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Interactions of Antibody Aromatic Residues with a Peptide of Cholera Toxin Observed by Two-Dimensional Transferred Nuclear Overhauser Effect Difference Spectroscopy†

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Received October 13, 1988; Revised Manuscript Received December 19, 1988

ABSTRACT: The interactions between a peptide of cholera toxin and the aromatic amino acids of the TE33 antipeptide antibody, cross-reactive with the toxin, have been studied by NOESY difference spectroscopy. The 2D difference between the NOESY spectrum of the Fab with a 4-fold excess of the peptide and that of the peptide-saturated Fab reveals cross-peaks growing with excess of the peptide. These cross-peaks are due to magnetization transfer between the Fab and neighboring bound peptide protons, and a further transfer to the free peptide protons by exchange between bound and free peptide (transferred NOE). Additional cross-peaks appearing in the difference spectrum are due to a combination of intramolecular interactions between bound peptide protons and exchange between bound and free peptide. Assignment of cross-peaks is attained by specific deuteration of antibody aromatic amino acids using also the resonance assignment of the free peptide, deduced from the COSY spectrum of the peptide solution. The antibody combining site is found to be highly aromatic. We have identified one or two histidine, two tyrosine, and two tryptophan residues and one phenylalanine residue of the antibody interacting with valine-3, proline-4, glycine-5, glutamine-7, histidine-8, and aspartate-10 of the peptide. The 2D TRNOE difference spectroscopy can be used to study protein–ligand interactions, given that the ligand off rate is fast relative to the spin–lattice relaxation time of the protein and ligand protons (about 1 s). The resolution obtained in the difference spectra implies that the technique is equally applicable for studying proteins having a molecular weight larger than 50000.

Short synthetic peptides corresponding in sequence to segments of proteins are used to raise antipeptide antibodies cross-reactive with native antigens. Such peptides can be helpful in identifying pathogen-neutralizing epitopes and are potential synthetic vaccines (Arnon, 1986; Steward & Howard, 1987). Jacob and Arnon raised monoclonal antibodies against the synthetic peptide CTP3 (residues 50–64 of the B subunit of cholera toxin) that was previously suggested as a possible epitope for a synthetic vaccine against cholera (Jacob et al., 1983). The obtained antipeptide antibodies differ in their cross-reactivity with cholera toxin, implying that the same

† This work was supported by grants from the U.S.–Israel Binational Science Foundation (85-00299) and by the fund for Basic Research administered by the Israel Academy of Science.
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0006-2960/89/0428-3360$01.50/0

peptide acquires different antigenic structures, with only one or a small number of them capable of eliciting the production of antibodies that bind the pathogen (Anglister et al., 1988). We have shown previously, by one-dimensional nuclear Overhauser effect (1D NOE)[1] experiments, that in two anti-CTP3 antibodies which are cross-reactive with cholera toxin (TE32 and TE33), histidine-8 of the peptide is buried in a hydrophobic pocket formed by a tryptophan and a tyrosine residue. Such a pocket was not observed in a third antipeptide antibody which does not bind cholera toxin (Anglister et al., 1988). In an earlier study, 1D TRNOE experiments with selectively deuterated Fab fragments were used to identify interactions between (dinitrophenyl)hapten and tryptophan residues of a monoclonal anti-DNP spin-label antibody (Anglister et al., 1987).

Two-dimensional NMR is used extensively to study the structure of small proteins and nucleic acids having molecular weights of up to 20 000 (Wuthrich, 1986). Studies of larger macromolecules are hindered by loss of resolution due to the considerable broadening of individual proton resonances, complicated by an increasing number of protons. An additional difficulty arises in studies of protein–ligand interactions, in that it is necessary to discriminate between cross-peaks due to intermolecular interactions and the numerous cross-peaks due to intramolecular interactions. Taking advantage of the exchange between bound and free antigen, we have developed a methodology that pinpoints the interactions between the antibody and the peptide antigen, using 2D TRNOE difference spectroscopy.

Materials and Methods

The sequence of the peptide CTP3 is Val-Glu-Val-Pro-Gly-Ser-Gln-His-Ile-Asp-Ser-Gln-Lys-Lys-Ala. The peptide and specifically deuterated forms were synthesized by using an automated peptide synthesizer (Applied Biosystems). Peptide purification and preparation of Fab fragments of labeled antibody have been described previously (Anglister et al., 1988). Deuterated amino acids were obtained from MSD Isotopes. Protected deuterated amino acids for peptide synthesis were prepared by Oz Chemicals, Jerusalem, Israel.

NMR Measurements. NOESY spectra were measured with a Bruker AM500 spectrometer in the phase-sensitive mode with a mixing time of 100 ms, which was found to be optimal for obtaining maximum intensities for the strong cross-peaks observed. In a preliminary experiment, about half the intensity of the strong cross-peaks was observed using a mixing time of 50 ms, thus excluding the possibility that the strong cross-peaks are due to spin diffusion. The carrier frequency was set on the HDO line, and a spectral width of 6000 Hz was used. The HDO line was presaturated, using minimal power, for 3 s before the first 90° pulse was applied. Spectra were Fourier transformed in both dimensions after application of a squared cosine window function. A considerable reduction in t_1 ridges was achieved by using the method of Otting et al. (1986), already incorporated in the standard 2D Fourier transformation in Bruker's software. Base-line distortions due to t_2 ridges were corrected for by subtracting from the 2D spectrum the value for a column with no signal at the intersection with the diagonal of the 2D spectrum (Klevit, 1985). The spectra were not symmetrized. The 2D spectrum of the Fab saturated with peptide was subtracted from the 2D spectrum of the Fab with peptide in excess, multiplied by a factor accounting for the dilution (due to peptide addition). The two spectra used in the difference calculation were measured consecutively, and the phases of the two were very carefully adjusted to yield minimal distortion in the difference spectrum base line. Only slight base-line correction of a few rows of the 2D difference spectrum was required, after good phasing had been achieved. Fab concentration was between 2 and 3.5 mM in 0.01 M phosphate-buffered D_2O, pD 7.15, and the temperature was 45 °C. Spectra were recorded with 256 values of t_1; for each value, 64 or 80 scans were collected which were preceded by 4 uncollected scans. The COSY spectrum was measured in the magnitude mode, using a spectral width of 4000 Hz and 512 increments of t_1. A sine window shifted by 5.6° was used in both dimensions. The COSY spectrum was not symmetrized, and additional correction of t_1 ridges was carried out by using the subtraction technique of Klevit (1985). A factor of 2 was used between consecutive contour levels for all contour plots.

Results

Methodology. In a previous study, the exchange rate between the CTP3 peptide bound to the TE33 antibody and the free peptide was found to be 30 s^{-1} at 47 °C, while the spin–lattice relaxation time of both peptide and Fab protons was about 1 s (Anglister et al., 1988). Therefore, magnetization transferred between Fab and bound peptide protons is efficiently transferred further to the free peptide by exchange.

The NOESY spectrum of the Fab–peptide complex yields, in principle, cross-peaks due to all dipolar interactions between neighboring protons within the complex. Each cross-peak is designated by two frequency values, corresponding to the resonance frequencies of the interacting nuclei. To discriminate between cross-peaks arising from intramolecular Fab interactions and those due to interactions between Fab and peptide protons, we calculated the difference between the NOESY spectrum of the Fab with a 4-fold excess of the peptide and that of the peptide-saturated Fab. It is expected that all cross-peaks due to intramolecular interactions in the complexed Fab are common to both spectra and will be canceled in the 2D difference spectrum. The cross-peaks appearing in NOESY difference spectrum are then due to one of the following reasons: (a) chemical exchange between bound and free peptide; (b) magnetization transfer between Fab protons and free peptide protons via bound peptide (intermolecular transferred NOE; Balaram et al., 1972; James & Cohn, 1974); (c) intramolecular magnetization transfer in the bound peptide via exchange with the free peptide (intramolecular transferred NOE; Albrand et al., 1979).

It should be noted that cross-peaks due to intermolecular interactions between Fab and bound peptide protons have weaker intensities when the peptide is added in excess and therefore will be only partially canceled out in the difference spectra. The residual will have a sign opposing that of the cross-peaks due to the reasons designated (a–c) above and will appear in the spectrum whenever there is a significant change in chemical shift between bound and free peptide resonances. No NOE was observed in the free peptide solution, since under the conditions of the measurement, the NOE is zero for the correlation time of dipolar interactions between the CTP3 protons. Therefore, intramolecular interactions in the free peptide do not contribute to the cross-peaks observed in the difference spectra.

Assignment of Cross-Peaks to Interacting Peptide Amino Acids. Assignment of the cross-peaks in the NOESY dif-

[1] Abbreviations: NMR, nuclear magnetic resonance; 2D, two dimensional; NOE, nuclear Overhauser effect; NOESY, 2D NOE spectroscopy; TRNOE, transferred NOE; COSY, 2D J-correlated spectroscopy; t_1, evolution time; t_2, acquisition time; F1, Fourier-transformed t_1 dimension; F2, Fourier-transformed t_2 dimension.

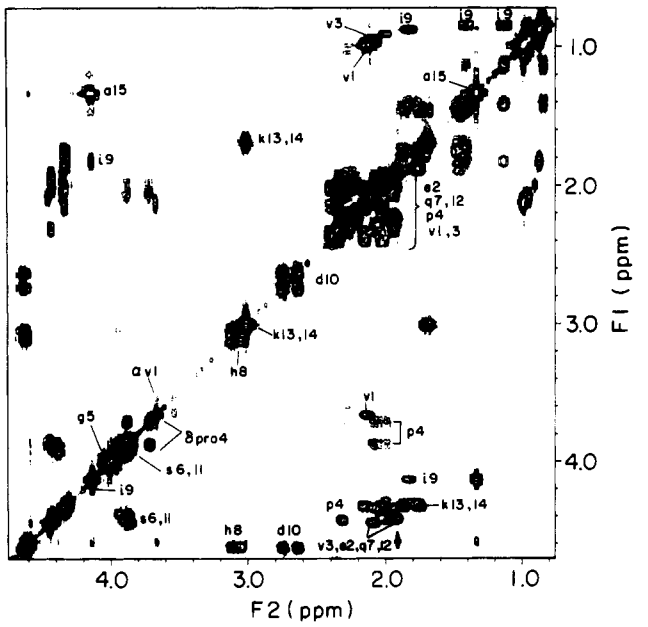

FIGURE 1: COSY spectrum of 15 mM CTP3 in 0.01 M phosphate-buffered deuterium oxide, pD 7.15, measured at 45 °C. A total of 256 points were acquired in the t_1 domain, with 16 free induction decays acquired for each t_1 value. A spectral width of 4000 Hz was used, and the free induction decay was digitized in 2K data points. The spectrum was Fourier transformed in both dimensions, after a squared sine bell shifted by 5.6° was used as a window function. The peaks and cross-peak assignments are marked on the spectrum.

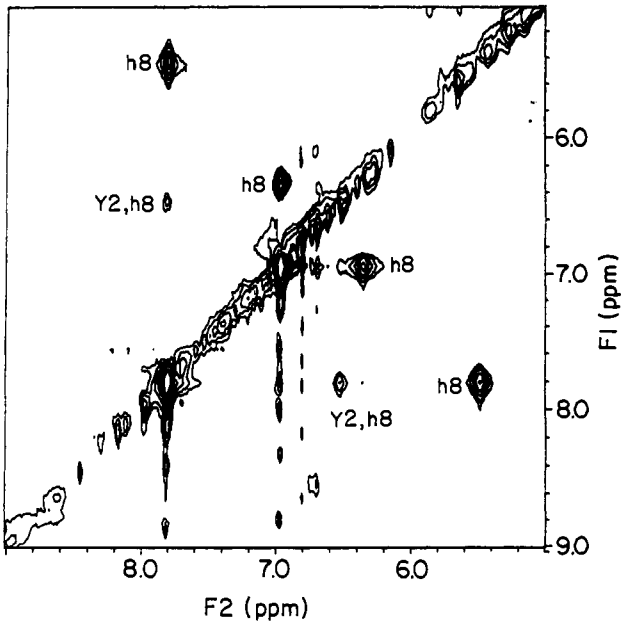

FIGURE 2: Aromatic portion of the 2D difference spectrum between the NOESY spectra of the Fab with 4-fold peptide excess and the peptide-saturated Fab. Only contributions of antibody $C_{\epsilon 1}$ and $C_{\epsilon 2}$ tyrosine protons and histidine aromatic protons of the Fab and the peptide are shown. Assigned antibody residues are marked by capital letters and arbitrary numbers; peptide residues are marked by small letters and their location in the sequence.

ference spectrum to the free peptide protons involved is based on the COSY spectrum of CTP3 (Figure 1). The spin systems of most peptide protons can be identified. The resonances of valine-1 were assigned according to the typical pattern of cross-peaks and the unusual chemical shift of the α-proton due to protonation of the N-terminus (valine-1) amino group. Further verification was provided by measurements of the 1D spectrum of CTP3 in which valine-3 was perdeuterated. The resonances of proline-4, glycine-5, serine-6 and -11, histidine-8, isoleucine-9, aspartate-10, lysine-13 and -14, and alanine-15 were identified on the basis of typical chemical shifts of their protons and the typical COSY cross-peak patterns. The resonances of serine-6 and serine-11 as well as lysine-13 and lysine-14 overlap. By measurements of the 1D spectrum of CTP3 at different pH values and then additional measurement of CTP3 in which glutamic acid-2 was perdeuterated, we were able to assign the resonances of the β- and γ-protons of glutamate-2. The resonances of glutamine-7 and glutamine-12 γ-protons and one of the proline-4 β-protons are clustered together between 2.25 and 2.42 ppm. The assignment of these resonances is based on the COSY spectrum of CTP3 and a COSY spectrum of a truncated peptide (residues 1–10 of CTP3). It should be noted that the dynamic equilibrium will average only proton resonances of bound and free peptide differing by less than 10 Hz, resulting in practically unobservable changes in the chemical shifts of the free peptide protons in the presence or absence of the antibody.

Interactions of Histidine and Tyrosine Residues of the Antibody. In order to simplify the spectra and improve resolution, tryptophan, phenylalanine, valine, and leucine residues of the antibody were perdeuterated, while tyrosine residues were deuterated at phenyl positions 2, 6 ($C_{\delta 1}H$, $C_{\delta 2}H$) leaving 3, 5 ($C_{\epsilon 1}H$, $C_{\epsilon 2}H$) unlabeled.

Figure 2 shows a section of the two-dimensional difference spectrum between the phase-sensitive NOESY spectrum of the Fab in the presence of a 4-fold peptide excess and that of the peptide-saturated Fab. This section reveals cross-peaks due to aromatic–aromatic proton interactions. Two very strong cross-peaks, and their symmetrical counter peaks, are identified at 6.98, 6.39 ppm and 7.82, 5.38 ppm. On the basis of the following observations, we conclude that these cross-peaks are due to the two histidine imidazole protons of the peptide exchanging between the bound and the free state: (a) The two cross-peaks appear in the NOESY spectrum even when antibody tyrosine residues are perdeuterated. (b) The two cross-peaks have the same integrated intensity, far stronger than the intensities of all other cross-peaks. (c) The transfer revealed by the cross-peak at 6.98, 6.39 ppm was previously assigned by 1D NOE measurements (Anglister et al., 1988) to the histidine-8 $C_{\delta 2}H$ of the peptide exchanging between the bound (6.39 ppm) and the free state (6.98 ppm). (d) The cross-peak at 7.82, 5.38 ppm connects a resonance having a chemical shift identical with that of the free peptide histidine $C_{\epsilon 1}H$ (7.82 ppm).

On the other hand, the weaker cross-peak at 6.53, 7.82 ppm disappears when tyrosine is deuterated. It is therefore assigned to transfer via chemical exchange between the Fab tyrosine and $C_{\epsilon 1}H$ of the free peptide histidine. The streaks in the lower half of the spectrum at 7.82, 6.98, and 6.82 ppm are most probably due to t_1 noise, a common problem in unsymmetrized 2D spectra.

Figure 3 presents a section of the 2D difference spectrum showing cross-peaks due to interactions between antibody tyrosine residues and antibody and peptide histidine aromatic protons with nonaromatic protons of CTP3. Assignment of the cross-peaks to the CTP3 protons involved is based on the COSY spectrum of the free peptide. The cross-peaks having a chemical shift of $F2 = 6.34$ and $F2 = 6.53$ ppm disappear when Fab tyrosine residues are perdeuterated, and therefore assigned to interactions between Fab tyrosine and peptide protons. Interactions between the tyrosine protons assigned to the resonance at 6.34 ppm and three residues of CTP3, Val-3, Pro-4, and Asp-10, are observed. A second tyrosine residue yields a weak cross-peak, 3.08, 6.53 ppm, due to in-

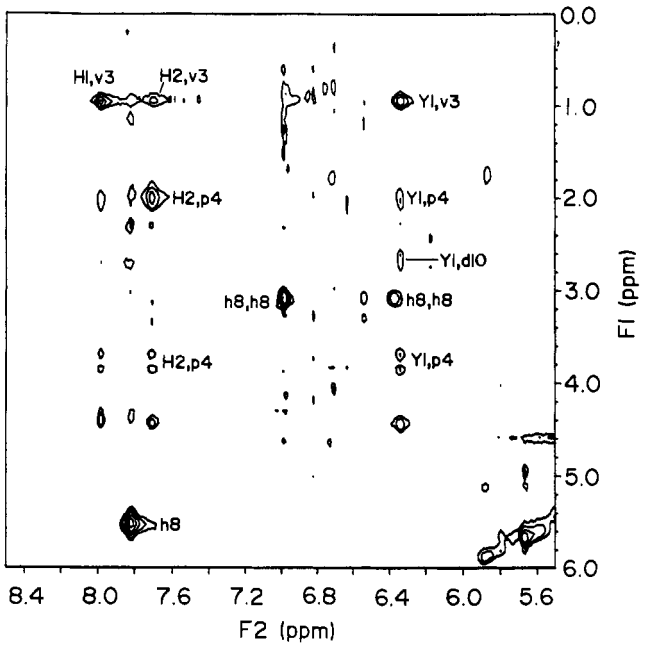

FIGURE 3: Portion of a 2D NOESY difference spectrum showing interactions of aromatic protons of antibody tyrosine and histidine residues of the antibody and the peptide with nonaromatic peptide protons. Assignments are marked as in Figure 2. Antibody phenylalanine and tryptophan residues are perdeuterated, while tyrosine residues are deuterated at $C_{\delta 1,\delta 2}$ positions (phenyl 2, 6 positions).

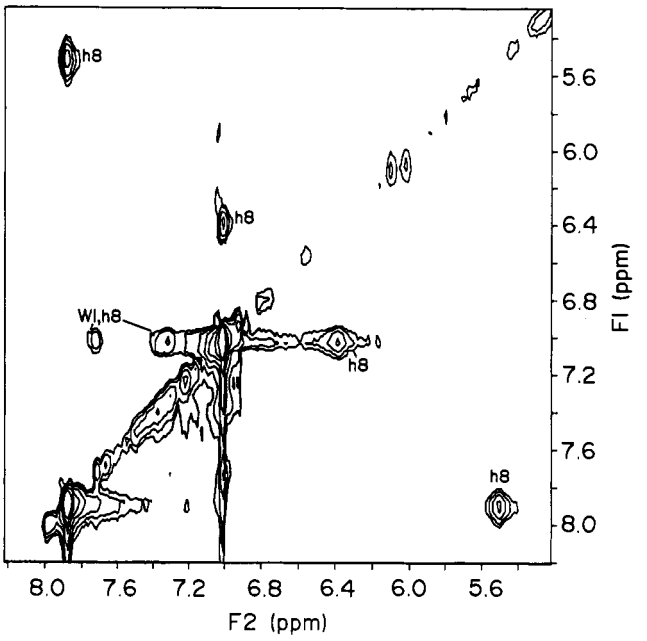

FIGURE 4: Section of the 2D NOESY difference spectrum between the NOESY spectra of the Fab with a 4-fold peptide excess and the peptide-saturated Fab. Antibody tyrosine and phenylalanine residues are perdeuterated. Assignments of cross-peaks are marked as in Figure 2.

teraction with the peptide histidine-8 β-protons. The same tyrosine interacts with $C_{\epsilon 1}H$ of the peptide histidine (Figure 2).

The other cross-peaks in Figure 3 are assigned to histidine protons of either the antibody or the peptide, interacting with nonaromatic peptide protons, since they remain after perdeuteration of all antibody aromatic amino acids except histidine. The cross-peak 3.08, 6.38 ppm (the chemical shift of the free peptide histidine β-protons and that of the bound CTP3 histidine $C_{\delta 2}H$, respectively) is due to intramolecular interaction in the bound peptide transferred by chemical exchange to the histidine β-protons of the free. The cross-peak at 3.08, 6.98 ppm has the chemical shifts of the β-protons and the $C_{\delta 2}H$ of the free peptide histidine, respectively. The cross-peak at 2.04, 7.71 ppm is assigned to the peptide proline-4 γ-protons and an antibody histidine, based on the chemical shift and the observation of weaker cross-peaks between the same antibody proton and the δ-protons of the peptide proline. The cross-peak at 0.96, 7.98 ppm is assigned to valine-3 methyl protons and another histidine proton of the Fab. A weaker cross-peak is observed between the same histidine proton and the proline γ-protons. The 1D difference between the spectrum of the peptide-saturated Fab and that of the Fab in which phenylalanine, tryptophan, and tyrosine residues are perdeuterated reveals four resonances of histidine imidazole protons changing their chemical shift upon peptide binding. The two resonances found at lower field have the same chemical shifts as those of the antibody histidine protons found to interact with the peptide (7.71 and 7.98 ppm). On the basis of the typical chemical shifts values, we assume that the two other resonances at 7.1 and 6.8 ppm are probably of the $C_{\delta 2}H$ (C4H) protons of the same histidine residues, the $C_{\epsilon 1}H$ (C2H) protons of which interact with the peptide.

In order to confirm our assignment of the cross-peaks due to intermolecular interactions in the complex, the NOESY experiments were repeated using a specifically deuterated peptide. Perdeuteration of valine-3 resulted in the disappearance of all cross-peaks previously assigned to valine-3. Perdeuteration of the peptide proline-4 resulted in the disappearance of those cross-peaks assigned as due to interaction between CTP3 proline-4 and antibody protons. The assignment of aspartate-10 β-proton and histidine-8 proton resonances is unambiguous, therefore, we did not find it necessary to label either of them.

Contribution of Antibody Tryptophan Residues to Peptide Binding. The interactions between tryptophan residues of the antibody and amino acids of the peptide were studied by measuring NOESY spectra of Fab in which all phenylalanine and tyrosine residues were perdeuterated. Figure 4 shows a portion of the 2D difference spectrum between the NOESY spectrum of the Fab with peptide excess and that of the Fab saturated with the peptide, revealing two cross-peaks due to interactions between antibody tryptophan and histidine-8 of the peptide. Examination of the NOESY spectrum of the saturated Fab reveals that there are actually three tryptophan protons interacting with the peptide histidine as was observed previously by 1D NOE experiments (Anglister et al., 1988). The cross-peak corresponding to the third tryptophan proton cannot be observed with peptide excess as it is too close to the resonance of the $C_{\delta 2}H$ of the free peptide histidine.

The portion of the 2D difference spectrum showing cross-peaks of aromatic protons of antibody tryptophan and histidine residues of both the antibody and the peptide with nonaromatic protons of the peptide is presented in Figure 5. In addition to cross-peaks previously assigned to histidine residues of either the Fab or the peptide, several new cross-peaks assigned to interactions between antibody tryptophans and peptide amino acids are observed. A group of at least three tryptophan protons yield cross-peaks with peptide protons with a chemical shift of 2.315 ppm. The chemical shift of these tryptophan protons differs from those of the tryptophan protons that interact with the peptide histidine and is therefore assigned to another (second) tryptophan in the antibody combining site. According to our assignment of the peptide resonances, that of the glutamine-7 γ-protons is at 2.31 ppm. Therefore, we assign the observed cross-peaks to interactions between antibody tryptophan and the peptide glutamine-7. This assign-

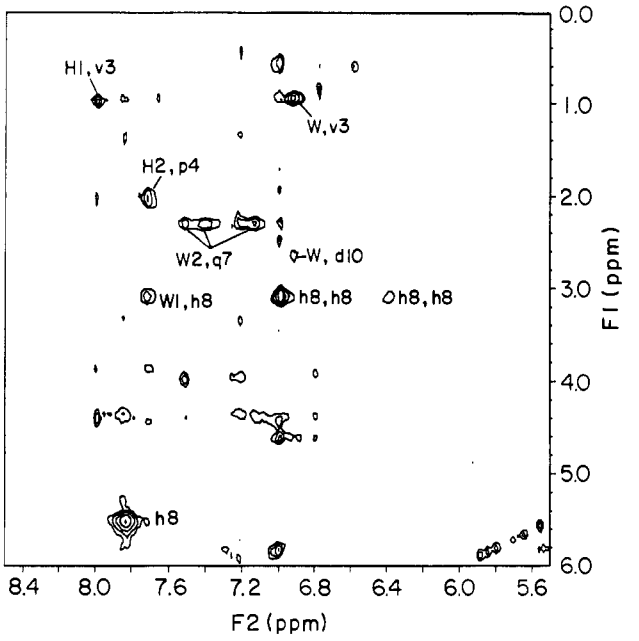

FIGURE 5: Section of the 2D NOESY difference spectrum showing interactions of aromatic protons of antibody tryptophan and histidine residues of the antibody and the peptide with nonaromatic protons of the peptide. Assigned antibody residues are marked with capital letters and arbitrary numbers. Peptide residues are marked by small letters and their location in the sequence. Antibody tyrosine and phenylalanine residues are perdeuterated.

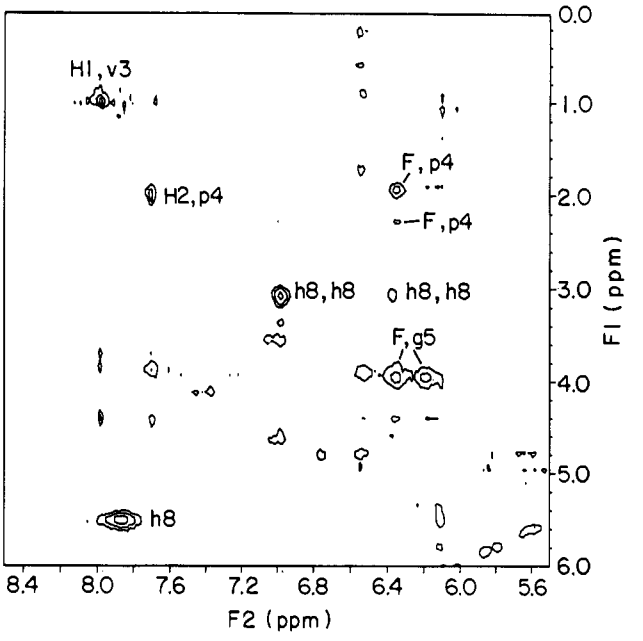

FIGURE 6: Section of the 2D NOESY difference spectrum showing interactions of antibody phenylalanine and antibody and peptide histidine aromatic protons with nonaromatic protons of the peptide. Assignments are marked as in Figure 5. Antibody tyrosine and tryptophan residues are perdeuterated.

ment was verified by observing the disappearance of the three cross peaks in the difference spectrum when using a peptide in which glutamine-7 was specifically deuterated.

The cross peak at 3.08, 7.71 ppm is assigned to the peptide histidine-8 β-proton interacting with a tryptophan proton of the antibody. The same tryptophan proton interacts with histidine-8 $C_{\epsilon 1}H$ and yields a cross-peak at 7.71, 7.01 ppm. A single tryptophan proton (6.92 ppm) yields a strong cross-peak with peptide protons at 0.94 ppm and a weaker cross-peak with peptide protons at 2.63 ppm. The peptide protons are assigned as valine-3 methyl protons and aspartic acid-10 β-protons, respectively, an assignment confirmed by experiments repeated with a peptide in which valine-3 was deuterated. In measurements repeated with partially labeled tryptophan (deuterated at indole positions $\phi 2$, $\eta 2$, and $\epsilon 3$), the two cross-peaks remain strong, indicating that it is tryptophan $C_{\delta 1}H$ that is involved in the interaction. At this stage, we cannot tell whether this proton is of the tryptophan interacting with the peptide histidine-8 or that interacting with the peptide glutamine-7.

Contribution of Antibody's Phenylalanine to Peptide Binding. The contribution of the antibody phenylalanine residues to peptide binding was studied by measuring NOESY spectra of Fab in which tryptophan and tyrosine residues were perdeuterated. The NOESY spectra of the peptide-saturated Fab and Fab with peptide excess were measured, and the 2D difference between the two was calculated. A portion of the 2D difference between the NOESY spectrum of the Fab with 4-fold peptide excess and that of the peptide-saturated Fab (Figure 6) reveals, in addition to cross-peaks previously assigned to histidine residues of either the Fab or the peptide, three new strong cross-peaks: (3.95, 6.35), (3.95, 6.18), and (1.92, 6.35) ppm. These cross-peaks are therefore assigned to interactions between antibody phenylalanine and peptide protons. The chemical shift of 3.95 ppm could be that of the peptide serine β-protons or glycine-5 α-protons. The resonance at 1.92 ppm could be assigned to proline-4 γ-protons, glutamate β-protons, or glutamine β-protons. We have found that deuteration of the peptide glycine-5 resulted in the disappearance of the cross-peaks at 3.95, 6.35 ppm and at 3.95, 6.18 ppm, while deuteration of proline-4 resulted in the disappearance of the cross-peak at 1.92, 6.35 ppm. We have not observed any interactions between antibody phenylalanine and the peptide histidine.

Table I: Summary of Interactions between CTP3 Residues and Aromatic Residues of the TE33 Anti-CTP3 Antibody, Observed by 2D TRNOE Difference Spectroscopy

peptide residue[a]	interacting antibody residue[b]
valine-3	histidine-1,[c] histidine-2,[c] tyrosine-1, tryptophan
proline-4	histidine-2, tyrosine-1, phenylalanine
glycine-5	phenylalanine
glutamine-7	tryptophan-2
histidine-8	tryptophan-1, tyrosine-3
aspartate-10	tyrosine-1, tryptophan

[a] Numbers indicate the position of the residue in the peptide's sequence. [b] Arbitrary numbers of antibody residues. [c] While histidine-1 and histidine-2 are probably two different histidine residues, we cannot rule out the possibility at this stage that they are the two different imidazole protons of the same histidine residue.

Discussion

The antibody combining site appears to be highly aromatic with one or two histidine, two tyrosine, and two tryptophan residues and one phenylalanine residue, interacting with six peptide residues. As summarized in Table I, most of the peptide residues interact with at least two antibody residues. A few more peptide residues may interact with nonaromatic protons of the antibody. Usually, observation of magnetization transfer between two protons implies that they are less than 5 Å apart. Under our experimental conditions, only strong NOE cross-peaks are observed in the difference spectra, and those are due to dipolar interactions between protons probably less than 4 Å apart.

The 2D TRNOE difference spectroscopy can provide us with detailed information about the interactions between peptide antigens and the aromatic amino acids of antipeptide

antibodies. This information, combined with amino acid sequence and crystallographic data on the three-dimensional structure of other antibodies, can be used to obtain a model for the binding site structure and the bound peptide conformation, so that structures of antibody–antigen complexes of antibodies differing in cross-reactivity with the native antigen can be compared. Such studies will contribute to an understanding of the molecular basis for the antigenic cross-reactivity between peptides and proteins.

The difference spectrum between the NOESY spectrum of saturated Fab and that of the Fab in the presence of peptide excess (TRNOE difference spectrum) spectacularly pinpoints that cross-peaks due to either Fab–peptide interactions or intramolecular interactions in the bound peptide. The constancy of the appearance of the cross-peaks assigned to histidine protons in the spectra of the various Fab preparations demonstrates the reliability of the technique. In comparison with 1D NOE experiments, the NOESY difference spectroscopy gives a far more complete and reliable picture of the interactions between the Fab and the peptide and allows us to discriminate between cross-peaks due to intermolecular interactions from those due to intramolecular interactions. Moreover, the specificity of irradiation and the Bloch–Siegert effect on the sharp resonances of the free peptide are severe problems in 1D NOE experiments.

Our approach can be widely applicable for elucidating antibody–antigen interactions in solution and determining the bound peptide conformation as long as the off rate of the antigen is fast relative to the t_1 relaxation time of antigen and antibody protons (1 s). A more stringent requirement is that a considerable exchange between bound and free peptide must occur during the NOESY mixing time (100 ms). Extensive deuteration simplifies the spectrum, increases resolution, permits the assignment of antibody resonances to specific types of amino acids, and, in addition, alleviates spin diffusion problems. Residual t_1 noise is still a problem in certain regions of the 2D spectrum, especially in the lower right quarter (lower field in both frequency axes). The 2D TRNOE difference spectrum can be used even for studying proteins that undergo conformational changes due to ligand binding. The resolution obtained in the difference spectrum implies that the technique is applicable also for larger proteins.

The contribution of the nonaromatic amino acids can be studied by examining other parts of the spectra that are far more complicated to analyze, due to the large number of proton signals in those regions and the very strong peptide proton signals. Only part of the nonaromatic amino acids can be efficiently labeled, and, therefore, assignment of cross-peaks to a specific antibody amino acid type by deuteration may not be possible.

Acknowledgments

We are greatly indebted to Prof. Ruth Arnon and Dr. Chaim Jacob for the gift of the TE33 hybridoma.

Registry No. CTP3, 89157-28-8.

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