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A binding site peptide fragment of the nicotinic acetylcholine receptor. Sequence-specific assignment of ^{1}H-NMR resonances in the dodecamer α185–196

Synthetic peptide fragments of the nicotinic acetylcholine receptor (nAChR)* are being used to explore and to delineate the ligand binding site of this physiologically important cell surface receptor. Previous work has shown that a number of small peptides corresponding in sequence to residues from within a 32-amino acid region of the α-subunit (α173–204) from the *Torpedo* nAChR bind the curaremimetic antagonist α-bungarotoxin with high affinity [1–3]. The elucidation of the structure of the complex formed between α-bungarotoxin and a peptide from the binding site region should provide insights as to which amino acids on the native receptor are involved in antagonist binding. Such studies should also help determine the mode of antagonist binding to the nAChR and may suggest the structural differences underlying agonist versus antagonist specificity.

To exploit fully modern two-dimensional NMR methods to determine the solution structure of the complex and to probe the molecular details of the interactions that mediate binding, it is first necessary to assign all of the ^{1}H resonances for both binding components. Fortunately, all the ^{1}H resonances of α-bungarotoxin have been assigned [4, 5], and many interproton distance constraints defining the solution structure for this protein have already been obtained. In this paper we present the complete sequence-specific ^{1}H-NMR resonance assignment for a 12-amino acid peptide (α185-peptide: residues 185–196 of the α-subunit). This peptide, in solution, formed a complex with α-bungarotoxin with an apparent K_d in the low micromolar range (1 to 2.5 μM) as determined in a number of solution assay systems.†

Methods

The 12-amino acid peptide corresponding to residues 185 to 196 of the α-subunit, designated α185-peptide, was synthesized and purified by reverse-phase HPLC in the Protein and Nucleic Acid Facility, Yale University School of Medicine, New Haven, CT. The sequence, KHWVYYTCCPDT, is that found in the *Torpedo* nAChR and is highly conserved among species. Both amino and carboxyl termini were prepared unblocked, and the cysteines were prepared and maintained in the reduced state. Susceptibility to covalent alkylation by *N*-ethylmaleimide, as indicated by a marked increase in retention time on HPLC chromatography, confirmed that >90% of the peptide contained cysteines in the free sulfhydryl form. The amino acid composition of the peptide was verified by ion exchange chromatographic analysis of performic acid oxidation products on a Beckman model 6300 amino acid analyzer. The mass of the peptide (M_r = 1515) was confirmed by fast-atom mass bombardment techniques.

Three peptides with sequences similar to the α185-peptide were used to facilitate the assignment process. These peptides were also prepared as described above and corresponded to the α185 peptide but with (1) a *p*-fluorophenylalanine substituted for Tyr-189 (α185{Fl-Phe-189}-peptide), (2) a deletion of Lys-185 (α186-peptide), and (3) a deletion of Lys-185 along with the substitution of an Ala for Cys-192 (α186{Ala-192}-peptide).

Peptide samples (0.35 mL) were prepared at pH 4 (at 1 mM) and at pH 2.8 (at 3 mM) in D_2O (99.8%) or in a mixture of 80% H_2O and 20% D_2O. The pH (pD) was adjusted with NaOD. ^{1}H-NMR spectra were acquired at 25° on a Bruker AM-500MHz NMR spectrometer. Two-dimensional correlated spectra (COSY) were recorded in magnitude absorption mode. Time domain data sets consisted of 128 to 256 t_1 increments, zero-filled in t_1 to 1024 data points, with a sweep width of 4132 Hz and 2048 complex data points in t_2. A recycle delay of 2 sec was used, and 176 transients were collected for each free induction decay determination. A continuous low power irradiation of the residual water signal was applied during the recycle time in all COSY spectra. For one-dimensional (1D) difference nuclear Overhauser effect (NOE) experiments, 16K data points were collected over a 7800 Hz sweep width with a 3-sec repetition delay. Resonances of interest were irradiated for 1 sec using the decoupler channel. Data sets corresponding to on- and off-resonance irradiation were interleaved every 16 scans for a total of 256 scans, and were then subtracted prior to Fourier transformation in order to obtain the NOE difference spectra. All data processing was performed on a Bruker spectrometer with the sine-bell window shifted $\pi/64$ in t_2 and $\pi/32$ in t_1.

Results and Discussion

The total assignment of the main and side chain ^{1}H resonances for the α185-peptide was achieved by a combination of COSY experiments together with 1D spin-decoupling studies of the well resolved resonances. Since we had previously prepared a number of related peptides with similar sequences but altered at single identified residues, we were able to use these peptides to identify the sequence-specific resonances for the two pairs of degenerate residues, Tyr-189 and -190, and Thr-191 and -196, within the α185-peptide. The complete assignment of the eleven amide resonances was made by selective spin decoupling of the identified CαH resonances in a peptide solution containing 80% H_2O/20% D_2O. Table 1 contains a compilation of the sequence-specific ^{1}H resonance assignments obtained from the studies described below.

Beginning with the N-terminal residue of the α185-peptide, the Lys-185 resonances were easily identified by comparing the 1D ^{1}H NMR spectra for the α185-peptide and the α186-peptide lacking Lys-185. In addition, all expected crosspeaks were clearly evident in the COSY spectrum of the α185-peptide. The assignment for His-186 was also straightforward from the COSY experiment. In addition to a COSY crosspeak between the C2(ε)H and C4(δ)H resonances of the histidine ring, we were also able to see a crosspeak due to the four-bond coupling between the C4(δ)H resonance and that of the two CβH resonances in the side chain. This assignment was confirmed by spin decoupling and NOE difference experiments. Figure 1 is a contour plot of the ^{1}H COSY spectrum of the α185-peptide which illustrates the ring-proton connectivities of His-186.

The assignment of the aromatic resonances for Trp-187 was readily obtained from the COSY spectrum, and the connectivity between the singlet C2H resonance and the CβH resonance was obtained by NOE difference experiments. The unique A_3B_3MX spin system of valine allowed for the identification of the diagnostic connectivity pattern for this residue, as shown in Fig. 1. A comparison of the NMR spectra for the α185-peptide and the α185{Fl-Phe-189}-peptide clearly identified the C2,6H and C3,5H ring proton resonances for Tyr-189 and Tyr-190, since the chemical shift of the C2,6H resonance of Tyr-190 was unaffected by the Fl-Phe substitution at position 189. The CαH and CβH resonances of the Tyr spin systems were

* Abbreviations: nAChR, nicotinic acetylcholine receptor; 1D, one-dimensional; COSY, correlated spectroscopy; Fl-phe, *p*-fluoro-phenylalanine; and NOE, nuclear Overhauser effect.

† Submitted manuscript.

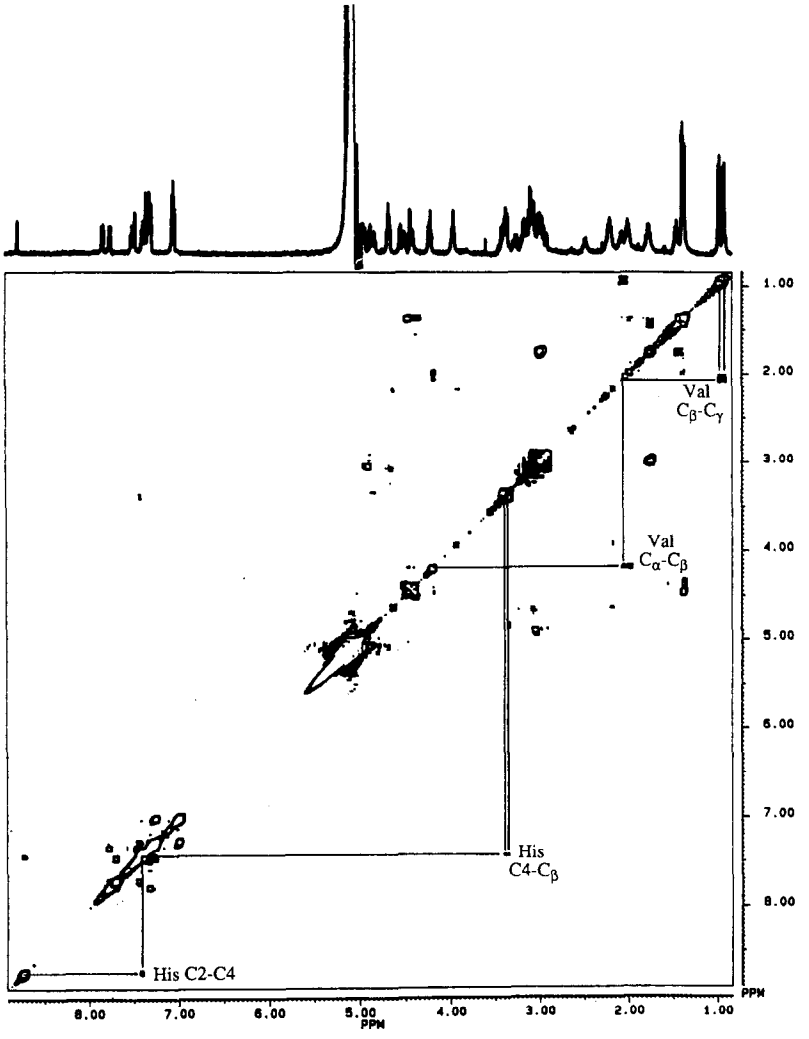

Fig. 1. Contour plot of the ^1H COSY spectrum of the α185-peptide in D_2O at pH 4 and 1 mM peptide. The x-axis is ω_2 and the y-axis is ω_1. Proton–proton J-coupling connectivities are indicated by the off-diagonal crosspeaks, and the connectivities of the His and Val spin systems are illustrated. The connectivity between the C4H and the CβH of His was confirmed by 1D NOE difference spectroscopy. In most cases, spin decoupling was used to confirm the CβH–CαH connectivities.

Table 1. ^1H Chemical shift assignments in ppm for the dodecamer, α185–196, at pH 4.0, 25°*

Residue	NH	CαH	CβH	Others
Lys-185	NO†	4.18	1.98	CγH 1.47; CδH 1.75; CεH 2.96
His-186	8.68	4.84	3.35, 3.39	CδH 7.41 (C4H); CεH 8.72 (C2H)
Trp-187	8.63	5.00	3.31, 3.35	C2H 7.32; C5H 7.34; C6H 7.46; C7H 7.71; C4H 7.79; NεH 10.28
Val-188	8.18	4.17	2.04	CγH 0.91, 0.97
Tyr-189	8.63	4.92	2.89, 2.96	C3,5H 7.01; C2,6H 7.28
Tyr-190	8.50	4.89	3.02, 3.09	C3,5H 6.99; C2,6H 7.26
Thr-191	7.94	4.40	4.45	CγH 1.38
Cys-192	8.26	4.64	3.05, 3.07	
Cys-193	8.63	4.64	3.05, 3.07	
Pro-194		3.91	2.12–2.24 (CβH–CγH)	CδH 3.10, 3.23
Asp-195	8.26	4.64	2.15, 2.48	
Thr-196	8.26	4.50	4.36	CγH 1.36

* All proton resonances for the dodecamer are assigned based on 2D-COSY with water suppression, spin decoupling, and the comparison of spectra with those of similar peptides with single amino acid sequence changes as described in Methods.

† NO, not observed.

identified by the infrequently observed COSY crosspeaks due to the four-bond coupling between the C2,6H and the CβH resonances. By comparing the chemical shifts of the two methyl resonances of Thr-191 and Thr-196 in the α185-peptide and in the α186{Ala-192}-peptide, we observed that the chemical shift of the methyl doublet at 1.36 ppm remained unchanged in the α185{Ala-192}-peptide, whereas the downfield methyl doublet (1.38 ppm) was shifted by about 0.015 ppm further downfield. Assuming that the Thr residue sequentially adjacent to the amino acid substitution at position 192 would be affected more than the Thr at position 196, we assigned the 1.38 methyl resonance to Thr-191. The remaining sequence-specific resonance assignments could all be obtained from the COSY spectrum. The simplified aliphatic region of the NMR spectrum for the α186-peptide lacking the N-terminal Lys facilitated the assignment and confirmation of many of the remaining aliphatic resonances, especially those from Pro and Asp. The two CαH as well as the two CβH resonances for the two Cys residues (192, 193) were degenerate (Table 1), suggesting that these two side chains are in a similar chemical environment.

Since random coil amino acids do not contain CαH resonances with chemical shifts greater than 4.8 ppm, it has been suggested that the difference in chemical shifts between those in a polypeptide and those in a random coil represents a conformation-dependent chemical shift [6]. Four of the CαH resonances (His, Trp, and both Tyr) have chemical shifts greater than 4.8 ppm, suggesting some amount of ordered structure in the α185-peptide. In addition, the amide resonance of Thr-191 remains a sharp doublet while the other amide resonances are broadened due to exchange. The observation of such a sharp resonance suggests that the Thr-191 amide proton is slowly exchanging due to some feature of an ordered structure within the peptide. For example, such an observation is thought to be consistent with the Thr-191 amide being buried and inaccessible to solvent or with it being involved in H-bonding.

In summary, the total sequence-specific ^{1}H assignment for the α185-peptide was accomplished by analysis of COSY spectra along with spin-decoupling and confirmatory NOE difference experiments. Some ambiguities in the assignments were successfully addressed utilizing additional peptides with selective amino acid substitutions. The chemical shifts of several of the CαH resonances, along with evidence for a slowly exchanging amide at Thr-191 suggest that the α185-peptide may contain a certain amount of non-random coil structure. The role of any such ordered structure in the mechanism of binding to α-bungarotoxin remains to be determined. The assignment of the peptide ^{1}H resonances will facilitate the analysis and identification of chemical shift perturbations observed upon formation of the complex between α-bungarotoxin and the α185-peptide [7].

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NMR studies of protein surfaces. The interaction of lysozyme with tri-*N*-acetylglucosamine

Methods to identify the surface residues of proteins have several potential applications. Since functional regions of a protein surface may be composed of residues not in continuous sequence, knowledge of surface structure is required for rational design of non-protein mimics of protein function. Also, as illustrated below, mapping exposed amino acid residues may aid in identifying substrate or inhibitor binding sites of enzymes. In addition, it is possible that independent identification of surface residues may supplement the nuclear magnetic resonance data usually employed in determination of a solution structure.

Several nuclear magnetic resonance methods have been used to identify solvent-exposed residues of proteins and peptides. These include measurement of amide proton