Antibodies against a Peptide of Cholera Toxin Differing in Cross-Reactivity with the Toxin Differ in Their Specific Interactions with the Peptide As Observed by ^{1}H NMR Spectroscopy†

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ABSTRACT: The interactions between the aromatic residues of the monoclonal antibody TE34, and its peptide antigen CTP3, have been studied by 2D TRNOE difference spectroscopy. The sequence of CTP3 corresponds to residues 50–64 of the B subunit of cholera toxin (VEVPGSQHIDSQKKA). Unlike two previously studied anti-CTP3 antibodies (TE32 and TE33), the TE34 antibody does not bind the toxin. The off-rate of CTP3 from TE34 was found to be too slow to measure strong TRNOE cross-peaks between the antibody and the peptide. Much faster off-rates, resulting in a strong TRNOE, were obtained for two peptide analogues: (a) CTP3 with an amide in the C-terminus (VEVPGSQHIDSQKKA-NH_2) and (b) a truncated version of the peptide (*N*-acetyl-IDSQKKA). These modifications do not interfere significantly either with the interactions of the unmodified part of the peptide with the antibody or with intramolecular interactions occurring in the epitope recognized by the antibody. The combined use of these peptides allows us to study the interactions between the antibody and the whole peptide. Two tyrosine residues and one or more tryptophan and phenylalanine residues have been found to interact with histidine-8, isoleucine-9, aspartate-10, lysine-13 and/or lysine-14, and alanine-15 of the peptide. In the bound peptide, we observe interactions of a lysine residue with aspartate-10 β protons. While the peptide epitope recognized by TE34 is between histidine-8 and the negatively charged C-terminus, that recognized by TE32 and TE33 is between residues 3 and 10 of the peptide. The strong interaction of TE34 with the negatively charged C-terminus of CTP3 is one of the main reasons for its lack of cross-reactivity with the native toxin. Similar use of modified peptides may extend the applicability of 2D TRNOE difference spectrscopy to the study of other antibody–peptide complexes involving slow peptide off-rates.

Peptides corresponding in sequence to segments of proteins are used to raise anti-peptide antibodies that are cross-reactive with native antigens (Van Regenmortel, 1987). The molecular basis for such antigenic cross-reactivity is still not fully understood, although it has been suggested that several factors relating to the structure of the epitopes play an essential role (Dyson et al., 1988). Synthetic peptides derived from the proteins of various pathogens have been used successfully to raise antisera that display neutralizing activity and may potentially be used as synthetic vaccines (Arnon, 1986; Steward & Howard, 1987).

The CTP3[1] peptide, corresponding to residues 50–64 of the B subunit of cholera toxin, elicits the production of antibodies cross-reactive with the toxin and other diarrheagenic toxins closely related in structure (Jacob et al., 1983). In addition, immunization of laboratory animals with CTP3 or its longer version (residues 50–75) has been shown to provide protection from cholera toxin (Pedoussaut et al., 1989). Jacob and Arnon (Anglister et al., 1988) obtained 80 anti-CTP3 monoclonal antibodies of which approximately one-third showed strong cross-reactivity with the toxin, while another one-third manifested no detectable affinity to it. Our studies concentrate on the combining site structure of three of these antibodies: TE32 and TE33, which are both cross-reactive with the toxin, and TE34, which does not bind the native protein. By comparing 1D difference spectra, calculated from the spectrum of the Fab, and that of the Fab complexed with CTP3, we previously concluded that the combining site structure of TE34 is very different from those of the two cross-reactive antibodies (Anglister et al., 1988). NOE experiments revealed strong interactions between a hydrophobic pocket formed from single tryptophan and tyrosine residues within the TE32 and TE33 antibodies and the imidazole group of the peptide histidine-8. Such interactions were not observed in the TE34/CTP3 complex. The binding constants of CTP3 for the TE32, TE33, and TE34 antibodies were found to be 0.5×10^6, 1.2×10^6, and 2.5×10^6 M^{-1}, respectively.

In more recent studies (Anglister et al., 1989; Levy et al., 1989), we used two-dimensional difference spectra, calculated from the NOESY spectrum of the peptide-saturated Fab and that of the Fab with a 4-fold excess of the peptide. This type of difference spectrum pinpoints TRNOE cross-peaks due to intermolecular interactions between the antibody and the peptide (thus termed "TRNOE difference spectrum"). In the two antibodies that are cross-reactive with the toxin, we found that two tryptophan residues, two tyrosine residues, and one

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[1] Abbreviations: Ac, acetyl; COSY, 2D *J*-correlated spectroscopy; CTP3, cholera toxin peptide 3; CTP3(*n*–*m*), truncated version of CTP3 comprised of residues *n*–*m*; Fab, antibody fragment containing the antigen combining site; NCA, *N*-acetyl-CTP3-amide; NMR, nuclear magnetic resonance; NOE, nuclear Overhauser effect; NOESY, 2D NOE spectroscopy; TRNOE, transferred NOE; 2D, two dimensional; 2D TRNOE difference spectrum, calculated 2D difference between the measured NOESY spectrum of the protein saturated with the ligand and the NOESY spectrum of the protein measured in the presence of a large excess of ligand.

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histidine residue interact with the peptide. In TE33, an additional phenylalanine residue is also involved. The epitope recognized by the antibody resides in the middle of the CTP3 sequence, and the residues interacting directly with the aromatic residues of the two antibodies include valine-3, proline-4, glycine-5, glutamine-7, histidine-8, and aspartate-10. Computerized molecular modeling was used to predict the three-dimensional structure of the Fv for TE32 and TE33. These models permitted specific assignment of those antibody aromatic residues interacting with the peptide.

For successful application of 2D TRNOE difference spectroscopy, it is essential that the exchange between bound and free ligand is fast relative to the spin–lattice relaxation time and the mixing time used in the NOESY experiment. While the peptide off-rate was favorable in complexes with the two antibodies cross-reactive with the toxin, it was too slow for TE34. Here we show how this problem was addressed, and how the interactions between the antibody aromatic amino acids and residues of the peptides were elucidated. The specific antibody–antigen interactions in the peptide complexes of the three antibodies are then compared.

Materials and Methods

The sequence of the CTP3 peptide is Val-Glu-Val-Pro-Gly-Ser-Gln-His-Ile-Asp-Ser-Gln-Lys-Lys-Ala. This peptide was synthesized on a (phenylacetamido)methyl (PAM) resin (Applied Biosystems) with a 430A peptide synthesizer (Applied Biosystems). The NCA peptide was synthesized on *p*-methylbenzhydryl resin (Applied Biosystems) with 1-hydroxybenzotriazole esters and *N*-methylpyrrolidone as a solvent. The N-terminal of the peptide was acetylated, and the peptide was cleaved and deprotected by cold hydrofluoric acid (0 °C for 1 h) in the presence of anisole and thioanisole as scavengers. The peptide was then purified by gel filtration over a G-25 column equilibrated with 0.5% acetic acid. Fab labeling and preparation were as described previously (Anglister et al., 1988). Perdeuterated aspartic acid, alanine, tryptophan, and phenylalanine and L-(hydroxyphenyl-2,6,d_2)alanine-2-d_1 were purchased from MSD Isotopes, Montreal, Canada. Deuterated isoleucine was obtained from CIL. The protection of deuterated amino acids for peptide synthesis was carried out by Oz Chemicals, Jerusalem, Israel.

Difference Spectra Calculations. The procedure for NOESY measurement was described previously (Anglister et al., 1989). The 2D TRNOE difference spectrum was obtained by subtracting the NOESY spectrum of the Fab saturated with the peptide from that of the Fab in the presence of a large excess of the peptide [a 10-fold excess for NCA and a 2–3-fold excess for CTP3 and *N*-acetyl-CTP3(9–15)]. The phases of the initial measurements (first t_1 values) of both 2D experiments were matched to obtain a 1D difference with minimal distortion to resonances and base line. This was accomplished first by visual comparison of the two spectra and then by fine numerical phase correction of one to match the other. The phases obtained were applied to the preliminary 2D Fourier transformation. From the NOESY spectrum of the Fab with excess peptide, we selected that row crossing the resonance of the peptide alanine-15 methyl protons on the diagonal. The same row was selected from the NOESY spectrum of the peptide-saturated Fab, and the two were subtracted. The phase of the resulting 1D difference spectrum was numerically corrected to obtain a pure Lorenzian for the resonance of the alanine-15 methyl protons. This phase correction was used to reprocess the two NOESY spectra, and the difference between these rows was reexamined to verify that the resonance of alanine-15 retained a pure Lorenzian shape. This procedure ensured the matching of phases between the two 2D spectra in the region containing cross-peaks between nonaromatic protons. The rows and columns crossing the two resonances of the histidine imidazole protons on the diagonal required additional slight phase correction. Further base-line correction of individual rows was carried out where necessary by fitting a base line with a fourth-order polynomial. The typical Fab concentration used was 3 mM, and measurements involving NCA were carried out at 37 °C.

Measurements of Binding Constants by Fluorescence Quenching. The Fab fluorescence was measured at room temperature on a Shimadzu RF-540 spectrofluorometer. The excitation wavelength was 282 nm with a slit bandwidth of 2 nm, and fluorescence was detected at 362 nm with a slit bandwidth of 20 nm. The typical Fab concentration used was 10^{-7} M. A 20-fold excess of CTP3–DNP (dinitrobenzene linked to the peptide's N-terminal) was added to the Fab to ensure its saturation with the ligand and to obtain the maximal quenching value. Bound CTP3–DNP quenches the fluorescence of antibody tryptophan residues by an energy-transfer mechanism. The Fab saturated with CTP3–DNP was then titrated with the peptide of interest, and the competition between the two ligands was followed by detection of the increased Fab fluorescence. The occupancy of the Fab with CTP3–DNP is expressed by the ratio between the quenching at each titration point and the maximal quenching. The occupancy with the truncated peptide was obtained by subtracting the concentration of the Fab occupied with CTP3–DNP from the total Fab concentration. These values were used to calculate the ratio between the binding constants of the two ligands. The binding constant of TE34 for DNP–CTP3, found previously to be 3×10^6 M^{-1} (Anglister et al., 1988), was used to calculate the binding constants of TE34 for the truncated and modified CTP3 analogues.

Results

Interaction of TE34 with CTP3. Figure 1A shows the difference between the NOESY spectrum of TE34 Fab in the presence of a 2.3-fold excess of CTP3 and that of the peptide-saturated Fab at 47 °C. Tryptophan and phenylalanine residues of the antibody are perdeuterated, while tyrosine residues are deuterated at the C_{δ_1} and C_{δ_2} positions. This TRNOE difference spectrum reveals two very strong cross-peaks at 7.03, 5.87 ppm and 7.90, 7.74 ppm. The intensities of these two cross-peaks are far stronger than those of all others, and they are not affected by further deuteration of antibody tyrosine residues. The resonances at 7.03 and 7.90 ppm correspond respectively to the histidine-8 C_{δ_2} and C_{ϵ_1} protons of the peptide. On the basis of these observations, the two cross-peaks are attributed to an exchange between bound and free peptide. The NOESY spectra were measured with Fab solutions containing 0.15 M sodium chloride; the presence of salt increased the peptide off-rate by a factor of 2.5 relative to that in sodium chloride free deuterium oxide buffered with phosphate. From the ratio of the height of the cross-peak to the height of the bound peptide resonance for a 0.2-s mixing time, the peptide off-rate was estimated to be less than 2.5 s^{-1}. Higher salt concentrations further increased the peptide off-rate; varying the pH from 4 to 9 however did not significantly alter the off-rate.

In addition to the exchange cross-peaks of the histidine imidazole protons, Figure 1A shows much weaker TRNOE cross-peaks due to interactions between tyrosine residues of the antibody and protons of the peptide. On the basis of the COSY spectrum of CTP3, the cross-peaks at 6.58, 1.36 ppm and 6.58, 1.74 ppm are respectively assigned to interactions

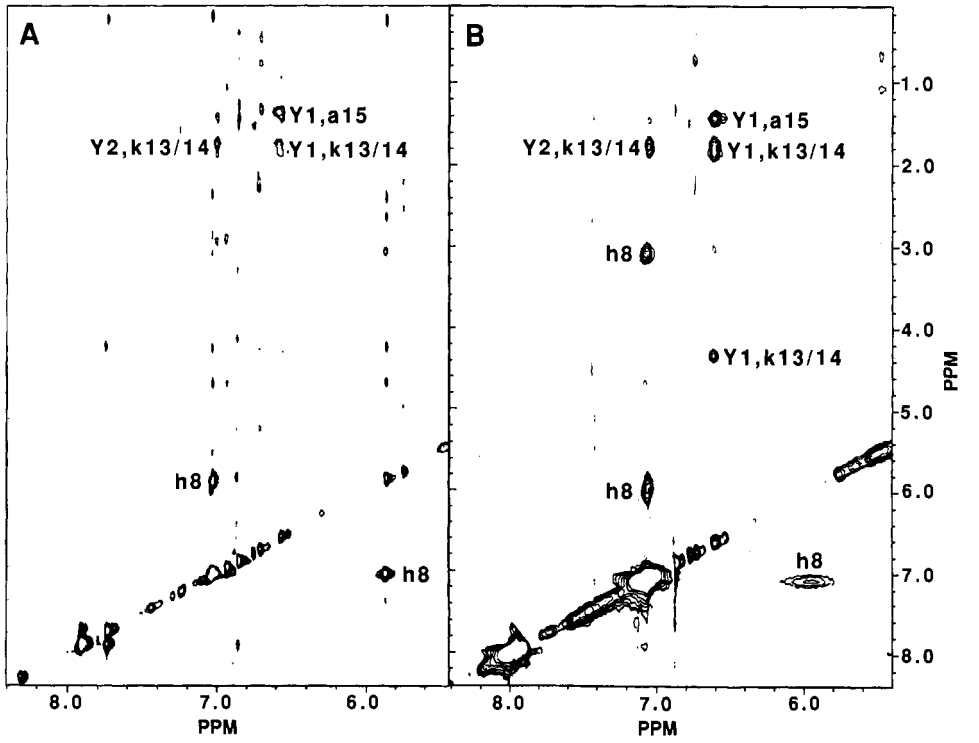

FIGURE 1: Section of a 2D TRNOE difference spectrum, showing interactions between tyrosine residues of the TE34 antibody and protons of the peptide, as well as exchange cross-peaks due to the imidazole protons of the peptide histidine (h8). Tryptophan and phenylalanine residues of the antibody are perdeuterated, while tyrosine residues are deuterated at the phenyl 2,6-positions. (A) Interactions of the antibody with CTP3. (B) Interactions of the antibody with the NCA peptide. Assigned antibody residues are marked by upper-case letters and arbitrary numbers, while peptide residues are marked by lower-case letters and their location in the sequence.

of the C_{ϵ_1} and C_{ϵ_2} protons of a tyrosine residue of the antibody with the methyl protons of the peptide alanine-15 and with the β (and possibly δ) protons of one of the peptide lysine residues (lysine-13 and/or lysine-14). Another cross-peak at 7.00, 1.74 ppm is assigned to the interaction of a second tyrosine residue with the β (and possibly δ) protons of peptide lysine.

Interactions of TE34 with NCA. The slow off-rate of CTP3 results in weak TRNOE cross-peaks. Therefore, the difference spectra calculated for the TE34/CTP3 complex exhibit poor signal to noise ratios and are dominated by the presence of residual signals arising from imperfections in the subtraction process. The elevated temperature at which these spectra were measured in an attempt to increase the intensity of TRNOE cross-peaks repeatedly caused massive precipitation of the antibody. The effect of salt addition, although not sufficient to elicit a strong TRNOE, indicates that electrostatic interactions between the antibody and the peptide are important to binding. Indeed, neutralization of the carboxy terminus by conversion into an amide decreases the binding constant of the peptide by 2 orders of magnitude. This binding constant was measured by following the change in antibody fluorescence on competition between NCA and CTP3–DNP. The modification of the C-terminal carboxyl involves only the substitution of O^- with NH_2 and should cause only minimal steric hindrance.

Figure 2 shows the 2D TRNOE difference spectrum calculated for nonlabeled TE34 in the presence of a 10-fold excess of NCA at 37 °C. Such a large excess of the NCA peptide was required to obtain sharp resonances for the free peptide protons, and this difference spectrum displays a good signal to noise ratio. The cross-peak at 7.07, 5.95 ppm, which is similar to that seen in Figure 1A, is assigned to $C_{\delta_2}H$ of histidine-8 and is due to exchange between bound and free peptide. This cross-peak remains even after perdeuteration

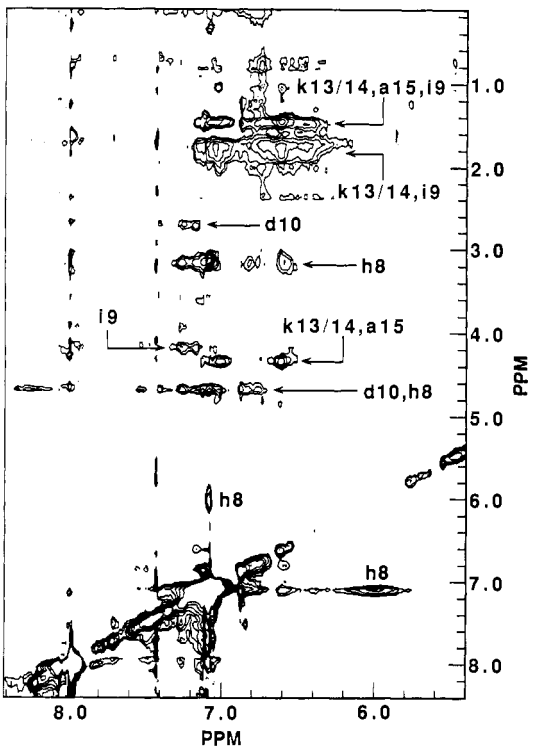

FIGURE 2: 2D TRNOE difference spectrum showing interactions of aromatic protons of the antibody (TE34) with the NCA peptide. The assignment of the NCA protons is based on the COSY spectrum of the Fab in the presence of a 10-fold excess of NCA. In the case of ambiguity, all possible assignments are listed. Assigned peptide residues are marked by lower-case letters and their location in the sequence.

of tyrosine, phenylalanine, and tryptophan residues of the antibody. Furthermore, the difference spectrum calculated from corresponding measurements carried out at 27 °C ex-

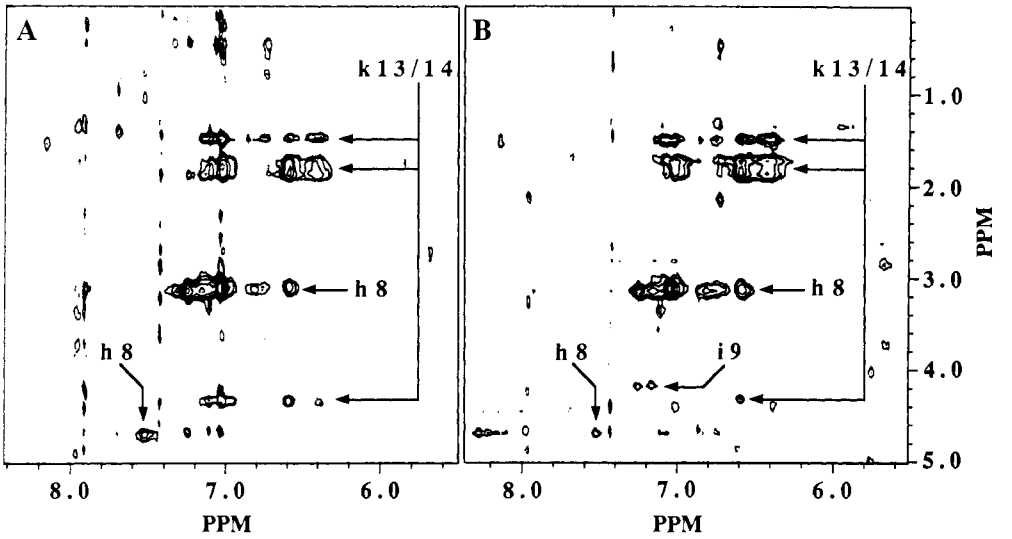

FIGURE 3: 2D TRNOE difference spectrum showing interactions of aromatic protons of the antibody with specifically deuterated NCA. (A) Isoleucine-9, aspartate-10, and alanine-15 are perdeuterated. (B) Isoleucine is unlabeled, and aspartate-10 and alanine-15 are perdeuterated. Comparison of these difference spectra with the spectrum in Figure 2 is used to complete the assignment of the cross-peaks to specific peptide protons. Assigned peptide residues are marked by lower-case letters and their location in the sequence.

hibited a line width of 50 Hz for the resonance at 5.95 ppm, 3 times narrower than that observed at 37 °C (147 Hz). We attribute the very pronounced narrowing of this resonance at lower temperature to the slower off-rate of the peptide. This narrowing, as opposed to the observed broadening of the protein resonances, justifies the assignment to the bound peptide histidine. The marked difference between the line widths of the resonance at ~5.90 ppm when compared between the TE34/CTP3 and TE34/NCA complexes, 11.5 Hz relative to 147 Hz, further verifies that this cross-peak is due to chemical exchange. Assuming that the major contribution to the line width of the bound NCA protons is from lifetime broadening, an estimate of 460 s^{-1} is obtained for the off-rate of the NCA peptide at 37 °C. The resonance of the $C_{\delta_2}H$ of the peptide histidine-8 undergoes a large upfield shift (1.15 ppm) on binding, indicating close proximity to aromatic residues of the antibody. However, the chemical shift difference of this resonance for bound CTP3 relative to bound NCA is extremely small, similar to the difference observed between the resonance of the same proton in the free peptides. These observations indicate that the chemical environments of the $C_{\delta_2}H$ of the peptide histidine are very similar in both complexes.

Some of the cross-peaks in Figure 2 are unambiguously assigned to specific peptide protons on the basis of the COSY spectrum of TE34 in the presence of a 10-fold excess of NCA. The COSY spectrum was measured under the same conditions applied in the NOESY measurements, in order to take into account possible averaging of the free and bound peptide resonances due to fast exchange. In this spectrum the contributions of NCA are observed almost exclusively, while those of the Fab vanish due to the line width of the Fab protons and because of the window function applied in processing (squared sine shifted by 11.25°). The cross-peaks along 2.71, 3.15, 4.17, and 7.07 ppm are respectively assigned to the interactions of the peptide protons of aspartate-10 β, histidine-8 β, isoleucine-9 α, and histidine-8 C_{δ_2} with protons of the antibody. Possible assignments for the remaining cross-peaks are indicated in the figure.

In order to unambiguously assign TRNOE cross-peaks to their interacting peptide protons, we measured NOESY spectra and calculated 2D TRNOE difference spectra for the Fab complexed with specifically deuterated peptide. Figure 3A shows the TRNOE difference spectrum for an unlabeled Fab obtained with NCA in which isoleucine-9, aspartate-10, and alanine-15 were perdeuterated. In this figure, cross-peaks due to extensive interactions between the lysine residues of the peptide and aromatic residues of the antibody are emphasized. Figure 3B shows the TRNOE difference spectrum obtained for the unlabeled Fab with NCA in which aspartate-10 and alanine-15 were perdeuterated. Comparison between the three difference spectra in Figures 2 and 3 allows unambiguous assignment of cross-peaks to the peptide protons interacting with the antibody. The strong cross-peak at 6.60, 1.44 ppm, absent in Figure 3, is assigned to alanine-15. Isoleucine-9 does not contribute to any of the cross-peaks between 1.4 and 2.0 ppm; however as confirmed by specific deuteration of the peptide, those at 4.17 ppm are due to interaction of the α proton of isoleucine-9 with the antibody (Figure 3B). The α proton of the peptide aspartate-10 interacts with at least two antibody protons (7.25 and 7.15 ppm). The cross-peak at 7.52, 4.68 ppm is due to interaction of the antibody with the α proton of histidine-8.

Interactions of Tyrosine Residues of the Antibody. In order to assign the TRNOE cross-peaks to those antibody protons interacting with the peptide, we repeated the NOESY experiments with different preparations of specifically deuterated Fab. Figure 1B shows the interactions between antibody tyrosine protons ($C_{\epsilon_1}H$ and $C_{\epsilon_2}H$) and protons of the NCA peptide. Tryptophan and phenylalanine residues of the antibody were perdeuterated and tyrosine residues deuterated at the C_{δ_1} and C_{δ_2} positions. One tyrosine residue (6.60 ppm) interacts with the methyl protons of the peptide alanine-15 (1.43 ppm) and with the α (4.25 ppm), β (1.86 and 1.76 ppm), and possibly δ protons (1.72 ppm) of a peptide lysine. The β (and possibly δ) protons of a peptide lysine also interact with a tyrosine proton asumed to be from another antibody tyrosine (7.05 ppm). The interactions of the tyrosine residues of TE34 with NCA are very similar to those observed with CTP3 (Figure 1A) except for the additional strong interaction of the α proton of lysine in NCA with one of the tyrosine residues of the antibody. Moreover, there are only minor differences in the chemical shifts of the interacting tyrosine protons between the two complexes. Comparison of panels A and B of Figure 1 clearly demonstrates the superior signal to noise ratios obtainable on increasing the ligand off-rate.

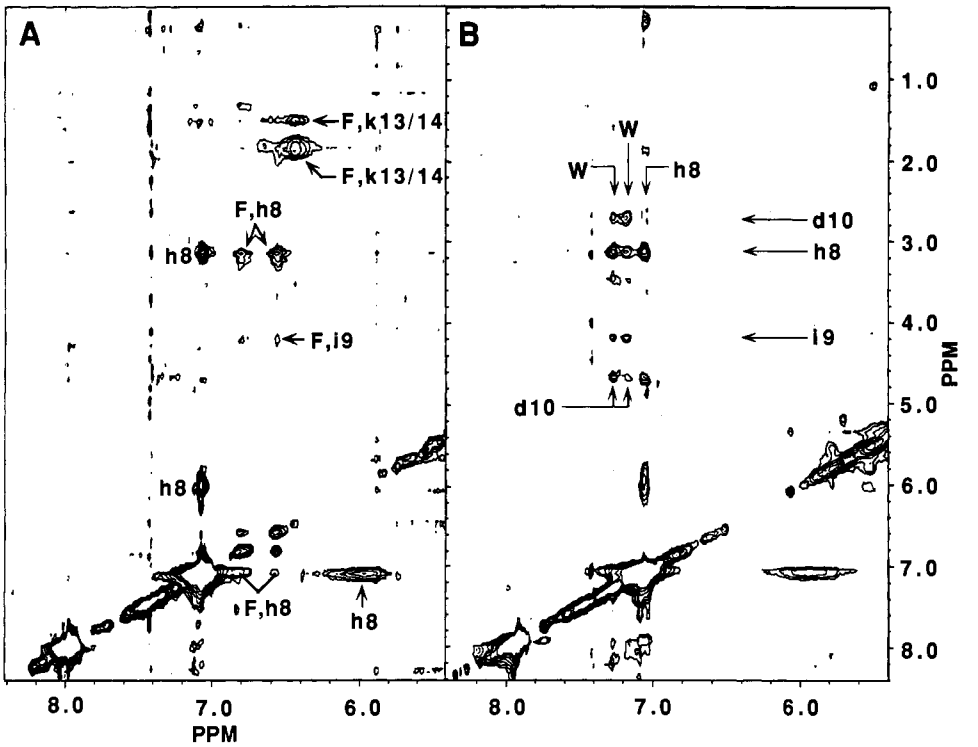

FIGURE 4: TRNOE difference spectrum showing interactions between specifically deuterated antibody and NCA protons. (A) Interactions of antibody phenylalanine residues with the peptide. Antibody tryptophan and tyrosine residues are perdeuterated. (B) Interactions of antibody tryptophan residues with NCA. Antibody phenylalanine and tyrosine residues are perdeuterated. Assigned antibody residues are marked by upper-case letters, while peptide residues are marked by lower-case letters and their location in the sequence.

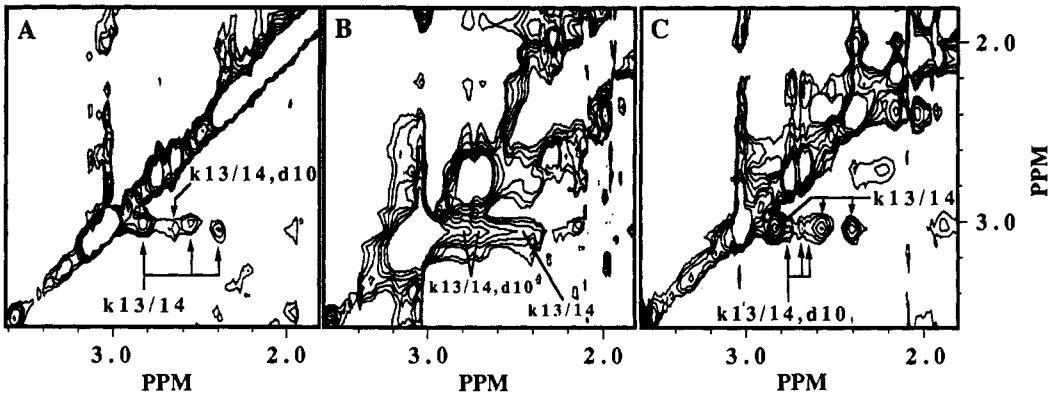

FIGURE 5: Sections of 2D TRNOE difference spectra showing intermolecular interactions of the ϵ protons of the peptide lysine residues with nonaromatic protons of the antibody, their exchange cross-peaks, and cross-peaks due to intramolecular interactions in the bound peptide. (A) Interactions observed in the TE34/CTP3 complex. (B) Interactions observed in the TE34/NCA complex. (C) Interactions observed in the TE34 complex with the truncated peptide AcIDSQKKA. Assigned peptide residues are marked by lower-case letters and their location in the sequence.

Interactions of Phenylalanine Residues of the Antibody. Figure 4A shows TRNOE cross-peaks due to interactions between antibody phenylalanine residues and protons of the NCA peptide. Tyrosine and tryptophan residues of the Fab used in this experiment were perdeuterated. At least one phenylalanine proton (6.45 ppm) interacts very strongly with the β protons of peptide lysine (1.76 and 1.86 ppm) and more weakly with its γ protons (1.48 ppm). The cross-peak at 6.64, 1.76 ppm is due to a weak interaction of another phenylalanine proton with a β proton of a peptide lysine. Two more phenylalanine protons (6.56 and 6.81 ppm) interact with histidine-8 β protons (3.13 ppm) and histidine-8 $C_{\delta_2}H$ (7.07 ppm), as well as very weakly with the α proton of isoleucine-9 (4.17 ppm). This difference spectrum also shows cross-peaks due to an intraresidue interaction between those antibody phenylalanine protons shown to interact with the histidine residue of the peptide. In principle, such cross-peaks should not appear in the TRNOE difference spectrum.

Interactions of Tryptophan Residues of the Antibody. Figure 4B shows interactions between tryptophan protons of TE34 and the protons of NCA. All phenylalanine and tyrosine residues of the antibody were perdeuterated. At least two tryptophan protons (7.18 and 7.27 ppm) interact with aspartate-10 α and β protons (4.68 and 2.71 ppm, respectively), the isoleucine-9 α proton (4.17 ppm), and histidine-8 β protons (3.13 ppm). Additionally, the cross-peaks at 7.07, 3.13 ppm and 7.07, 4.68 ppm are due to intraresidue interactions of the histidine-8 C_{δ_2} proton (7.07 ppm) with histidine-8 β and α protons, respectively, occurring in the bound peptide.

Interactions of the Peptide Lysine Residues with Nonaromatic Protons of the Antibody. Figure 5A presents another section of the 2D TRNOE difference spectrum given in Figure 1A, showing intra- and intermolecular interactions of the lysine residues of the CTP3 peptide. The cross-peaks at 3.01, 2.56

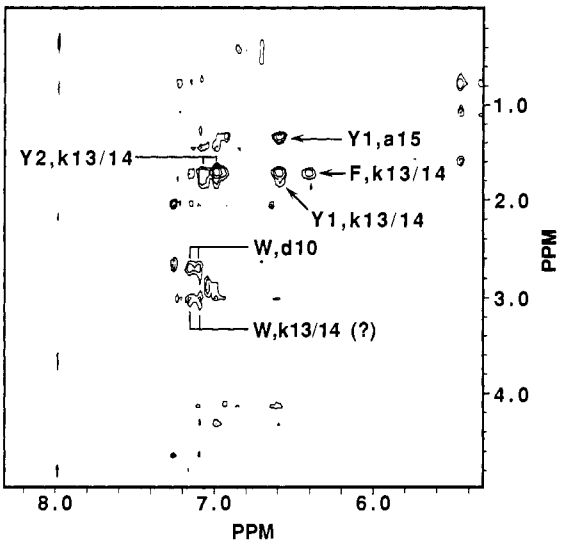

FIGURE 6: 2D TRNOE difference spectrum showing interactions of the aromatic residues of TE34 with the truncated peptide AcID-SQKKA. Assigned antibody residues are marked by upper-case letters and arbitrary numbers, while peptide residues are marked by lower-case letters and their location in the sequence.

ppm, 3.01, 2.83 ppm, and 3.06, 2.40 ppm are assigned to interactions of the C_ϵ protons of the peptide lysine residues with antibody protons or due to exchange between bound and free peptide. On the basis of the COSY spectrum of CTP3, another cross-peak at 3.06, 2.65 ppm is probably due to interaction of the C_ϵ protons of a peptide lysine with the β protons of aspartate-10, respectively. It should be noted that the NOESY spectra of CTP3 and NCA by themselves do not show any cross-peaks due to intrapeptide interactions because of the NOE dependence on the rotational correlation time. The 2D TRNOE difference spectrum showing interactions between nonaromatic protons in the TE34/NCA complex is shown in Figure 5B. The resonances of the ϵ protons of the lysine residues are very much broadened by the fast exchange of the peptide and partially mask cross-peaks due to interactions of lysine-13/14 with aspartate-10 β protons.

Use of Truncated Peptides. The NCA peptide was modified at its C-terminus to yield a peptide off-rate favorable for TRNOE measurements. In order to verify that this modification did not alter the interactions occurring between the antibody and the C-terminal residues of the peptide, we repeated the TRNOE experiment using an analogue of CTP3 with its C-terminus intact but with eight residues truncated from its N-terminus to increase the off-rate. The N-terminus of the truncated peptide was N-acetylated. The truncation shortened the peptide epitope recognized by TE34 by the histidine residue and subsequently resulted in a 10-fold decrease in binding affinity to the antibody. However, the resulting increase in the peptide off-rate was still insufficient for the observation of strong TRNOE cross-peaks. Thus, measurements of the NOESY spectra were carried out in the presence of 0.3 M sodium chloride at 42 °C with 0.2-s mixing time. Figure 5C presents a section of the 2D TRNOE difference spectrum showing interactions between the nonaromatic protons of TE34 in the antibody complex with AcID-SQKKA. Again, an interaction between the ϵ protons of a peptide lysine with the β protons of aspartate-10 (possibly indicating an intramolecular salt bridge between the two residues) is clearly observed. In this spectrum, the multiplicity of the cross-peak due to this interaction further verifies our initial assignment. The other three strong cross-peaks assigned to exchange or to interactions of the peptide lysine with antibody protons are similar in chemical shift to those observed in Figure 5A; however, they manifest stronger magnetization transfer due to the faster ligand off-rate.

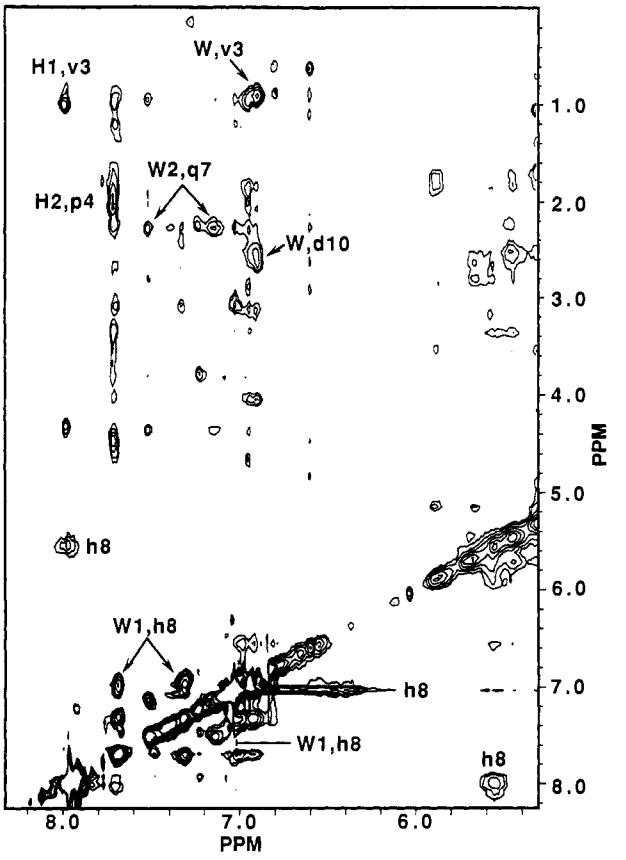

FIGURE 7: 2D TRNOE difference spectrum showing interactions of tryptophan and histidine residues of the TE33 antibody with a truncated version of CTP3, comprised of CTP3 residues 1–10 with the C-terminal carboxyl converted into an amide. Tyrosine and phenylalanine residues of the antibody are perdeuterated. Assigned antibody residues are marked by upper-case letters and arbitrary numbers, while peptide residues are marked by lower-case letters and their location in the sequence.

Figure 6 presents a section of the 2D TRNOE difference spectrum showing the interactions between nonlabeled TE34 and the truncated peptide. The interactions of the antibody with the three carboxy-terminal residues are the same as those observed in the TE34/NCA complex, although the truncated peptide has a C-terminal carboxyl whereas NCA has a C-terminal amide. In the N-terminal part of the peptide, the interaction of the antibody with histidine-8 is absent since this residue is omitted in the truncated peptide. While the expected interaction between the α proton of isoleucine-9 and an antibody tryptophan residue is undetected, that between the aspartate β proton (2.74 ppm) of the truncated peptide and an antibody tryptophan (7.15 and 7.25 ppm) is clearly observed. These observations indicate that the effect of truncation on the antibody–peptide interactions is not significant beyond the residue nearest to the modified end.

In order to further probe the applicability of modified peptides for future studies, we carried out TRNOE measurements with the TE33 antibody using a truncated peptide. The peptide CTP3(1–10)-amide incorporates residues 1–10 of CTP3, with its C-terminal carboxyl converted to an amide to neutralize its charge. The binding constant of this truncated peptide for TE33 is 5 times lower than that of the unmodified peptide. On the basis of the line width of the resonances of the histidine imidazole protons of the bound peptide, its off-rate is approximately 200 s^{-1}. Figure 7 shows the interactions of

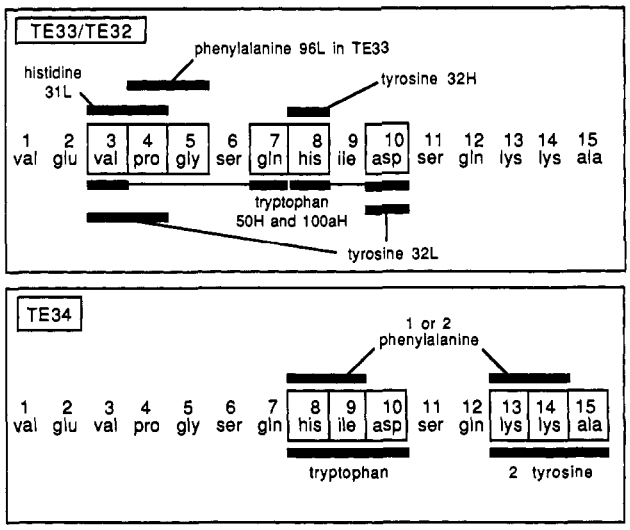

FIGURE 8: Schematic comparison of the antibody–antigen interactions observed in the complexes of the three anti-CTP3 antibodies.

tryptophan and histidine residues in TE33 with protons of the truncated peptide (tyrosine and phenylalanine residues of the antibody were perdeuterated). These interactions are the same as previously observed in the CTP3/TE33 complex, and the overall chemical shift values of the interacting antibody protons in the two complexes are very similar if not identical (Anglister et al., 1989). Even the interaction of aspartate-10 (at the C-terminus of the modified peptide) with a tryptophan proton of the antibody is not affected by removal of the five carboxy-terminal residues. This finding shows that mild truncation of the epitope recognized by TE33 does not interfere significantly with the interactions of the remaining part of the epitope with the antibody.

Discussion

This study offers a strategy that permits the application of TRNOE difference spectroscopy to the study of antibody–antigen interactions when the antigen off-rate is too slow to yield strong TRNOE cross-peaks. Minor modifications of the epitope recognized by the antibody increased the ligand off-rate sufficiently to permit the detection of strong TRNOE cross-peaks due to antibody–peptide interactions and intramolecular interactions in the bound peptide itself. The interactions of the whole peptide with the antibody have been elucidated with two analogues of the peptide, one modified at the N-terminus [*N*-acetyl-CTP3(9–15)] and the other modified at the C-terminus (NCA). The binding constants of TE34 to CTP3, NCA, and *N*-acetyl-CTP3(9–15) were found to be 2.5×10^6, 2×10^4, and 2.5×10^5 M^{-1}, respectively. The specificity of the antibody–peptide interactions is ensured by choosing an antibody with high affinity to the unmodified peptide. Thereafter, this antibody serves as a template for a fixed conformation of the peptide. Since the antibody combining site exhibits limited flexibility and is unable to drastically rearrange itself, slightly truncated or modified versions of its antigen interact with the antibody in a manner similar to that of the corresponding part of the unmodified antigen. However, drastic changes in the length of the epitope recognized by the antibody, or the introduction of steric hindrance, may modify the antibody–antigen interactions.

The following observations support our contention that slightly modified or truncated peptides can be used to elucidate peptide–antibody interactions when the peptide off-rate is slow: (a) Exactly the same intramolecular interactions within the bound peptide are observed in the complexes of TE34 with CTP3, with truncated CTP3, and with NCA. (b) The three C-terminal residues of both NCA and the truncated peptide share common interactions in association with the antibody, although in NCA the C-terminus was converted into an amide. (c) The only antibody–peptide interactions observed in the TE34/CTP3 complex were observed in the antibody complexes with NCA and with the truncated peptide *N*-acetyl-CTP3-(9–15). (d) The truncation of eight residues from the N-terminus of CTP3, thus removing one residue from the peptide epitope recognized by the antibody, affected only the antibody interaction with the residue nearest to the truncation (isoleucine-9). (e) The residues of the truncated peptide CTP3-(1–10)-amide interact with the TE33 anti-CTP3 antibody in the same way as when they are part of the full-length peptide.

A comparison between the interactions observed in the TE32 and TE33 antibody complexes with CTP3 and the interactions deduced for the TE34/CTP3 complex is presented in Figure 8. The two antibodies cross-reactive with the toxin, TE32 and TE33, recognize an epitope in the middle of the peptide that includes valine-3, proline-4, glycine-5, glutamine-7, histidine-8, and aspartate-10. The anti-CTP3 antibody TE34, which does not bind cholera toxin, recognizes a different epitope that includes histidine-8, isoleucine-9, aspartate-10, lysine-13/14, alanine-15, and the C-terminal carboxyl. Histidine-8 and aspartate-10 are recognized by all three antibodies, probably due to a combination of their central location in the peptide's sequence and their chemical nature. The recognition of the C-terminal carboxyl by TE34 is demonstrated by the 2 orders of magnitude drop in the binding constant when the C-terminal carboxyl was modified into an amide, as in NCA. In contrast, the affinities of TE32 and TE33 for NCA are very similar to their affinities for CTP3 (Hiller and Anglister, unpublished results). The replacement of the CTP3 C-terminal carboxyl by an amide bond when the peptide sequence is part of cholera toxin is probably one of the major reasons for the lack of cross-reactivity between TE34 and the native toxin. A feature common to all three antibodies is the extensive contribution of aromatic residues toward binding of the peptide.

It should be noted that all three antibodies were obtained after immunization with CTP3 coupled through its N-terminus to tetanus toxoid, (*p*-aminophenyl)acetic acid being used as a linker (Anglister et al., 1988). This linking abolished the charge on the peptide's N-terminus, leaving the peptide's C-terminal carboxyl intact and negatively charged at neutral pH. A better simulation of the native protein's antigenic determinant can be obtained by conversion of the peptide's C-terminal carboxyl into an amide. Immunization with such peptides will probably result in stronger cross-reactivity with the native antigen.

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Registry No. CTP3(50–64), 89157-28-8; CTP3(50–64) amide derivative, 124042-26-8; *N*-acetyl-IDSQKKA, 124042-27-9.

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Crystal Structure of Two Covalent Nucleoside Derivatives of Ribonuclease A[†,‡]

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ABSTRACT: Crystal structures of two forms of ribonuclease A with deoxynucleosides covalently bound to respectively His12 and His119 have been solved. One form, T-H12-RNase, has a deoxythymidine bound to N_{ϵ_2} of His12, while the other one, U-H119-RNase, has a deoxyuridine bound to N_{δ_1} of His119. The two crystal forms are nearly isomorphous, with two molecules in the asymmetric unit. However, the modified ribonucleases differ both in their enzymatic activities and in the conformation of the catalytic site and of the deoxynucleoside–histidine moiety. T-H12-RNase is characterized by complete loss of enzymatic activity; in this form the deoxynucleoside completely blocks the catalytic site and forms intramolecular contacts with residues associated with both the B1 and B2 sites. U-H119-RNase retains 1% of the activity of the unmodified enzyme, and in this form His119 adopts a different orientation, corresponding to the alternate conformation reported for this residue; the deoxynucleoside–histidine moiety points out of the active site and does not form any contacts with the rest of the protein, thus allowing partial access to the catalytic site. On the basis of these structures, we propose possible mechanisms for the reactions of bromoacetamido nucleosides with ribonuclease A.

We report here the X-ray structures of two modified forms of bovine pancreatic ribonuclease A (RNase A),[1] with deoxynucleosides covalently bound to respectively His12 and His119. These two histidine residues, together with Lys41, form the catalytic site of RNase A. This study continues the already large body of crystallographic studies of this enzyme and of its complexes with various inhibitors, performed in order to understand the details of the mechanism of reaction catalyzed by RNase A [see review by Wlodawer (1985) and references cited therein; Campbell & Petsko, 1987; Wlodawer et al., 1988].

RNase A reacts rapidly with a variety of bromoacetamido pyrimidine nucleosides to form covalent derivatives in which either His12 or His119 is alkylated (Hummel et al., 1987). 3′-(Bromoacetamido)-3′-deoxythymidine forms four derivatives; the major product is [N^{ϵ_2}-[[[(3′-deoxy-3′-thymidinyl)-amino]carbonyl]methyl]histidine-12]ribonuclease A (T-H12-RNase) and is enzymatically inactive. However, T-H12-RNase can react further with the nucleoside alkylating agent to produce a disubstituted derivative in which both His12 and His119 are alkylated. The production of a disubstituted derivative suggests that the nucleoside moiety in the monosubstituted derivative binds at more than one site with only partial occupancy of the B_1–R_1–p_1 site or that the nucleoside exhibits conformational flexibility which limits residency time in the active site. These two alternatives may be distinguished by examining the high-resolution X-ray crystal structure of T-H12-RNase. Alternate binding sites may exist for the deoxythymidinyl moiety in T-H12-RNase, one of which permits further binding of bromoacetamido nucleoside and subsequent alkylation of His119. Alternatively, the deoxythymidinyl residue may be highly mobile, in which case the structure would appear disordered.

3′-(Bromoacetamido)-3′-deoxyuridine alkylates RNase A only at His119 and produces three major derivatives. The product formed in highest yield is [N^{δ_1}-[[[(3′-deoxy-3′-uridinyl)amino]carbonyl]methyl]histidine-119]ribonuclease

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[1] Abbreviations: RNase A, ribonuclease A; T-H12-RNase, [N^{ϵ_2}-[[[(3′-deoxy-3′-thymidinyl)amino]carbonyl]methyl]histidine-12]ribonuclease A; U-H119-RNase, [N^{δ_1}-[[[(3′-deoxy-3′-uridinyl)amino]-carbonyl]methyl]histidine-119]ribonuclease A.

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