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Phosphorescence and ODMR study of the binding interactions of acetylcholine receptor α-subunit peptides with α-cobratoxin

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Optical detection of magnetic resonance (ODMR) and phosphorescence spectroscopy have been applied to synthetic peptides derived from the α-subunit of the nicotinic acetylcholine receptor of *Torpedo californica* and their complexes with α-cobratoxin (CBTX). The CBTX Trp phosphorescence is strongly quenched by the proximal disulfide linkage, while the emission wavelengths and ODMR frequencies of the 18-mer α181–198 indicate a more hydrophobic Trp environment than in the 12-mer α185–196. Binding to CBTX produces a subtle increase in the hydrophobicity of the Trp environment for the peptides, in qualitative agreement with a recently proposed binding model, in which a receptor Trp residue interacts strongly with a hydrophobic cleft of the toxin.

Phosphorescence; ODMR spectroscopy; Neurotoxin-acetylcholine receptor interaction

1. INTRODUCTION

The nicotinic acetylcholine receptor (AChR) is a supramolecular array of 5 protein subunits, of stoichiometry $\alpha_2\beta\gamma\delta$, which plays a key role in muscular contraction by controlling transmembrane ion currents [1]. Snake venom neurotoxins such as α-bungarotoxin (BGTX), α-cobratoxin (CBTX), and erabutoxin inhibit neuromuscular transmission by binding competitively with the agonist acetylcholine [1] to the α-subunit. Recent studies have shown that synthetic peptide fragments which reproduce sequences from the α-subunit retain strong binding for the toxins [2–4]. In particular, studies have established the binding of α-bungarotoxin by the α173–204 fragment from *Torpedo californica* [2], and the BGTX binding of the shorter fragment α185–196 from *Torpedo* [3]. More recently, the binding of the peptide α179–191 from calf and human AChR to CBTX and to erabutoxin were studied [4] by fluorescence methods. These studies with synthetic peptides in principle make possible a detailed characterization of the role of particular residues in the binding process.

Based on computer graphics and physical model studies, a model for the binding of toxins to the alpha subunit of the AChR has been proposed [5,6], in which a specific tryptophan residue (Trp-187 or Trp-184) from the α-subunit is pictured as being inserted into a hydrophobic cleft in the toxin, a structural feature common to several long chain and short chain toxins. The possibility of direct hydrophobic interactions of the single Trp residue of the toxin with the above mentioned receptor Trp residues has also been proposed [5]. Recent fluorescence investigations of calf and human α179–181 peptide binding to CBTX and erabutoxin [4] have provided support for this model.

In the present work, we use triplet state optically detected magnetic resonance (ODMR) to study the binding to CBTX of two synthetic *Torpedo* sequence peptides α185–196 and α181–198, which include respectively, one or both of the aforementioned Trp residues at positions 184 and 187, which have been suggested as interacting with a hydrophobic toxin cleft. ODMR has been shown to be a useful probe of the microenvironment of Trp residues in proteins and peptides [7,8]. In particular, the phosphorescence wavelengths and frequencies of zero field splitting parameters, which arise from the dipole–dipole interaction of the two unpaired electron spins in the triplet state, have been shown to be sensitive to the hydrophobic vs. solvent exposed nature of the environment [9,10] and to aromatic stacking interactions [11,12]. In the present report, we characterize the Trp environments in the toxin and peptides by ODMR and phosphorescence decay measurements. We do find evidence for a small increase in the hydrophobic character of the environment of the peptide Trp residues upon CBTX binding, particularly in the case of the dodecamer. There is no evidence, however, for direct stacking with an aromatic residue.

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2. EXPERIMENTAL

CBTX was obtained from the Miami Serpentarium, Salt Lake City, UT, while the α185–196 dodecamer and α181–198 18-mer were synthesized at the Protein Synthesis Facility, Yale University School of Medicine, New Haven, CT. The complexes were prepared by dissolving the peptide and toxin in a 1:1 molar ratio in 5 mM phosphate buffer, pH 7.4 as previously described for α-bungarotoxin [13].

The apparatus and procedures for ODMR and phosphorescence decay measurements have been described previously [14]. To account for errors in ODMR peak frequencies which might arise from the delayed response of the Trp phosphorescence to microwave irradiation, we carried out sweeps in both directions (toward increasing and decreasing frequencies) and report in Table I the average peak position observed for the two sets of sweeps. In the phosphorescence decay determinations, a sum of three decaying exponentials plus a baseline was least squares fit to the data by the program KINFIT (OLIS, Inc., Bogart, GA).

3. RESULTS AND DISCUSSION

3.1. α-Cobratoxin

The phosphorescence of the single Trp-25 of CBTX (Fig. 1) is less well resolved than that of the dodecamer, or than that typically observed from Trp containing peptides and proteins [7,8]. The triplet properties of this residue are anomalous in other respects as well. While the phosphorescence (0,0) wavelength of 411.6 nm falls in the 410–415 nm range typically shown by Trp residues in hydrophobic regions, the 2E resonance frequency at 2.47 GHz is significantly lower than the range above 2.55 GHz which usually characterizes this class of residues [9]. Moreover, the usually strong D–E resonance is not observed with our broadband (Hg arc lamp plus Corning 7-54 filter) optical excitation. Furthermore, the phosphorescence decay shows two components (45% of total initial amplitude) with lifetimes significantly shorter than the normal 5.5–6.5 s lifetime range typically observed for Trp in proteins [15].

It is quite likely that the short lifetime components result from triplet quenching by the Cys-26–Cys-30 disulfide bridge, which is nearly in van der Waals contact with Trp-25 in CBTX [16]. From model studies [17] and studies with disulfide containing proteins [18], it is known that disulfide is an efficient quencher of Trp excited states, most likely by electron transfer processes. Moreover the red-shifted phosphorescence of this Trp might also be attributable to the proximity of the polarizable disulfide linkage, rather than to a generalized hydrophobic environment. The crystal structure of CBTX [16] shows that the toxin does not possess a true hydrophobic core, and that the Trp-25 residue is not part of a hydrophobic domain. On the other hand, electrostatic interactions of polarizable disulfide linkages with aromatic side chains in proteins have been cited as an important factor in their conformational stability [19].

The interactions with disulfide can also be used to rationalize the broadness of the CBTX Trp phosphorescence and the distribution of lifetimes seen. It has been

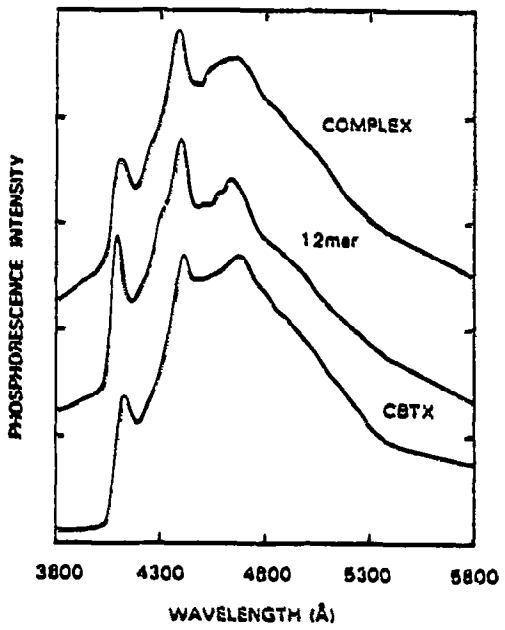

Fig. 1. Phosphorescence of CBTX, 12-mer and CBTX–12-mer complex at 1.7 K.

shown by model studies [17] that the Trp-disulfide interaction falls off rapidly with increasing distance; a change of 0.03–0.04 nm in this distance can cause a factor of two change in the Trp triplet decay rate [18]. Moreover, NMR studies of CBTX [20] have shown that the resonances of Trp-25 are very sensitive to temperature, and have provided evidence for two conformational ensembles in the range of 4–88°C; this work has suggested that Trp-25 resides in a conformationally 'mobile' region [20]. Thus it is likely that a distribution of conformations may exist in the cryogenic solvent, giving rise to a spread in Trp-disulfide distances, which in turn causes the spread in phosphorescence lifetimes observed, the poorly resolved phosphorescence and the lack of D–E resonance. When narrower band optical excitation is used, the D–E transition can be seen, and its resonance frequency depends on excitation wavelength [21].

3.2. Receptor peptides and complexes with CBTX

The α185–196 dodecamer, which contains a single Trp at position 187, shows a much better resolved phosphorescence spectrum than the toxin (Fig. 1), with a (0,0) at 409 nm and a D–E ODMR frequency of 1.73 GHz (Fig. 2a), which falls within the range of λ_{phos} = 405–409 nm and D–E = 1.7–1.8 GHz usually associated with solvent-exposed tryptophan residues [9]. The phosphorescence decay profile is dominated (86%) by a long lifetime (6.10 s) component, which falls in the range typical of Trp residues in proteins. The 14% short-lived contribution might be attributed to the close proximity

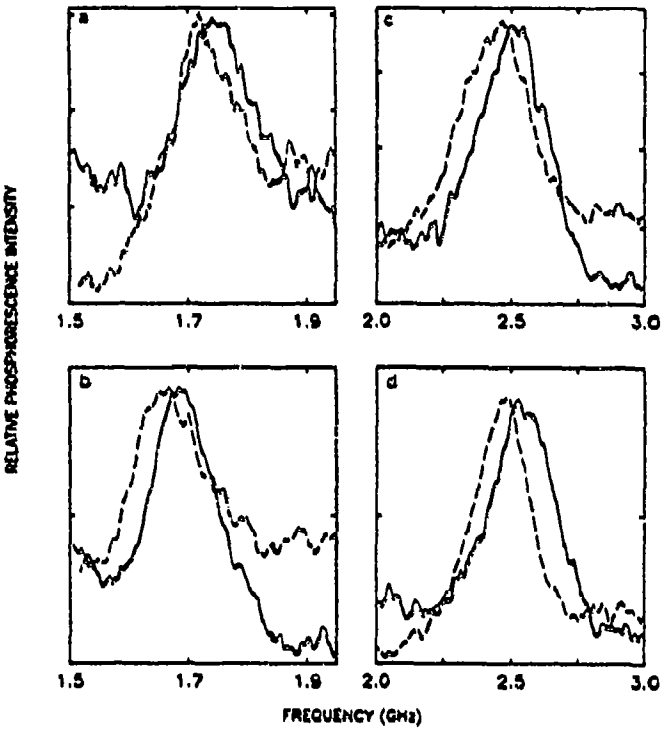

Fig. 2. ODMR spectra of (a) 12-mer, D–E transition, (b) 18-mer, D–E transition, (c) 12-mer, 2E transition, and (d) 12-mer–CBTX complex, 2E transition. Solid and dashed traces denote forward and backward microwave sweeps, respectively. Transition frequencies quoted in Table I are averages of peak positions for forward and backward sweeps.

of His-186; histidine is a known quencher of Trp *fluorescence* [22].

In the dodecamer-CBTX complex, with two Trp residues, there is the problem of assigning the emission which is seen. Since the CBTX phosphorescence is qualitatively much weaker than the peptide phosphorescence at similar concentrations, we will make the assumption that the phosphorescence of the complex is primarily due to Trp-187 in the 12-mer. This assumption would be invalidated if, in the complex, CBTX were to undergo a conformational change which would increase the distance of Trp-25 from the quenching disulfide linkage. However, on the contrary, it has been shown that CBTX binding of calf [4] and *Torpedo* peptides [23] produce quenching of fluorescence. Thus it is reasonable to assign the phosphorescence of the complex primarily to Trp-187 in the dodecamer.

With this assumption, we can state that the dodecamer phosphorescence (0,0) undergoes a slight, but measurable red shift, the 2E resonance frequency increases by 27 MHz (Fig. 2c,d) and the D–E decreases by 18 MHz when the complex forms (Table I). All these changes are indicative of a slight increase in the hydrophobicity of the Trp-187 environment upon binding to CBTX. There is however no evidence for aromatic stacking interactions, which would be expected to lower the value of the D parameter, which is related to the degree of separation of the two unpaired spins in the direction perpendicular to the indole ring, by at least 30 MHz [11,12]. In the complex with CBTX, the D of Trp-187 is lowered by only 4 MHz.

The phosphorescence spectrum of the 18-mer (not shown) is less well resolved than that of the 12-mer, and shows a high proportion (41%) of rapidly decaying components. These components *might* be assignable to triplet *tyrosinate*, for which decay times in the range of 1.3 s [24] to 1.6 s [25] have been reported. Tyrosinate *fluorescence* has been observed from the 18-mer; the generation of a negatively charged center on this side chain has been correlated with the strength of binding of this peptide to neurotoxins [26]. Another possible source of rapidly decaying phosphorescence components is quenching by the charged residue lysine-185, which is immediately adjacent to Trp-184 in the 18-mer; the trimer Lys–Trp–Lys at 77 K shows a biexponential phosphorescence decay [11].

The phosphorescence (0,0) of the 18-mer at 412 nm is strongly red shifted from that of the 12-mer (409 nm), the D–E resonance (Fig. 2b) occurs at a noticeably lower frequency (1670 MHz vs. 1732 MHz), and the 2 E resonance frequency is significantly higher (2550 MHz vs. 2488 MHz). All these shifts are indicative of a more hydrophobic environment for the emitting Trp

Table I

Triplet state properties of α-cobratoxin (CBTX), the α185–196 dodecamer, the α181–198 18-mer, and their complexes with CBTX

	Phos (0,0) (nm)	2E (MHz)	D–E (MHz)	Decay parameters*
CBTX	411.6	2474	-	3.5 ± 0.7 (15.8)(0.286) 0.75 ± 0.14 (29.3)(1.33) 0.19 ± 0.01 (53.5)(5.3)
12-mer	409.0	2488	1732	3.2 ± 0.8 (4.2)(0.313) 0.72 ± 0.1 (9.4)(1.39) 0.164 ± 0.001 (86.5)(6.10)
12-mer complex	410.2	2515	1714	3.4 ± 0.17 (13.1)(0.294) 0.7 ± 0.04 (16.7)(1.43) 0.173 ± 0.001 (70.4)(5.78)
18-mer	412.0	2550	1670	3.7 ± 0.23 (18.2)(0.270) 0.81 ± 0.06 (23.1)(1.23) 0.179 ± 0.004 (58.8)(5.59)
18-mer complex	413.0	2541	1663	2.7 ± 0.11 (11.4)(0.370) 0.62 ± 0.03 (16.4)(1.61) 0.173 ± 0.001 (72.2)(5.78)

Phosphorescence wavelengths and ODMR frequencies were measured at 1.7 K in 1:1 by volume 5 mM phosphate buffer, pH 7.4 and ethylene glycol; phosphorescence decay curves were determined at 77 K in 70%:30% by volume glycerol-buffer. The concentrations for CBTX, the dodecamer, and the dodecamer–CBTX complex were 1 mM, while the concentrations for the 18-mer and 18-mer–CBTX complex were 0.5 mM. Measurements for the latter two samples were carried out at pH 5.5

* Data presented in the order: decay constant in s^{-1} (% amplitude in triexponential fitted decay) (corresponding decay time in s).

residues in the 18-mer, as compared to the dodecamer, in agreement with recent room temperature *fluorescence* studies which showed a blue shift with increasing chain length in these peptides [13]. Complexation with the toxin leads to very small changes in the phosphorescence and ODMR spectra. There is a slight (1 nm) red shift in the phosphorescence, suggestive of increasing hydrophobicity, but the ODMR frequencies of the 18-mer and 18-mer–toxin complex are nearly the same within limits of error.

In summary, our phosphorescence and ODMR results for these α-subunit peptides and their complexes with α-cobratoxin are indicative of a slight but measurable increase in hydrophobicity of the peptide Trp residues upon toxin binding. This trend is certainly clearer for the dodecamer, for which the phosphorescence is better resolved. These findings are qualitatively consistent with the binding model put forth by Low and Corfield [5,6], in which a Trp peptide residue is inserted into a hydrophobic toxin cleft. However, the changes in the D parameter are much too small to indicate any aromatic stacking interactions upon binding. Qualitatively similar conclusions regarding the absence of Trp stacking interactions were reached in recent studies of *Torpedo* peptide binding to α-bungarotoxin using fluorescence [13], ODMR [27] and two-dimensional NMR [28].

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