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α-Bungarotoxin binding to two acetylcholine receptor α-peptides and their methylmercury-modified analogs: intrinsic phosphorescence and optically detected magnetic resonance studies

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Phosphorescence and optically detected magnetic resonance (ODMR) have been used to characterize two synthetic peptides, α181–198 and α185–196, of the major binding determinant of the α-acetylcholine receptor (AChR) of *Torpedo californica* and its interaction with α-bungarotoxin (BgTX) using Trp as an intrinsic probe. BgTX conformational changes are suggested upon complexation with the peptides. Methylmercury-modified peptides show conformational heterogeneity which brings some of the modified Cys residues into proximity of peptide Trp(s). These modified peptides, when bound to BgTX, undergo structural changes which remove the tagged Cys from its close contact with the Trp residue(s) of the peptide.

ODMR spectroscopy; Phosphorescence; Neurotoxin-acetylcholine receptor interaction; Heavy-atom modification

1. INTRODUCTION

The nicotinic acetylcholine receptor (AChR) is a pentameric transmembrane protein assembled from four structurally related subunits, $\alpha_2\beta\gamma\delta$. It functions as a ligand-gated channel protein mediating signalling in vertebrate skeletal muscle. The α-subunit is largely responsible for specifying the cholinergic ligand binding sites which number two per receptor. A segment, α173–204, of the α-subunit of the AChR from electroplaque of *Torpedo californica* has been shown to contain the main binding determinant [1]. Small segments within this region, α181–198 and α185–196, display micromolar affinity towards the cholinergic antagonist α-bungarotoxin (BgTX) [2].

In this work we describe the use of low temperature phosphorescence and optically detected magnetic resonance (ODMR) to investigate the environment of Trp residues present in two α-subunit synthetic peptides when the peptides are either unbound or bound to BgTX. Two peptides are investigated, one that is 18, and the other 12 residues long. The 18mer, Y^{181}RGWKHWVYYTCCPDTPY^{198}, contains two Trp residues, Trp^{187} and Trp^{184}, and the 12mer, K^{185}HWVYYTCCPDT^{196}, contains only Trp^{187}. An elegant model has been proposed previously which suggests that a direct hydrophobic interaction occurs between an aromatic residue of the toxin and a Trp residue of the AChR peptide upon complexation [3,4]. A primary motivation for undertaking this investigation was to test the validity of this model. A further inducement is the presence of the highly conserved Cys residues at adjacent positions 192 and 193. These cysteines form a highly strained and easily reduced vicinal disulfide bond in the intact AChR [5,6]. The presence of cysteine is not, however, a requirement for BgTX binding [7], although several studies using synthetic peptides or the intact AChR in which Cys^{192} and Cys^{193} are chemically modified or substituted by other amino acids have shown a decreased BgTX affinity [8–10]. The fortuitous placement of Cys at the binding site allows for the attachment of a thiol-specific heavy-atom probe. If a Trp residue is located in the immediate proximity of a heavy-atom-modified peptide thiol, when the peptide is either unbound or in a complex with BgTX, then a Trp heavy-atom effect (HAE) may be used to further elucidate toxin-peptide interactions.

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2. EXPERIMENTAL

The unblocked synthetic peptides, 18mer and 12mer, were synthesized at the Protein Chemistry Facility, Yale University School of Medicine, New Haven, CT. Greater than 90% of the 12mer and 18mer peptide thiols were in the reduced state. Solutions were prepared in triply distilled water and subsequently adjusted to pH 8. BgTX, also dissolved in water, was a generous gift from Professor Mark MacNamee of the Department of Biochemistry and Biophysics, University of California, Davis, CA. One to one molar ratio peptide-toxin

complexes were prepared and incubated for 15 min at room temperature. CH_3Hg-modified peptides were prepared by addition of concentrated CH_3HgI dissolved in ethylene glycol to peptide (a 3:1 molar ratio was used) followed by incubation at 37°C for ca. one hour. Binding of the modified peptides to the toxin followed the procedure described above. All samples were adjusted to 30% ethylene glycol (v/v) prior to spectroscopic measurements.

The procedures for obtaining phosphorescence spectra, triplet state lifetimes and ODMR spectra have been described previously [11]. All data presented here were obtained with sample excitation centered around 300 nm with 16 nm bandpass.

3. RESULTS AND DISCUSSION

3.1. *AChR model peptides*

The phosphorescence of both the 12mer and the 18mer are typical of samples containing both Trp and Tyr in that only the well-resolved emission of the former is observed. As shown in Table I the 18mer phosphorescence is shifted by ca. 1.4 nm to lower energies relative to the emission of the 12mer. This may be due to the added contribution of Trp^{184} to the emission or to a more polarizable environment for either or both of the 18mer's Trps. The major lifetime component (ca. 6.7 s) of both peptides (Table I) is assigned to Trp. The minor component may be due to Tyr or it may arise from a Trp in close proximity to an intrapeptide disulfide, Cys^{192}–Cys^{193}, which is present in small amounts (<10%) in our samples. Trp-disulfide interactions are known to reduce phosphorescence lifetimes (see discussion below).

The zero-field ODMR peak frequencies of the 12mer (Fig. 1A) and 18mer are virtually identical (Table I). The frequencies of the slow passage ODMR transitions can be empirically correlated with triplet state energy and also with some success to the degree of solvent exposure of Trp [12]. In the present case, the degree of Trp solvent exposure in the small peptides is similar to that observed for the solvent exposed Trp of the hormones glucagon or somatostatin [13]. Another indicator of site homogeneity is reflected in the linewidth of the D–E signal [12]. The 18mer D–E linewidth of 150 MHz is significantly less than the observed linewidth of 220 MHz for this transition in the 12mer and may be interpreted to be a consequence of a smaller distribution of Trp–peptide interactions in this significantly larger model peptide. Its red-shifted emission is consistent with a more polarizable environment.

3.2. *Model peptide complexes with BgTX*

When the model peptides are bound to BgTX the triplet state energies, lifetimes and ODMR peak frequencies are affected noticeably in comparison with the unbound peptides. The D–E transition linewidth of the unbound 12mer, 220 MHz, decreases considerably to 150 MHz upon complexation with BgTX (Fig. 1C). Similar decreases in ODMR linewidths were observed for 18mer binding to BgTX (Table I). This suggests a more ordered environment for Trp(s), as expected, upon complex formation.

In previous work [11,14] we have demonstrated that Trp^{28} of BgTX is quenched in the triplet state by the presence of the disulfide bridge between Cys^{29} and Cys^{33}. Triplet state Trp–disulfide interactions are known to be extremely short-range in nature [15] (an effective Bohr radius of $L = 0.8$ Å has been determined). For BgTX the triplet quenching was manifested by the observation of a low phosphorescence to fluorescence quantum yield ratio (0.02 compared with 0.28 for Trp in frozen solutions), an anomalously shortened triplet lifetime and the presence of an unusual negative polarity D–E signal upon excitation at 300 nm (Fig. 1B). The absence of a shortened Trp lifetime (Table I) for the peptide–BgTX complexes suggests that BgTX undergoes a conformational change upon peptide binding resulting in an increase of the disulfide–tryptophan distance, thereby reducing the quenching of Trp^{28}. Similar arguments were used previously to explain phosphores-

Table I

Triplet state properties of AChR model peptides and their complexes with α-bungarotoxin

Sample[a]	λ_{00}[b] (nm)	Lifetimes[c] (s)		D–E[d] (GHz)	2E[d] (GHz)
12mer	409.9	(15%) 2.43	(85%) 6.79	(+) 1.74 (220)	(+) 2.51 (300)
18mer	411.3	(8%) 2.78	(92%) 6.66	(+) 1.71 (150)	(+) 2.55 (270)
BgTX[e]	412.2	(45%) 1.1	(55%) 5.5	(−) 1.68 (160)	(+) 2.51 (170)
12mer:BgTX	410.1	(20%) 2.63	(80%) 6.51	(+) 1.73 (155)	(+) 2.59 (260)
18mer:BgTX	410.9	(15%) 2.50	(85%) 6.20	(+) 1.67 (130)	(+) 2.56 (250)

[a]Samples were dissolved in water and contained 30% (v/v) ethylene glycol.
[b]Assigned 0,0-band of phosphorescence at which all kinetic and ODMR measurements were made using excitation centered at 300 nm with 16 nm bandpass.
[c]Lifetimes were extracted from the observed decays at 77 K using a least-squares technique. The percentage of the total emission is in parentheses immediately preceding the extracted lifetime.
[d]The parentheses preceding the ODMR peak frequencies indicate whether the observed transition is an increase, (+), or decrease, (−), in phosphorescence intensity with respect to the total steady-state emission. The parentheses following the ODMR peak frequencies contain the observed linewidth of the transition in MHz. Observations were made at 1.2 K.
[e]Data from [11].

cence lifetime changes of disulfide-perturbed Trp residues of hen egg white lysozyme and the Fab' fragment of the anti-galactan antibody J539 upon ligand binding [16]. This explanation is in line with the qualitative observation of an overall AChR peptide–toxin complex phosphorescence intensity which is larger than the sum of the intensities obtained for the isolated toxin and peptide. This effect on phosphorescence quantum yield parallels the fluorescence enhancement observed in solution upon binding the weakly emitting BgTX (ϕ_f = 0.036) to the 12mer and the 18mer [2]. Since disulfides are very efficient singlet state quenchers, a displacement of the Trp^{28} residue of BgTX with respect to the Cys^{29}–Cys^{33} disulfide may account in part for changes in both room temperature fluorescence and the low temperature phosphorescence upon binding. BgTX conformational changes have been suggested previously by NMR studies of its binding to receptor prototopes [17]. Flexibility in the end of the β-pleated structure which includes Trp^{28} has been postulated to account for differences in BgTX crystal and solution structures [18].

Low and Corfield have proposed a dynamic model of toxin–receptor binding [3,4] in which a receptor Trp fits tightly into a toxin hydrophobic 'Trp' cleft. According to this model, shifts in neurotoxin residues would make possible direct hydrophobic binding interactions between the receptor Trp and one of two toxin aromatic residues, Trp^{28} or Tyr^{24} [3]. Model studies suggest that of the two Trps in the main binding determinant of the α-subunit, Trp^{187} is the more stereochemically probable residue to be entrapped by the toxin [4]. One expects that this type of a stacking interaction would be accompanied by a decrease in the D ZFS parameter resulting from triplet wavefunction expansion normal to the Trp aromatic plane [12]. For example, the hydrophobic interactions of Lys–Trp–Lys with polydeoxythymidic and polyriboadenylic acids result in reductions in D of 30 and 36 MHz, respectively, for the Trp residue [19,20]. It was suggested above that the Trp^{28} emission of BgTX is most probably unmasked upon peptide binding so that its contribution to the spectral properties of the complex cannot be ignored. We will analyze the phosphorescence and ODMR of the peptide-BgTX complexes by considering the contribution of each Trp separately. Ignoring for the moment the contribution from Trp^{28} of the toxin, changes in emission of the 12mer Trp^{187}, in the complex with BgTX, result in a slight increase in D of 30 MHz. If we assume that there is a hydrophobic interaction between Trp^{187} and Tyr^{24} we expect to observe a reduction in the D ZFS parameter. If on the other hand, we ignore any contribution from Trp^{187} of the 12mer, we find that BgTX Trp^{28} shows an increase in D of 90 MHz upon 12mer binding. Both interpretations appear to be inconsistent with the direct hydrophobic interaction of Trp^{187} of the 12mer and either BgTX Trp^{28} or Tyr^{24}. The 18mer shows a decrease in D of 30 MHz, a small decrease in ODMR linewidth, but a slight blue-shift in phosphorescence upon binding BgTX. It is thus possible that Trp^{184} of the 18mer undergoes a hydrophobic interaction with a toxin aromatic residue. It may also be possible to interpret the decrease in D as a reflection of the greater than four-fold higher affinity that BgTX displays for the 18mer relative to its affinity towards the 12mer [2] if we assume that binding affinity is enhanced by increased hydrophobic interaction upon complex formation. Recent NMR evidence supports the lack of direct stacking interactions between toxin and peptide aromatic residues [21], in agreement with the interpretation above of phosphorescence and ODMR observations.

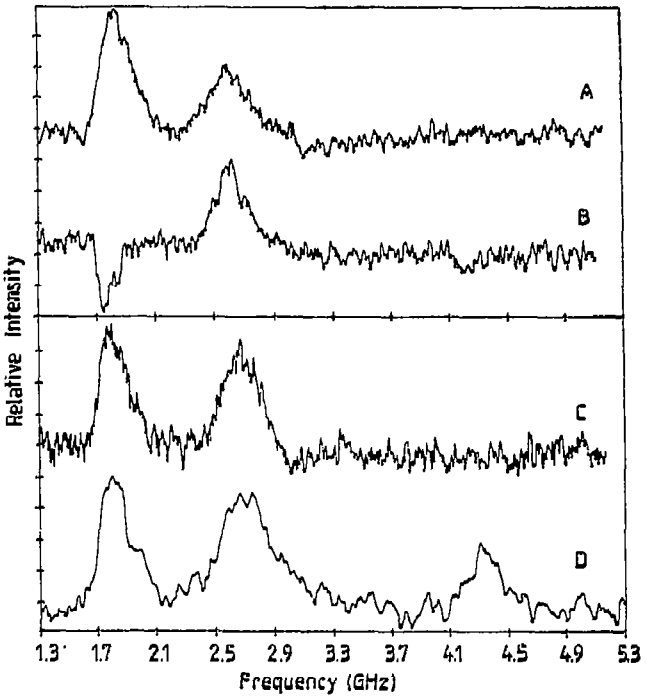

Fig. 1. Slow passage ODMR spectrum of (A) 12mer, (B) BgTX, (C) 12mer complexed with BgTX, and (D) 12mer complexed with CH_3Hg^+. Samples were dissolved in a water:ethylene glycol, 30% (v/v), solution. (A), (B) and (C) were recorded at 1.2 K with a sweep rate of 67 MHz·s^{-1}. (D) was obtained at 4.2 K with a sweep rate of 5 GHz·s^{-1}, conditions under which the signals of (A), (B) and (C) were not observed. Each sample was excited at 300 nm using 16 nm bandpass.

Table II

Triplet state kinetics of CH_3Hg-modified AChR model peptides and their complexes with α-bungarotoxin

Sample[a]	λ_{00}[b] (nm)	Lifetimes[c] (s)		
12mer:CH_3Hg	416.1	(15%) 0.06	(30%) 0.60	(55%) 4.36
18mer:CH_3Hg	416.1	(22%) 0.18	(31%) 1.65	(47%) 5.57
12mer:CH_3Hg:BgTX	411.2	(10%) 1.42	(90%) 6.56	
18mer:CH_3Hg:BgTX	411.3	(12%) 1.90	(88%) 6.00	

[a,b,c] See footnotes to Table 1

3.3. *CH_3Hg-modified peptides and their complexes with BgTX*

CH_3HgI is a thiol-specific reagent [22,23] and under the experimental conditions used here, it forms a complex with the free Cys at positions 192 and 193. The observation in the CH_3Hg-modified peptides of reduced lifetimes, significant red-shifted phosphorescence (Table II) and ODMR spectra (Fig. 1D) obtained under conditions in which unperturbed Trp does not show, ODMR (rapid microwave sweep rates and a temperature of 4.2 K compared with slow sweep rates and a temperature of 1.2 K normally required for unperturbed Trp) are a clear indication of an external HAE on peptide Trp(s). This is a short-range interaction with a reciprocal sixth power dependence on heavy-atom-perturber to chromophore distance, indicating that Trp^{187} of the 12mer is in close proximity to modified Cys^{192} and/or modified Cys^{193}. The spread in the observed lifetimes of the single tryptophan-containing 12mer (Table II) and especially the significant contribution of a 4–5 s lifetime component suggest that there may be a distribution of conformers present in the CH_3Hg-modified peptide. The proportion of the long-lived component in the total decay is greater than one would expect if the CH_3Hg-unmodified thiols, i.e. those involved in vicinal disulfide linkages (comprising <10%), are taken into account. Similar arguments suggest that conformational disorder is present in the CH_3Hg-modified 18mer (Table II). On the other hand, when the CH_3Hg-modified peptides are bound to BgTX, the HAE on Trp is no longer present. The lifetimes and phosphorescence for 12mer:CH_3Hg:BgTX and for 18mer:CH_3Hg:BgTX (Table II) are similar to those observed for the free, unmodified peptides (Table I). The disappearance of the HAE in the peptide complexes indicates implicitly that the modified peptides bind to BgTX. Furthermore, a structural change must occur upon binding in both 12mer and 18mer AChR peptides that removes the peptide Trp(s) from the neighborhood of the CH_3Hg-tagged peptide cysteines. Conformational change of the AChR [24] and also of model peptides [17] have been observed previously upon neurotoxin binding. The absence of a HAE in the complex also implies that neither of the heavy-atom modified peptide thiol groups are placed in the immediate proximity of Trp^{28} of BgTX.

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