Chapter 3

Epitope Mapping of Antibody–Antigen Complexes by Nuclear Magnetic Resonance Spectroscopy

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Summary

Nuclear magnetic resonance (NMR) is a very powerful tool for determining the boundaries of peptide epitopes recognized by antibodies. NMR can be used to study antibodies in complexes that exhibit a wide range of binding affinities from very weak and transient to very tight. Choice of the specific method depends upon the dissociation constant, especially the ligand off-rate.

Epitope mapping by NMR is based on the difference in mobility between the amino acid residues of a peptide antigen that interact tightly with the antibody and residues outside the epitope that do not interact with the antibody. The interacting peptide residues become considerably immobilized upon binding. Their mobility will resemble that of the antibody's residues. Several NMR methods were developed based on these characteristics. In this chapter we discuss some of these methods, including dynamic filtering, comparison of ^{1}H-^{15}N HSQC peaks' intensities, transverse relaxation time, measurements of ^{1}H-^{15}N nuclear Overhauser effect (NOE) values, and measurements of $T_{1\rho}$ relaxation time.

Key words: Epitope mapping, NMR, Antibody, V3, gp120, Acetylcholine receptor, α-bungarotoxin, Dynamic filtering, Relaxation times, Peptide antigen.

1. Introduction

Nuclear magnetic resonance (NMR) spectroscopy has become a powerful tool in the study of protein–protein interactions and the dynamics of protein–ligand complexes. NMR can be used to study protein complexes exhibiting a wide range of binding affinities from very weak and transient binding to very tight. The particular method that is selected depends upon the dissociation constant and especially on the ligand off-rate. NMR can also be used to determine the structure of antibody–antigen complexes

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and to study antibody–antigen interactions. However, for complete structure determination, the antibody Fv fragment is used, and the analysis is limited by the size of the Fv–antigen complex. The development of *E. coli* systems for expression of Fv fragments, combined with uniform isotope labeling with ^{15}N and ^{13}C, has enabled structure determination of a lyzozyme–antilyzozyme Fv complex *(1)* and a complex of the Fv of a HIV (human immunodeficiency virus)-1-neutralizing antibody with a third variable loop (V3) peptide derived from the HIV-1 envelope protein gp120 *(2)*. It also allowed structure determination of different V3 peptides bound to the Fv fragment of a HIV-1-neutralizing antibody *(3–6)*.

Even in the absence of an Fv fragment, NMR can still be used to study the interactions of the larger Fab antibody fragment with peptide antigens or haptens. However, structure determination of such Fab–antigens complexes is beyond current capabilities of NMR spectroscopy. Transferred nuclear Overhauser effect (NOE) was used to study the structure of a cholera-toxin peptide bound to three different antibodies *(7, 8)*. The structure of a V3 peptide bound to the Fab fragment of 0.5β, an HIV-1-neutralizing antibody, was studied using specific deuteration and NOE spectrometry (NOESY) difference spectroscopy *(9–11)*.

NMR is a very powerful tool for determining the boundaries of epitopes recognized by antibodies, and, for this purpose, a Fab fragment or perhaps even the entire antibody molecule can be used. Epitope mapping by NMR is based on the difference in mobility between those peptide antigen residues that interact tightly with the antibody and the residues that are outside the epitope recognized by the antibody. The peptide residues that interact with the antibody become considerably immobilized upon binding, their mobility being comparable to that of the antibody residues. As a result, their transverse relaxation time, T_2, will be shortened considerably in comparison with the free peptide. Peptide residues outside the epitope retain considerable mobility and their T_2 relaxation times are noticeably longer than those of the protons of the peptide interacting with the antibody. Different NMR techniques can differentiate between mobile and rigid segments of a protein or peptide on the basis of differences in T_2 and $T_{1\rho}$ relaxation times. The section below discusses the application of these techniques for epitope mapping.

1.1. Dynamic Filtering

The dynamic filtering approach uses the homonuclear Hartmann Hahn (HOHAHA or TOCSY) and rotating-frame Overhauser enhancement spectroscopy (ROESY) experiments to differentiate between mobile and immobile residues of the antigen. Both are two-dimensional (2D) homonuclear spectra that do not require isotopic labeling of either the antibody or the antigen. The signal-to-noise ratio of the observed cross-peaks in these spectra depends

on the $T_{1\rho}$ relaxation time of the protons that is practically equal to T_2 in most cases. The mixing period in the NMR experiments is tuned to discriminate between ligand protons that interact with the antibody and are thus immobilized and ligand protons that do not interact with the antibody and are flexible. When a long enough mixing period is used in HOHAHA and ROESY experiments, most of the cross-peaks of the protein protons as well as those of the immobilized residues of the epitope are canceled out, so that only cross-peaks of the mobile residues are observed. Thus, the dynamic filtering technique enables us to map accurately the segment of the peptide antigen interacting with the antibody or another protein. Measurement of HOHAHA and ROESY spectra in H_2O takes advantage of the dispersion in the amide protons' chemical shift and enables the sequential assignment of the mobile segments of the peptide antigen. In addition, peptide residues interacting with the antibody undergo significant changes in chemical shift upon binding to the antibody or another protein. Residues that do not participate in binding have identical or nearly identical chemical shifts in the free and antibody-bound form of the peptide antigen. Hence, peptide residues inside the epitope will have broader peaks and exhibit different chemical shift whereas those residues outside the interacting region will give narrow resonance lines and will have chemical shifts identical to those of the free ligand.

The dynamic filtering approach was applied to map the epitopes of several V3 peptides in complex with anti-gp120 HIV-neutralizing antibodies. To map the antigenic determinant recognized by 0.5β antibody, a complex of this antibody with a 24-residue $V3_{IIIB}$ (V3 of the HIV-1 IIIB strain) peptide corresponding to residues N301-G324 of $gp120_{IIIB}$ was studied using NMR *(10)*. A combination of HOHAHA and ROESY experiments of the free peptide as well as of the complex with 0.5β Fab were measured in H_2O. Comparison of the HOHAHA spectrum of the free peptide (**Fig. 1a**) with that of the complex (**Fig. 1b**) allowed us to assign the residues that are outside the epitope recognized by the 0.5β antibody. Superposition of the HOHAHA and ROESY spectra of the complex enabled sequential assignment of the segments that are outside the epitope and retained considerable mobility (**Fig. 2**). In this manner, a 14-residue segment corresponding to residues S306-T319 of $gp120_{IIIB}$ was shown to be immobilized beyond detection in the HOHAHA and ROESY spectra and therefore was determined as being part of the antigenic determinant recognized by the antibody 0.5β (the numbering is according to the HXB2 HIV-1 strain). K305 and I320 were found to retain considerable mobility in the bound peptide while their amide protons underwent significant change in chemical shift upon binding. This observation suggested that these two residues were at the boundaries of the determinant recognized by the antibody

(10, 12). The NMR structure determination of the $V3_{IIIB}$–peptide complex with 0.5β Fv indeed verified that K305 and I320 were at the boundaries of the epitope and had a few interactions with the antibody *(2)*.

In another study, the same procedure was applied for epitope mapping of a different V3 peptide, $V3_{MN}$ (V3 of the HIV-1 MN strain), in complex with 447–52D anti-gp120 HIV-neutralizing antibody *(5)*. In this case, seven residues of the C-terminal region of the V3 peptide corresponding to the segment T319-G325 of $gp120_{MN}$ and two of the N-terminal segment, N302 and R304,

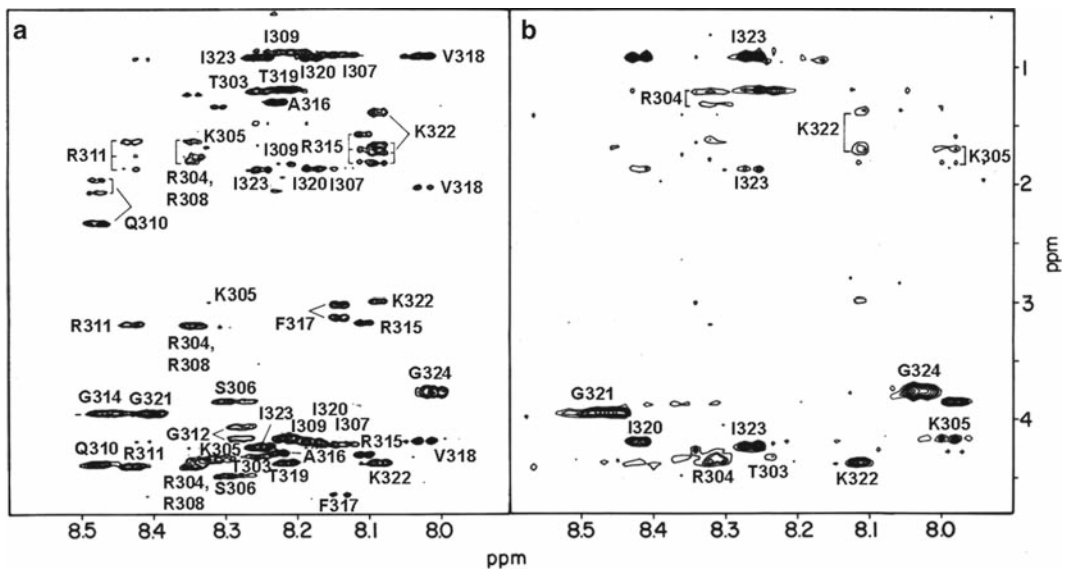

Fig. 1. HOHAHA spectra of free and antibody-bound $V3_{IIIB}$ peptide showing amide proton connectivities with amino acid side-chains. (**a**) Free $V3_{IIIB}$ peptide. (**b**) Complex of $V3_{IIIB}$ peptide with 0.5β Fab. (Reproduced from *(10)*).

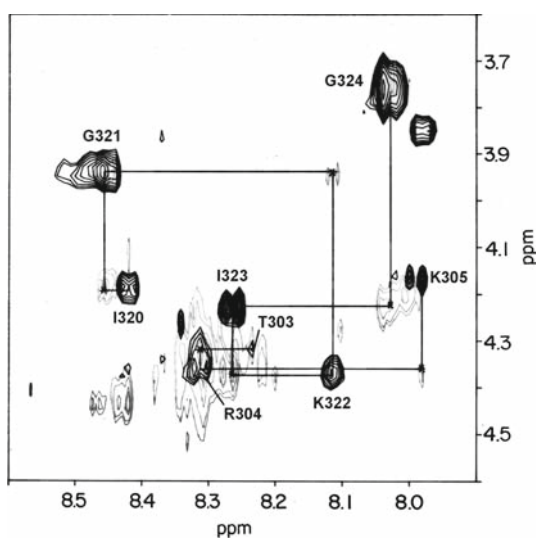

Fig. 2. Superposition of the NH-CαH region of HOHAHA (*black*) and ROESY (*gray*) spectra of $V3_{IIIB}$ peptide in complex with 0.5β Fab showing sequential connectivities. (Reproduced from *(10)*).

were observed. The proton chemical shifts of these residues were identical to those observed for the free peptide, confirming that they do not interact or at most have only very minor interactions with the antibody. The HOHAHA cross-peaks of the residues in the peptide segment corresponding to K305-R315 of $gp120_{MN}$ were undetectable in the spectra, implying strong interactions with the 447–52D antibody. The cross-peaks of residues A316-Y318 of $gp120_{MN}$ were weak, indicating that these three residues are part of the epitope. By this method, the epitope recognized by the 447–52D Fv was mapped to K305-Y318 and was later confirmed by other methods.

The dynamic filtering approach can be used to map segments of large proteins, such as membrane proteins, that are recognized by other proteins. The power of the approach is illustrated by mapping the determinant of the nicotinic-acetylcholine-receptor α-subunit (α-AChR) that is recognized by the snake neurotoxin α-bungarotoxin (α-BTX) *(13)*. In this study we used two overlapping synthetic peptides corresponding to segments $^{\alpha\text{-AChR}}$K-79-D200 and $^{\alpha\text{-AChR}}$R182-T202 that were complexed with α-BTX. Two glutamic acid residues were added on each side of the latter peptide to increase its solubility. To locate the N-terminus of the α-AChR epitope recognized by α-BTX, a set of HOHAHA spectra with different mixing times was acquired for the $^{\alpha\text{-AChR}}$K179-D200/α-BTX complex. The cross-peaks of the mobile part of the $^{\alpha\text{-AChR}}$K179-D200 retained good signal-to-noise ratio in the HOHAHA and ROESY spectra measured with a mixing time of 400 ms, while the contribution of the α-BTX toxin cross-peaks to the spectra was minimal (**Fig. 3**). Using these spectra, five residues, $^{\alpha\text{-AChR}}$E180-W184, as well as the cross-peaks arising from $^{\alpha\text{-AChR}}$K185-$H^{N\varepsilon}$, could easily be assigned. Proton chemical shifts of residues $^{\alpha\text{-AChR}}$E180-G183 were practically identical to those of the free peptide, indicating that these residues were flexible and did not participate in binding. The chemical shifts of $^{\alpha\text{-AChR}}$W184-H^{α} and $^{\alpha\text{-AChR}}$K185-$H^{N\varepsilon}$ differed from those of the free peptide, and their HOHAHA cross-peaks were very weak, indicating that these residues were within the AChR determinant recognized by α-BTX. The cross-peaks of $^{\alpha\text{-AChR}}$H186-Y198 were undetectable in the spectra. We therefore concluded that N-terminal residues $^{\alpha\text{-AChR}}$K179-G183 lie outside the determinant recognized by α-BTX. To locate the C-terminal residues of the α-AChR epitope recognized by α-BTX, a set of HOHAHA spectra with different mixing times was acquired for the $^{\alpha\text{-AChR}}$R182-T202/α-BTX complex. A 250-ms mixing time yielded a spectrum showing a number of peptide cross-peaks with high signal-to-noise ratio while only a limited number of α-BTX cross-peaks were observed. The peptide cross-peaks corresponding to residues $^{\alpha\text{-AChR}}$E180-W184 and $^{\alpha\text{-AChR}}$I201-T202 (and the EE tag) could be assigned. We therefore concluded that the determinant recognized by α-BTX comprised residues $^{\alpha\text{-AChR}}$W184-D200.

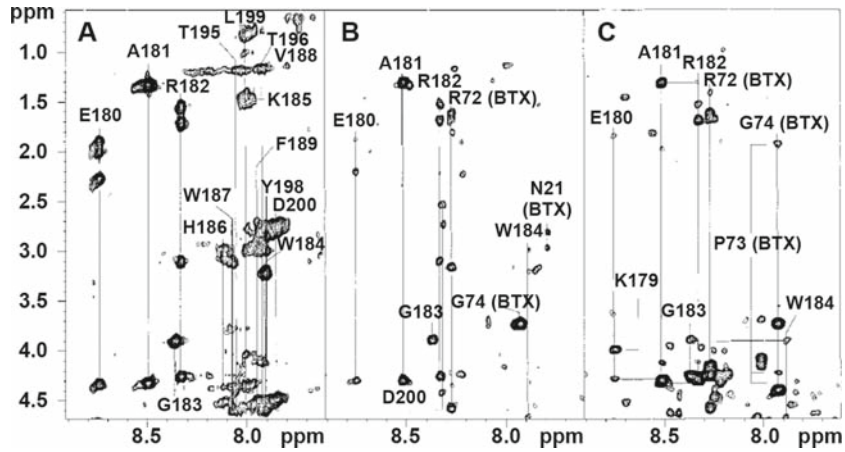

Fig. 3. Dynamic filtering spectra. (**a**) HOHAHA spectrum of free $^{\alpha\text{-AChR}}$K179-D200 peptide acquired with a mixing time of 150 ms. (**b**) HOHAHA spectrum of the α-BTX/$^{\alpha\text{-AChR}}$K179-D200 complex acquired with a mixing time of 400 ms. (**c**) ROESY spectrum of the α-BTX/$^{\alpha\text{-AChR}}$K179-D200 complex acquired with a mixing time of 400 ms. The sequential assignment for the mobile segment $^{\alpha\text{-AChR}}$K179-W184 is presented. (Reproduced from *(13)*).

1.2. Epitope Mapping by Comparison of ^{1}H-^{15}N HSQC Peaks' Intensities

Expression of peptide antigens in *E. coli* enables uniform labeling with ^{15}N and ^{13}C. This labeling allowed us to use several heteronuclear NMR experiments to map the segment of peptide antigens recognized by antibodies and eventually enabled structure determination of peptide antigen bound to the antibody Fv. The simplest experiment is the edited ^{1}H-^{15}N HSQC of uniformly ^{15}N-labeled peptide in its free form and in complex with the antibody Fv or the Fab, followed by comparison of the cross-peaks' intensities. The edited experiments show only the cross-peaks of the labeled heteronuclei and the hydrogen atoms bonded to them. Since the peptide is labeled and the Fv is not, the edited spectra show only the cross-peaks originating from the peptide while cross-peaks of the Fv are canceled out. A prerequisite for this approach is that all ^{1}H-^{15}N cross-peaks are assigned to the corresponding antigen residues. The sequential backbone assignment can be accomplished using conventional isotope-edited 3D experiments such as HNCO, CBCACONH, HNCA, and HNCACB *(14)*. The sequential assignment must be carried out for both the free and Fv-bound peptide. In these experiments the Fv is unlabeled.

The signal-to-noise ratio and the line-width of the cross-peaks in the ^{1}H-^{15}N HSQC spectrum of the ^{15}N-labeled peptide in complex with the Fv depend on the T_2 relaxation times of the peptide's amide protons. Examination of the ^{1}H-^{15}N HSQC spectra of the Fv–peptide complex revealed that residues that did not interact with the Fv exhibit intense and narrow cross-peaks that did not change their chemical shifts in comparison with the free peptide. However, peptide residues that interact with the

antibody Fv exhibit considerably broader and weaker ^{1}H-^{15}N HSQC cross-peaks and also exhibit chemical shift changes in comparison with the cross-peaks of the free peptide.

In our studies on the interactions between $V3_{IIIB}$ peptide and 447–52D antibody we used the above method to determine the epitope recognized by the antibody as shown in **Fig. 4**. This measurement indicates that the core epitope S306-F317 is immobilized and K322 is completely outside the epitope. The C-terminal segment V318-G321, although part of the epitope, is more mobile than the N-terminal half of the $V3_{IIIB}$ peptide, which is part of the core epitope. This is due to much less interactions between the C-terminal half of the $V3_{IIIB}$ peptide and the antibody Fv in comparison with the large number of interactions observed between the N-terminal half of the $V3_{IIIB}$ peptide and the Fv. K305 exhibits reduced intensity because of faster solvent exchange of its amide proton. K305 is the third residue of the peptide. The amide protons of the first two residues T303-R304 are not observed at all due to of fast exchange with the solvent.

1.3. Mapping of the Epitope by Transverse Relaxation Time, T_2 Measurements

Measurements of NMR relaxation times can provide detailed information about the dynamics of proteins and the changes in dynamics upon ligand binding. In recent years, ^{15}N relaxation has been used extensively to describe the dynamics of the protein backbone. NMR relaxation times are influenced by the global tumbling of the molecules as well as by local motions. Transverse relaxation time, T_2, also known as spin–spin relaxation, also depends upon conformational and chemical exchange. Thus,

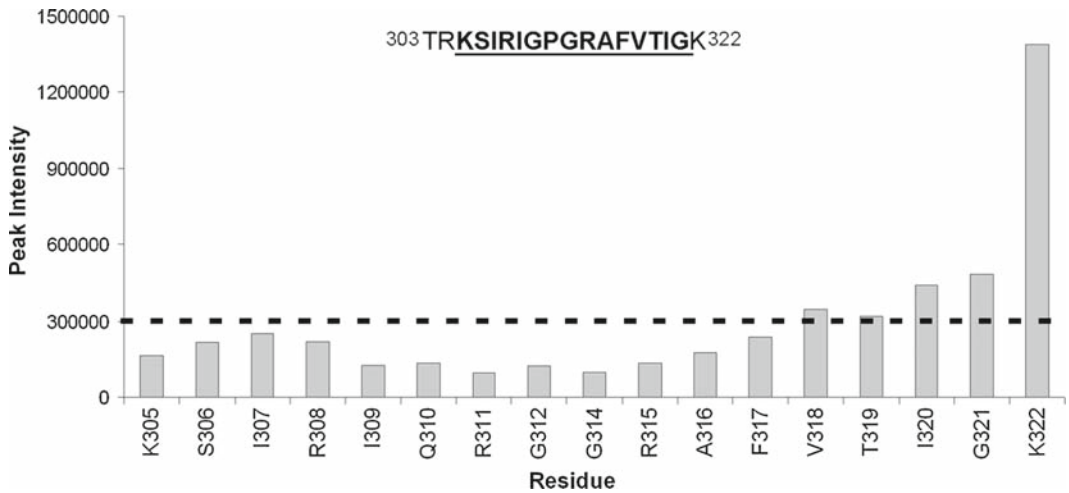

Fig. 4. The variations in the $^{1}H/^{15}N$ cross-peak intensities in the ^{1}H-^{15}N HSQC spectrum recorded with uniformly ^{15}N-labeled $V3_{IIIB}$ peptide bound to unlabeled 447–52D Fv. The intensity is given in arbitrary units. The $V3_{IIIB}$ sequence is shown and the epitope is in *bold* and *underlined*. The first two residues are not observed in the spectrum. The arbitrary 300,000 threshold, used to differentiate between residues interacting with the Fv and those that do not, is drawn as a *horizontal dashed line*. According to this analysis I320 and G321 are at the border of the epitope and K322 is outside.

measurements of T_2 can provide more quantitative insight into the dynamics of proteins in solution and the changes that occur upon ligand binding. We used this technique and determined the T_2 values of all $V3_{IIIB}$ residues. As shown in **Fig. 5**, when applied to the peptide corresponding to residues T303-K322 of $gp120_{IIIB}$ in complex with 447–52D, short ^{15}N T_2 relaxation times were measured for residues S306-T319, indicating backbone immobilization for these residues upon binding the antibody. K305, I320, and G321 exhibit slightly increased T_2 relaxation times, indicating that they are at the "borders" of the epitope. The much longer relaxation time of K322 suggests that this residue is flexible and completely outside the antibody epitope.

1.4. Epitope Mapping by ^1H-^{15}N NOE Measurements

The heteronuclear 2D ^{15}N-{^1H} nuclear Overhauser effect (hetNOE) is the most commonly used NMR experiment to study protein dynamics on fast time scales (picoseconds to nanoseconds). hetNOE allows quantification of thermal fluctuations in a protein on a per residue basis. The ^1H-^{15}N NOE values can range from −3.5 for very flexible segments to 1 for very rigid segments *(15)*. This wide range of the NOE effect allows sensitive discrimination between different residues in a peptide antigen complexed with an Fv or Fab molecule according to their mobility. The NOE values are determined by taking the ratio of the ^{15}N signal intensities recorded in the presence and absence of proton saturation prior to excitation of ^{15}N magnetization *(15)*. At least three critical factors require special attention for reliable NOE measurements: First, water–amide proton exchange can affect NOE values if the water-flip pulse is not applied. Second, ^1H saturation is also important for quantitatively reliable NOE values. Third, complete magnetization recovery during the pulse repetition delay is critical for accurate measurements.

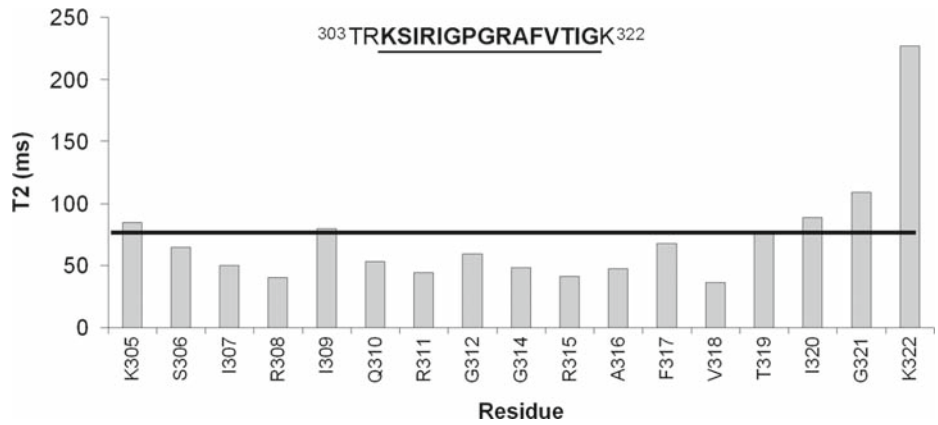

Fig. 5. The variations of the ^{15}N T_2 relaxation times of the $V3_{IIIB}$ peptide bound to 447–52D Fv along the peptide sequence. The 75-ms threshold for T_2, used to differentiate between residues interacting with the Fv and those that do not, is drawn as a *horizontal line*. According to this analysis K305, I320, and G321 are at the border of the epitope and K322 is outside.

The measurements of ^{1}H-^{15}N NOE of a $V3_{IIIB}$ peptide bound to the 447–52D, as shown in **Fig. 6**, revealed that only the core epitope encompassing residues I307-V318 is strongly immobilized. K305-S306 and T319-I320 revealed some mobility as a result of their location at the edge of the epitope. K322 is completely outside the epitope.

1.5. Quantitative Measurements of $T_{1\rho}$ for Epitope Mapping Using Homonuclear Spectra

Dynamic studies usually focus on measurements of the relaxation parameters of ^{15}N and ^{13}C nuclei bonded to ^{1}H using labeled proteins, as described above. Proton relaxation times in unlabeled proteins have not been investigated as thoroughly. This can be attributed to several factors, including the difficulty in measurements and data analysis due to spectral overlap. It is also difficult to interpret the T_2 data and analyze the type and time scale of motions contributing to relaxation because each proton may relax by several different mechanisms such as scalar couplings and dipole–dipole interactions with multiple nearby protons *(16)*. In our laboratory, we developed a simple homonuclear 2D method for measuring proton $T_{1\rho}$ relaxation times based on the HOHAHA experiment. This method can be used for epitope mapping without requiring ^{15}N or ^{13}C labeling. This technique was applied to α-BTX in complex with α-AChR peptide *(17)*. To calculate the $T_{1\rho}$ values, the decay in the intensity of the H_N-H_α cross-peaks as a function of the duration of the spin-lock pulse was fitted to a monoexponential curve with minor deviations. Long relaxation times, 155 ms on average, were measured for the free peptide. After binding, peptide residues outside the binding determinant, namely, $^{\alpha\text{-AChR}}$E181-R182, $^{\alpha\text{-AChR}}$W184, and $^{\alpha\text{-AChR}}$I201-E204 (and the EE tag), exhibited $T_{1\rho}$ values above an

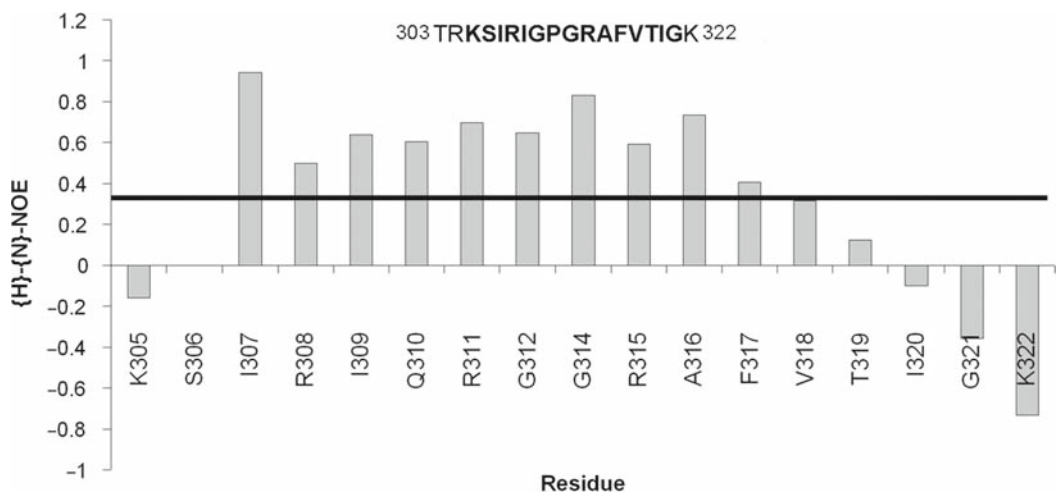

Fig. 6. The variations of the ^{1}H-^{15}N NOE ratios of the $V3_{IIIB}$ peptide bound to 447–52D Fv along the peptide sequence. The 0.35 threshold used to differentiate between the core epitope and the rest of the peptide is drawn as a *horizontal line*.

arbitrary threshold value of 45 ms. Residues within the binding determinant, $^{\alpha\text{-AChR}}$K185-D200, with the exception of $^{\alpha\text{-AChR}}$D195, displayed $T_{1\rho}$ values of <45 ms. In general, the change in the exposed surface upon binding of the AChR peptide is correlated with the change in $T_{1\rho}$ times, as presented in **Fig. 7.**

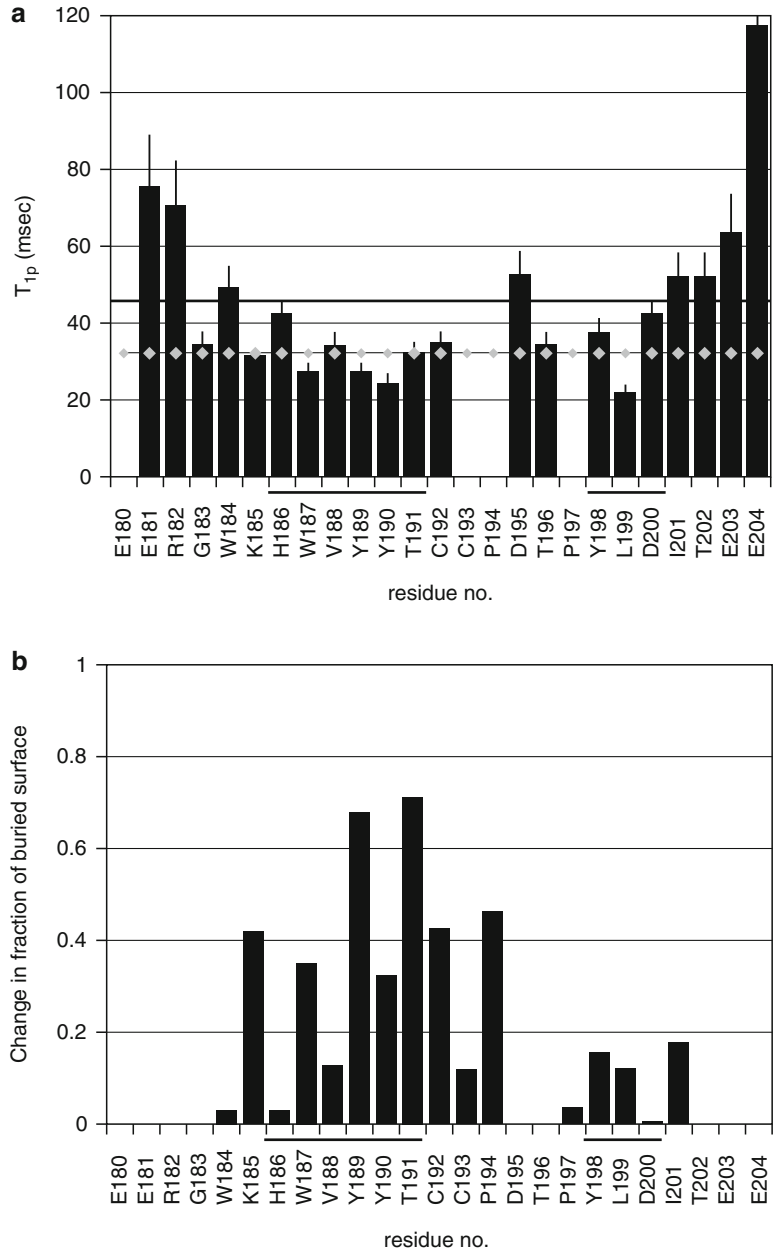

Fig. 7. $T_{1\rho}$ values of Hα protons in the bound AChR peptide and changes in the exposed surface upon binding. (**a**) $T_{1\rho}$ values for each residue in the AChR peptide in complex with the toxin. The 45-ms threshold used to differentiate between the peptide residues interacting with the toxin and those that do not is drawn as a *horizontal line*. (**b**) Fractional decrease in the exposed surface for residues in the AChR peptide. (Reproduced from *(17)*).

2. Materials

2.1. Antibody's Fv Expression and Purification

1. BL21-Gold(DE3)pLysS strain (Stratagene, La Jolla, CA)
2. pET-27b vector (Novagen, Madison, WI)
3. SBP medium
4. Isopropyl-β-D-thiogalactoside (IPTG): 1 M stock solution
5. Ethylene diamine tetraacetic acid (EDTA): 0.5 M stock solution
6. Isotope-enriched Celtone-rich medium (Martek Biosciences, Columbia, MD)
7. RPMI 1640 vitamin cocktail (Gibco-Invitrogen, Paisley, SC)
8. *N*-ethylmaleimide, iodoacetamide, benzamidine, and *N*-tosyl-L-phenylalanine chloromethyl ketone (Sigma-Aldrich, Israel) as protease inhibitors
9. 25 mM Tris-HCl buffer, pH 8.0
10. 50 mM Tris-HCl buffer (pH 8.0), 0.05% NaN_3, 5 mM EDTA
11. 50 mM Tris-HCl buffer (pH 8.0), 0.05% NaN_3, 0.5 M NaCl
12. 25 mM Tris-HCl buffer (pH 7.5), 125 mM NaCl, 0.02% NaN_3
13. 25 mM Tris-HCl
14. Acetic acid
15. Triton X-100
16. Buffer A: 25 mM acetate buffer, pH 4
17. Washing buffer I: 50 mM Tris (pH 7.9), 0.5 mM EDTA, 50 mM NaCl, and 5% glycerol
18. Guanidine hydrochloride (GuHCl): 6 M solution
19. Chromatography columns (Q-Sepharose Fast Flow, Sepharose 4 Fast Flow, SP-Sepharose, HiLoad Superdex 75 26/60), beads for the columns, and ÄKTA chromatography system (Amersham Biosciences, Uppsala, Sweden)
20. Vivaspin concentrator with a 10-kDa cutoff membrane (Vivascience, Westfold, MA)

2.2. Peptide Expression and Purification

1. BL21-Gold(DE3)pLysS strain (Stratagene)
2. Isotope-enriched Celtone-rich medium and RPMI 1640 vitamin cocktail (*see* **Subheading 2.1**, **item 7**)
3. Ampicillin and chloramphenicol
4. Isopropyl-β-D-thiogalactoside (IPTG): 1 M stock solution

5. Protease inhibitor stock solutions: aprotinin (10 mg/mL), leupeptin (10 mg/mL), and PMSF (17.4 mg/mL)
6. Triton X-100
7. 8 M urea, 50 mM glycine, and 450 mM NaCl in water
8. Washing buffer II: 50 mM Tris-HCl (pH 8.0), 0.5 mM EDTA, 0.5 M NaCl, and 5% glycerol
9. NH_4HCO_3: 50 mM solution in water
10. Cyanogen bromide (CNBr)
11. Formic acid: at least 70%
12. Trifluoro acetic acid: at least 70%
13. Dimethyl sulfoxide
14. Vydac polymer (polystyrene–divinylbenzene) reversed-phased semipreparative (25 × 250 mm^2), Vydac (10 × 250 mm^2), and analytical Vydac (4 × 250 mm^2) columns (Amersham Biosciences)
15. pET11d vector (Novagen)
16. Restriction enzymes and *E. coli* DNA polymerase I (New England Biolabs, Beverly, MA)
17. T4 DNA ligase (Gibco Life Technologies, Gaithersburg, MD)
18. Vivaspin concentrator (*see* **Subheading 2.1**, **item 14**)

2.3. Fv–Peptide Complex and NMR Sample Preparation

1. Vivaspin concentrator (*see* **Subheading 2.1**, **item 15**)
2. Shigemi NMR tube (Shigemi, Allison Park, PA)

2.4. Binding Interface Mapping Protocol

1. DMX-500 Bruker Avance NMR spectrometer equipped with triple resonance probe with Z-gradient
2. DRX-800 Bruker Avance NMR spectrometer equipped with triple resonance inverse cryoprobe with Z-gradient
3. Software to process and analyze spectra: NMRView, NMRD-raw, and NMRPipe

3. Methods

3.1. Antibody's Fv Expression and Purification

3.1.1. Expression and Purification of 447–52D V_L

1. Transform BL21-Gold(DE3)pLysS cells with the 447Lb27b plasmid.
2. Inoculate a 1-L flask containing 50 mL of SBP medium with starter culture that was diluted 1:100 and incubated with constant shaking at 37°C.
3. Add IPTG to a final concentration of 1 mM (1 mL of 1 M stock to 1-L culture) when the culture reaches an optical

density of 0.7–0.9 at 595 nm and incubate the culture overnight at 20°C with shaking at 225 rpm.

4. Pellet the bacteria by centrifugation at 3,200×*g* for 30 min at 4°C and collect the culture medium containing the V_L protein. Inhibit endogenous protease activity by adding 2 mL of each: 0.5 M EDTA, 0.1 M *N*-ethylmaleimide, 0.1 M iodoacetamide, 0.1 M benzamidine, and 4 mL of 3 mg/mL of *N*-tosyl-l-phenylalanine chloromethyl ketone to every 50 mL culture medium.
5. Adjust the culture medium solution to pH 8 and centrifuge at 18,500×*g* for 30 min at 4°C; then dilute the supernatant fourfold with HPLC-grade water.
6. Purify the protein on a Q-Sepharose Fast Flow anion exchange column equilibrated with 25 mM Tris-HCl, pH 8. Collect the flow-through, adjust to pH 4 with acetic acid, and centrifuge at 18,500×*g* for 30 min at 4°C.
7. Collect the supernatant and load onto a Sepharose 4 Fast Flow column connected in series with a SP-Sepharose column. Equilibrate both columns with buffer A. Wash the SP-Sepharose column with 5 bed volumes of buffer A containing 100 mM NaCl.
8. Elute the protein with 15 column volumes of a 0.1–0.7 M NaCl linear gradient in buffer A. Adjust the pH of the eluted light-chain protein to pH 8 by adding Tris-HCl solution at pH 9 to a final concentration of 73 mM of the buffer to prevent adverse effects of the acidic pH on the protein.

3.1.2. Expression and Purification of 447–52D V_H

1. Transform BL21-Gold(DE3)pLysS cells with the 447Hb27b plasmid.
2. Inoculate a 1-L flask containing 50 mL of SBP medium with starter culture that was diluted 1:100 and incubate with constant shaking at 37°C.
3. Add IPTG to a final concentration of 1 mM when the culture reaches an OD_{595} of 0.7–0.9 and incubate the culture for 4 h at 25°C with shaking at 225 rpm.
4. Pellet the bacteria by centrifugation at 3,200×*g* for 30 min and collect the V_H protein, which sequesters in inclusion bodies, by suspending the cell harvest in 5 mL washing buffer I (50 mM Tris (pH 7.9), 0.5 mM EDTA, 50 mM NaCl, and 5% glycerol).
5. Sonicate for 5 min on ice and remove membrane components by adding Triton X-100 to a final concentration of 1%. Then incubate the slurry on ice for 10 min.
6. Centrifuge at 18,500×*g* for 10 min at 4°C. Wash the pellet containing the inclusion bodies twice with 5 mL washing

buffer I containing 1% Triton X-100 and a third time with washing buffer I only.

7. Suspend the inclusion bodies from 50 mL of bacterial growth in 1.5 mL of 6 M guanidine-hydrochloride (GuHCl) and centrifuge the extracted protein.
8. Purify the V_H by size fractionation of the denaturated protein using a HiLoad Superdex 75 26/60 column, with 4 M GuHCl as a running buffer.

3.1.3. Recombination of the 447–52D V_L and V_H Chains

1. Combine light- and heavy-chain proteins by mixing the V_H protein (dissolved in 4 M GuHCl) and the V_L protein (in the SP-Sepharose elution buffer) in a molar ratio of 1:1 (equal to 1:1.25 mg ratio).
2. Dialyze the Fv overnight against 50 mM Tris-HCl (pH 8), 0.05% NaN_3, and 5 mM EDTA at 4°C.
3. Concentrate to 0.6 mg/mL using a Vivaspin concentrator and purify the Fv further by size-exclusion chromatography on a HiLoad Superdex 75 26/60 column equilibrated with 125 mM NaCl, 25 mM Tris-HCl (pH 7.5), and 0.02% NaN_3.
4. Load the eluted Fv onto a benzamidine column equilibrated with 0.5 M NaCl, 50 mM Tris-HCl (pH 8), and 0.05% NaN_3 to remove serine proteases. The Fv fraction is eluted in the flow-through.

3.2. Peptide Expression and Purification

3.2.1. Expression of $V3_{MN}$ Fusion Protein in E. coli

1. Transform *E. coli* competent cells of the BL21-Gold(DE3) pLysS strain with the pM4-$V3_{MN}$ plasmid containing the sequence for the $V3_{MN}$ peptide.
2. Inoculate growth medium at 1:100 dilution and grow the cells at 37°C in Celtone containing 100 μg/mL ampicillin, 25 μg/mL chloramphenicol, RPMI 1640 vitamin cocktail, 1 mM $MgSO_4$, and 50 mM K_2HPO_4 to a cell density of OD_{595} 1.0–1.2.
3. Induce expression by adding 1 mM IPTG, followed by incubation for 3 h at 37°C and shaking at 225 rpm. Then collect the bacteria by centrifugation (8,000×*g* for 30 min). The over-expressed protein is obtained in large quantities in the form of inclusion bodies.
4. Suspend the cells in 20 mL of washing buffer II and add a mixture of protease inhibitors (4 μL of 10 mg/mL aprotinin, 4 μL of 10 mg/mL leupeptin, and 200 μL of 17.4 mg/mL PMSF) to the cooled buffer, and sonicate the mixture for 90 s on ice.
5. Add 1% Triton X-100 in order to remove membrane components and incubate the cell lysate on ice for 10 min.
6. Spin the cell lysate at 20,200×*g* for 10 min at 4°C and wash the pellet containing the inclusion bodies twice with 20 mL

washing buffer II and 1% Triton X-100 to dispose of cell debris. Remove traces of Triton X-100 by a third wash with the washing buffer II.

7. Gradually solubilize the inclusion bodies in 8 M urea, 50 mM glycine, and 450 mM NaCl in washing buffer II (*see* **Note 1**). Collect the protein from the supernatant by centrifugation at 20,200×g for 10 min and subject the supernatant to an additional centrifugation at 30,900×*g* for 30 min to pellet the remaining DNA.
8. Dialyze the supernatant against 50 mM NH_4HCO_3 (four changes) and lyophilize. This yields a 90% pure preparation of the fusion protein.

3.2.2. $V3_{MN}$ Peptide Cleavage and Purification

1. Release the peptide from the fusion protein using CNBr (*see* **Note 2**), which cleaves peptide bonds at the C-terminus of a methionine residue *(18)*. Cleavage requires 24 h in 70% formic acid at room temperature in a darkened flask (*see* **Note 3**).
2. Add 10 volumes of HPLC-grade water upon completion of the reaction and lyophilize to complete dryness. Then dissolve in 100% dimethyl sulfoxide.
3. Dilute the cleaved protein solution to 30% dimethyl sulfoxide and purify by HPLC on a Vydac polymer reversed-phased semipreparative column (25 × 250 mm^2) and then on a Vydac (10 × 250 mm^2) column.
4. Lyophilize and detect the purity on an analytical Vydac column (4 × 250 mm^2), using an acetonitrile water gradient. All solvents contain 0.1% trifluoroacetic acid.

3.2.3. Construction of the $V3_{IIIB}$ Expression Vector

1. Use the unique NdeI and BamHI restriction sites, found at the 5′ and 3′ ends of the $V3_{MN}$ construct in pM4-$V3_{MN}$, for the removal of the $V3_{MN}$ gene and the introduction of the $V3_{IIIB}$.
2. Prepare the nucleotide sequence of the $V3_{IIIB}$ gene, then digest it with *Nde*I and *Bam*HI and ligate with the pM4-$V3_{MN}$ vector, previously cut with the same enzymes.
3. Transform *E. coli* TG1 strain with this new construct to obtain the recombinant clones.
4. Transform *E. coli* BL21(DE3)pLysS strain with the plasmid and express in a similar manner as the $V3_{MN}$ peptide.
5. For $V3_{IIIB}$, which has threonine as the first amino acid, carry out the CNBr cleavage in 70% trifluoro acetic acid instead of 70% formic acid. The efficiency of the cleavage in formic acid when a threonine residue follows methionine is very low *(19)*.

3.3. Fv–Peptide Complex and NMR Sample Preparation

1. Prepare the complex of the 447–52D Fv fragment and a $^{13}C/^{15}N$ V3 peptide (~28 kDa) by shaking the Fv and the labeled peptide in a 1:1.2 molar ratio respectively for 3 h at 37°C.
2. Remove excess peptide and concentrate the sample by subjecting to centrifugal filtration in Vivaspin tubes (Vivascience) with a 10-kDa membrane cutoff.
3. Insert the recovered solution into a Shigemi NMR tube (total volume of 350 μL). All samples contained 10 mM deuterated acetate buffer (pH 5.0), 95% H_2O/5% D_2O, 1 mM EDTA, and 0.02% NaN_3.

3.4. Binding Interface Mapping Protocol

3.4.1. NMR Dynamic Filtering

1. The experimental and processing parameters are given in **Note 4** (for α-BTX) and **Note 5** (for V3 peptides).
2. Perform sequential assignment of $^{\alpha\text{-AChR}}$K179-D200 and $^{\alpha\text{-AChR}}$R182-T202 peptides according to the well-established method of Wüthrich *(20)*. This is achieved by measuring HOHAHA, ROESY, and NOESY spectra of the free peptides.
3. Measure HOHAHA and ROESY spectra of the α-BTX/$^{\alpha\text{-AChR}}$K179-D200 and α-BTX/$^{\alpha\text{-AChR}}$R182-T202 complexes with mixing times of 400 and 250/200 ms respectively.
4. Compare the HOHAHA spectrum of the complex with that of the free peptide to identify cross-peaks corresponding to the peptide residues that do not interact with the receptor, thus retaining their mobility and exhibiting chemical shift values similar to those found for the free peptide. As a result of the long mixing period used in the HOHAHA and ROESY spectra, the cross-peaks of peptide protons interacting with the receptor as well as of most receptor protons vanish.
5. For the V3: Measure HOHAHA, ROESY, and NOESY spectra for the free V3 peptide in H_2O. Completely assign all cross-peaks observed for the free peptide according to the well-established method of Wüthrich *(20)*.
6. Measure ROESY and HOHAHA spectra with long mixing times (90 ms) for the peptide–Fv (or peptide–Fab) complex in H_2O. The mixing time is adjusted to discriminate between cross-peaks of peptide protons that are immobilized in the complex because of interactions with the antibody and therefore have a short $T_{1\rho}$ relaxation time and those protons that do not interact with the Fv and, therefore, retain considerable mobility and have a long $T_{1\rho}$. The combination of the HOHAHA and ROESY spectra is used for sequential assignment of the mobile segments of the peptide in the Fv–peptide complex.
7. Compare the HOHAHA spectrum of the complex **(step 6)** with that of the free peptide **(step 5)** to identify cross-peaks corresponding to the peptide residues that do not interact with the Fv, thus retaining their mobility and exhibit chemical

shift values similar to those found for the free peptide. Cross-peaks of peptide protons interacting with the Fv as well as of most Fv protons vanish as a result of the long mixing period used in the HOHAHA and ROESY spectra.

3.4.2. ^{1}H-^{15}N HSQC Intensities

1. Measure the ^{15}N TROSY HSQC spectrum of the uniformly ^{15}N-labeled peptide in complex with unlabeled Fv.
2. Measure cross-peak intensities and draw intensities vs. peptide residues. Examine the variation in peak intensity. Those residues interacting with the antibody have low intensity while those outside the epitope have higher values.

3.4.3. ^{15}N T_2 Relaxation Times

1. The experimental and processing parameters are given in **Note 6**.
2. Measure a series of HSQC spectra modified in a way that the magnetization will remain on the nitrogen for a different amount of time in each measurement. The delay time, during which the ^{15}N relaxes, is increased gradually in the series of experiments.
3. Process the spectra in a manner similar to that of HSQC spectra and then extract the T_2 relaxation time values by fitting the decline in signal strength over time to a decreasing exponential.
4. The major factor influencing T_2 is the tumbling rate of the protein. Rapid tumbling results in a long T_2, whereas slow tumbling results in a short T_2. Therefore, V3 residues interacting with the Fv will tumble slower and have short T_2.

3.4.4. Proton $T_{1\rho}$ Relaxation Times

1. The experimental and processing parameters are given in **Note 7**.
2. Measure HOHAHA, ROESY, and NOESY spectra for the toxin and its peptide complex in H_2O. Sequentially assign all cross-peaks observed according to the well-established method of Wüthrich *(20)*.
3. Measure a series of HOHAHA spectra for the unlabeled complex. The HOHAHA experiment is based on the HOHAHA pulse sequence *(21)* and incorporates a spin lock (SL) pulse after the initial 90° pulse. Measure six experiments with SL durations varying from 0 to 25 ms in 5-ms increments.
4. Determine proton $T_{1\rho}$ values by fitting the decay in cross-peak intensity as a function of the duration of the SL pulse to a single-exponential decay [$I(t) = I_0 \exp(-t/T_{1\rho})$] using the modelXY package *(22)*. Obtain error values to be included in the calculation from signal-to-noise ratios.
5. A decrease in the relaxation times and the mobility of residues involved in binding is exhibited, while residues not implicated in binding retain considerable mobility. The quantitative $T_{1\rho}$ measurements enable to corroborate the mapping of boundaries of binding determinant by other methods.

3.4.5. ^{1}H-^{15}N NOE

1. Record pairs of interleaved ^{1}H-^{15}N NOE spectra with and without proton saturation during the recycle delay. Since the longitudinal magnetization of the heteroatom is to be measured for calculating the hetNOE, magnetization must originate from the ^{15}N spin. Magnetization is subsequently transferred via a refocused INEPT sequence to the directly coupled NH proton for observation.
2. hetNOEs are evaluated by calculating the ratio of intensities in spectra recorded with and without proton saturation.

4. Notes

1. The yield of the purified V3 peptide varied from 6–13 mg/L of Celtone medium. To obtain higher yields, the inclusion bodies (**Subheading 3.2.1**, **step 7**) can be repeatedly solubilized.
2. Since CNBr is volatile and toxic (**Subheading 3.2.2**, **step 1**), the V3 peptide cleavage reaction from the fusion protein should be carried out in a hood and all tips and tubes used must be neutralized with 10 M NaOH.
3. CNBr cleavage should be done in a darkened flask using a 400-fold molar excess of CNBr over methionine residues (**Subheading 3.2.2**, **step 1**).
4. All NMR spectra were acquired on Bruker DMX 500-MHz and DRX 800-MHz spectrometers (**Subheading 3.4.1**, **steps 2**, **3**). HOHAHA, ROESY, and NOESY spectra of $^{\alpha\text{-AChR}}$K179-D200 were acquired at 20°C using mixing times of 150, 150, and 300 ms, respectively. For $^{\alpha\text{-AChR}}$R182-T202 peptide, HOHAHA spectra were acquired at 30 and 47°C. The HOHAHA and ROESY spectra of the α-BTX/$^{\alpha\text{-AChR}}$K179-D200 peptide and α-BTX/$^{\alpha\text{-AChR}}$R182-T202 complexes were acquired with varying mixing times of 100–400 ms, 2–4K points in the F2 and 256–600 increments in the F1 dimensions at 20 and 30°C. For the complete sequential assignment of the α-BTX/$^{\alpha\text{-AChR}}$R182-T202 complex, HOHAHA and NOESY spectra with 8K data points in F2 and 800 increments in F1 were acquired with mixing times of 70 and 150 ms, respectively, at 30 and 37°C. For assignment of the aliphatic region in 99.99% D_2O, HOHAHA and NOESY spectra with water presaturation were acquired at 37°C with mixing times of 70 and 150 ms, respectively.
5. The long mixing time (90 ms) in the ROESY and HOHAHA spectra (**Subheading 3.4.1**, **step 6**) was adjusted to discriminate

between cross-peaks of peptide protons that are immobilized in the complex because of interactions with the antibody and have a short $T_{1\rho}$ relaxation time and those of protons that do not interact with the Fv and, therefore, retain considerable mobility and have a long $T_{1\rho}$. Hence, the combination of the HOHAHA and ROESY spectra was used for sequential assignment of the mobile segments of the peptide in the Fv–peptide complex. NMR spectra were acquired at 35°C on Bruker DMX 500 and DRX 800 spectrometers. Two-dimensional ROESY and HOHAHA spectra of the unlabeled complex were measured at 30°C, 20°C, and 10°C and at pH 7, 5, and 4.25.

6. T_2 ^{15}N relaxation time measurements (**Subheading 3.4.3**, **step 2**) were carried out with a total of 182 transients. Six time points were collected with parametric delays of 8, 16, 24, 32, 48, and 72 ms on a Bruker DRX-800 MHz spectrometer, with a 2-s delay between scans.
7. NMR spectra of α-BTX and its complex were acquired at 30°C. The $T_{1\rho}$-filtered HOHAHA spectra were measured using the following pulse sequence: $90_x°$-SL_y-evolution (t_1)-(WALTZ)-acquisition (t_2) (**Subheading 3.4.4**, **step 3**). Isotropic mixing was realized using a WALTZ *(23)* pulse sequence with a short duration of 30 ms. The spectra were acquired using sensitivity enhancement and TPPI, and the water signal was suppressed by the WATERGATE pulse sequence (water suppression by gradient-tailored excitation) *(24)*. The numbers of complex points acquired on the 500-MHz spectrometer were 2,048 and 256 in the F_2 and F_1 dimensions, respectively, with spectral width of 6,000 Hz. The numbers of complex points acquired on the 800-MHz spectrometer were 8,192 and 800 in the F_2 and F_1 dimensions, respectively, with spectral widths of 11,160 Hz. All spectra were processed and analyzed using NMRDraw and NMRPipe *(22)*. Unresolved peaks at frequencies close to that of water required additional baseline correction using polynomial water subtraction in all experiments. Linear prediction in the F_1 dimension was also done to increase resolution and improve the automated peak picking. Automated peak picking in these spectra was achieved using NMRPipe and in-house Perl scripts.

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