tryptophan protons of the Fab. From the initial slope of the dependence of the transfer on the irradiation period (Figure 2b), and assuming a correlation time of 2.2×10^{-8} s, we estimate that the tryptophan protons are 3.3–3.5 Å away from the histidine C4H. As shown in Figure 8c, a very similar transfer was observed in TE32 in which tyrosine and phenyl-

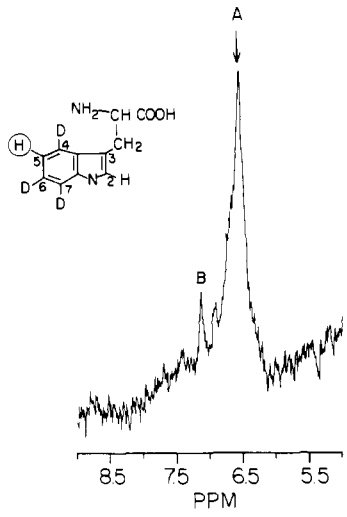

FIGURE 9: Magnetization transfer difference spectrum showing transfer between the peptide histidine and a partially deuteriated tryptophan residue of the TE33 Fab. Tyrosine and phenylalanine residues of the antibody are perdeuteriated while tryptophan is deuteriated at the indole 4-, 6-, and 7-carbons. Positions 2 and 5 are substituted 40% with deuterium and 60% with hydrogen. TE33 is saturated with the peptide. Irradiation of resonance A, assigned to the C4 proton of the histidine residue of the bound peptide, resulted in a magnetization transfer to resonance B, assigned to the tryptophan indole C5 hydrogen. Irradiation time was 400 ms.

alanine were perdeuteriated. When resonances B–D are irradiated in a sample of the labeled Fab containing a large excess of CTP3, a transfer to the histidine of the free CTP3 is clearly observed. Irradiation of TE34 with the same labeling does not reveal any transfer between tryptophan residues and the histidine of the free CTP3.

Fab was prepared in which all tyrosine and phenylalanine residues were perdeuteriated and all tryptophan protons deuteriated except at indole positions 2 and 5 where only 40% deuteriation occurred. The Fab was saturated with CTP3, and the resonance of the C4H of the histidine of the bound peptide was irradiated. The magnetization transfer difference spectrum is shown in Figure 9. It shows transfer to only one tryptophan proton, with a chemical shift the same as resonance B in Figure 8a. It is very unlikely that resonance B is of the indole C2 tryptophan proton since it is impossible for the histidine proton to be this close (3.5 Å) to the indole C2 proton ($C_{\nu 1}H$) and to two other indole protons of the same tryptophan simultaneously. If we assign resonance B to the indole C5 proton ($c_{\zeta 3}H$), resonances C and D can be assigned to tryptophan indole protons C4 and C6 ($C_{\epsilon 3}H$ and $C_{\eta}H$, respectively). The histidine proton can be placed in a position such that it is approximately equidistant to these three tryptophan protons and further away from the remaining two indole protons of the tryptophan. The magnetization transfer difference spectrum given in Figure 9 is further evidence that the transfer observed is not between tryptophan protons of a Fab residue. Such transfer is impossible during the short irradiation period used due to the long distance between protons 2 and 5 of the tryptophan.

DISCUSSION

In the present paper, we describe for the first time NMR study of the specific interactions between a peptide and its monoclonal antibodies. We demonstrate the applicability of the difference spectra technique and magnetization transfer experiments with specifically labeled Fab fragments to the comparative study of the structures of monoclonal antibodies and their complexes with the antigen. X-ray crystallography

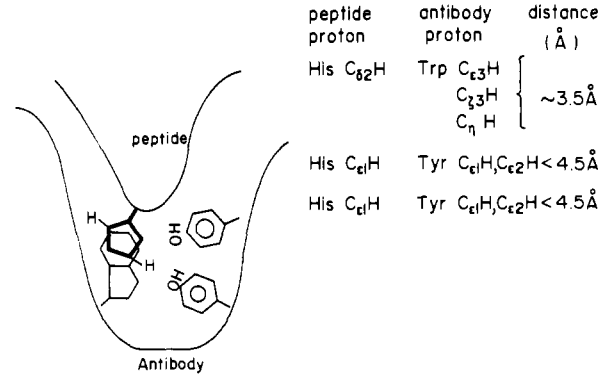

FIGURE 10: Schematic drawing of the hydrophobic pocket for the histidine residue of the peptide CTP3 in the combining sites of the two anti-CTP3 antibodies TE33 and TE32, both cross-reactive with cholera toxin.

has provided us with only one detailed structure of a complex between a monoclonal antibody and a protein (Amit et al., 1986), and there is no crystallographic structure of a complex between a monoclonal antibody and a peptide. A comparative study of antibodies is not feasible by X-ray crystallography since it is difficult to crystallize antibody molecules. The NMR methods that are presented can also be applied to the comparative study of genetically engineered protein molecules.

By comparing NMR spectra and the difference spectra obtained between the NMR spectra of the Fab complexes (with the peptide) and those of the Fab, we deduce that the two anti-CTP3 peptide antibodies cross-reactive with cholera toxin are similar in their structure and show even greater resemblance in the structure of their combining sites. The magnetization transfer experiments support this conclusion; in both antibodies, there is a hydrophobic pocket for the histidine residue of the peptide. This hydrophobic pocket is formed by two tyrosine residues and one tryptophan as schematically shown in Figure 10. Proton distances between tryptophan and the histidine C4H position, as well as between one of the tyrosine residues (resonances D and D′ in Figure 6) and the peptide histidine, are similar in TE32 and TE33. As for the second tyrosine residue involved in the pocket, a magnetization transfer 3.5 times stronger is observed in TE32 relative to that observed in TE33. This indicates that in TE32 the H3,5 tyrosine protons are closer to the histidine C2H proton than the equivalent TE33 protons. The third anti-CTP3 antibody (which does not bind cholera toxin) was found very different from TE32 and TE33. The difference spectra of specifically labeled antibodies can be used as a fast technique for the classification of antibodies against the same antigen according to the structure of their combining sites.

It should be noted that the magnetization transfer to protons of the free peptide can be observed only for those antibodies for which the off rate of the peptide is fast relative to the T_1 relaxation time of the peptide. In such a case, the magnetization is transferred effectively from the bound peptide to the free peptide by chemical exchange.

The fluorescence quenching that is observed after the binding of CTP3 to the TE32 and TE33 antibodies is surprisingly high. This quenching is not due to fluorescence energy transfer since CTP3 does not contain a chromophoric group with an absorption overlapping the absorption or fluorescence of tryptophan. The quenching is most probably due to a drastic change in the immediate environment of the combining site tryptophan. This may be attributed to π–π complex formation between the tryptophan and the histidine residue of the peptide. There is a considerable difference in the extent of fluorescence quenching between TE32 and TE33.

Most of the difference is due to the increased fluorescence of TE33 Fab relative to that of TE32. On the other hand, no quenching is observed in TE34 as a result of saturating the antibody with CTP3. However, DNP-CTP3 quenches the TE34 fluorescence by 60% due to fluorescence energy transfer between the tryptophans of the antibody and the DNP. The transfer occurs because of the overlap between the absorption spectrum of DNP and the fluorescence spectrum of tryptophan. Since the fluorescence energy transfer is effective over a distance longer than 50 Å, the fluorescence of all tryptophans that are in this range to the DNP is quenched.

The variable domains of TE33 are now being sequenced. We have already found one tryptophan in the third hypervariable region of the heavy chain and four tyrosines in the three hypervariable regions of the heavy chain. Once we finish the sequencing of the three antibodies, a model for the combining site will be constructed.

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