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Photolabeling reveals the proximity of the α-neurotoxin binding site to the M2 helix of the ion channel in the nicotinic acetylcholine receptor

(epitope mapping/functional domains/cross-linking/matrix-assisted laser-desorption-ionization mass spectrometry/protein–protein interactions)

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ABSTRACT **A photoactivatable derivative of neurotoxin II from *Naja naja oxiana* containing a ^{125}I-labeled *p*-azidosalicylamidoethyl-1,3′-dithiopropyl label at Lys-25 forms a photoinduced cross-link with the δ subunit of the membrane-bound *Torpedo californica* nicotinic acetylcholine receptor (AChR). The cross-linked radioactive receptor peptide was isolated by reverse-phase HPLC after tryptic digestion of the labeled δ subunit. The sequence of this peptide, δ-(260–277), and the position of the label at Ala-268 were established by matrix-assisted laser-desorption-ionization mass spectrometry based on the molecular mass and on post-source decay fragment analysis. With the known dimensions of the AChR molecule, of the photolabel, and of α-neurotoxin, finding the cross-link at δAla-268 (located in the upper part of the channel-forming transmembrane helix M2) means that the center of the α-neurotoxin binding site is situated at least ≈40 Å from the extracellular surface of the AChR, proximal to the channel axis.**

The nicotinic acetylcholine receptor (AChR) is a member of the superfamily of ligand-gated ion channels (type I receptors), with a pentameric structure $\alpha_2\beta\gamma\delta$ (for reviews, see refs. 1–4).

In the absence of crystals suitable for x-ray analysis, information concerning the structure of the receptor's functional domains is mainly obtained by using two complementary methods, site-directed mutagenesis and affinity labeling. There are two binding sites for agonists and competitive antagonists located on the α subunits (1, 2), more precisely at the α–δ and α–γ interfaces (5–8). A number of amino acid residues have been identified at or near the binding site: αCys-192, αCys-193, αTyr-198, αTyr-190, αTyr-93, and αTrp-149 (for recent reviews, see refs. 9 and 10). The sequence α-(180–200), which contains the tandem cysteine residues αCys-192/193, seems to be of special importance for the binding of agonists and competitive antagonists. By using a synthetic bifunctional affinity reagent, it was shown that these residues are ≈9 Å away from a negatively charged side chain of the δ subunit (11).

In the present investigation, to map the architecture in the agonist/competitive antagonist-binding site environment, we use as a tool an α-neurotoxin that carries a photoactivatable group in a defined position. High-resolution three-dimensional structures of α-neurotoxins are known from x-ray analysis of crystalline toxins (see, for example, ref. 12) and from NMR studies of the toxins in solution (13).

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α-Neurotoxins possess a relatively rigid structure. This allows us to use them for estimating distances on the toxin-binding surface of the receptor. In this investigation we further introduce matrix-assisted laser-desorption-ionization (MALDI) mass spectrometry (MS) to identify products of photo cross-linking.

MATERIALS AND METHODS

Synthesis and Isolation of ^{125}I-Labeled *p*-Azidosalicylamidoethyl-1,3′-dithiopropyl (ASED) Derivatives of the Neurotoxin II (NT-II) from the Venom of the Central Asian Cobra *Naja naja oxiana*, Photo-Induced Cross-Linking of ASED–NT-II to the Membrane-Bound AChR, and Analysis of Subunit Labeling. The preparative and analytical aspects are described in detail elsewhere (14). AChR-rich membranes from *Torpedo californica* electric tissue were prepared as described (15).

Isolation of the Labeled δ Subunit and Cross-Linked Peptide. Membrane samples (≈10 mg of protein) were suspended in 2.0 ml of 0.1 M sodium phosphate (pH 7.4) and incubated with 0.105 mg (15 nmol) of ASED-NT-II for 60 min in the dark. The subsequent steps, including UV-irradiation, were performed as described (14). The subunits were separated by preparative SDS/PAGE under nonreducing conditions in a tube-gel apparatus, Prep Cell model 491 (Bio-Rad). A 2% stacking gel and a 7.5% separation gel were used. The proteins were eluted with 75 mM Tris·HCl, pH 8.3/0.1% SDS/0.75 M glycine. Fractions (2 ml) were collected. The radioactive fractions containing the δ-subunit dimers were concentrated and precipitated from the concentrated solution with a 1:4 (vol/vol) mixture of chloroform and methanol. The pellet was dissolved and separated by slab gel SDS/PAGE under reducing conditions. The slab gels were 0.75 mm or 1.0 mm thick. They were stained with 0.1% Serva Blue R in 25% (vol/vol) 2-propanol/10% (vol/vol) acetic acid and destained with the same solvent without dye. The band corresponding to the δ subunit was cut out and submitted to tryptic digestion and internal sequence determination (16).

Determination of the Structure of the Cross-Linked Peptide by MALDI-MS. After HPLC, the radioactive fractions, as well as a number of neighboring fractions, were dried and dissolved in 10 μl of 70% (vol/vol) formic acid. Aliquots (3 μl) were mixed with the same volume of 10 mM dihydrobenzoic acid and transferred to a gold-plated sample holder. The solution was dried with gently blowing air, leaving behind a 3- to 4-mm

Abbreviations: AChR, nicotinic acetylcholine receptor; ASED, *p*-azidosalicylamidoethyl-1,3′-dithiopropyl; MALDI, matrix-assisted laser-desorption-ionization; NT-II, neurotoxin II; PSD, post-source decay; u, unified atomic mass unit(s).

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patch of microcrystalline dihydrobenzoic acid matrix. Typical sample loads were 1–3 pmol.

MS was performed on two instruments in parallel. While a linear time-of-flight (TOF-MS1) machine was used for quick identification of prevalent analyte ions, a home-built reflectron type time-of-flight (ReTOF) instrument (TOF-MS2) was employed for high-precision (≈0.02%) molecular weight determination of precursor ions and for post-source decay (PSD) fragment ion mass analysis (for a full technical description, see ref. 17). Both instruments were equipped with a model VSL-337ND (Laser Science, Cambridge, MA) nitrogen laser.

Spectral averaging (up to 1000 spectra) with a duty cycle of 10 Hz was performed by a LeCroy (Chestnut Ridge, NY) recorder prior to data transfer to and processing in a 386 PC running the software ULYSSES, version 7.2 (Chips at work, Bonn). For the characterization of the PSD peptide product ions, the nomenclature of refs. 18 and 19 has been used. Compilation of peptide product ion tables and subsequent searching routines were performed on a 486 PC by using the software THE MALDI-PSD PEPTIDE SEQUENCER, version 2.0 (copyright by Frank Lützenkirchen, Düsseldorf, Germany).

RESULTS

Photoactivatable α-Neurotoxin Derivative. In this investigation we used the ^{125}I-labeled ASED derivative of NT-II with the label attached to Lys-25 (ASED-NT-II). The important advantage of this derivative is the cleavable -S–S- group, which allows the removal of the toxin (Fig. 1) after cross-linking and separation of the receptor subunits. The site of the cross-linking on the AChR can be analyzed more easily because only part of the label (that which includes the radioactivity) remains at the receptor.

Specificity and Yield of Photo Cross-Linking with ASED-NT-II. Photoaffinity labeling of membrane-bound AChR resulted in predominantly cross-linking the δ subunit (Fig. 2). Considerably lower amounts of radioactivity were incorporated into the other subunits. Incubation of AChR with ASED-NT-II and photoactivation in the presence of a 40-fold excess of underivatized toxin prevented the photolabeling of the receptor virtually completely. Photolabeling could also be prevented by *d*-tubocurarine, the IC_{50} being 320 μM (14).

The cross-linking yield was 5.9% for the δ subunit. The yield is quite high compared to what is usually observed with nitrene-generating arylazide photolabels. However, the majority of the δ-subunit protein remains unlabeled, a fact that complicates the further analysis considerably.

Preparative Isolation of Labeled δ Subunit. Purifying the cross-linked δ subunit started with a preparative SDS/PAGE under nonreducing conditions. Four major radioactive fractions I–IV were obtained. Fractions I and II were eluted close to the gel front, representing free iodine and photoinactivated ASED-NT-II. Fraction III contained mostly δ-subunit monomer but was contaminated with other subunits. As shown by analytical SDS/PAGE, the predominant component of fraction IV was the dimeric δ subunit [dimerized due to a disulfide bridge between the δ subunits of the neighboring AChR molecules (20)]. The further experiments were performed with this fraction only.

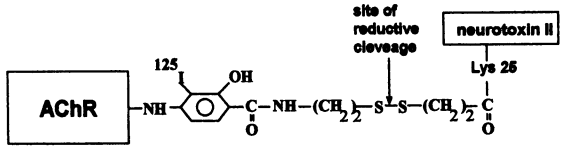

FIG. 1. Scheme of AChR labeling with a snake venom neurotoxin containing a cleavable photoactivatable group.

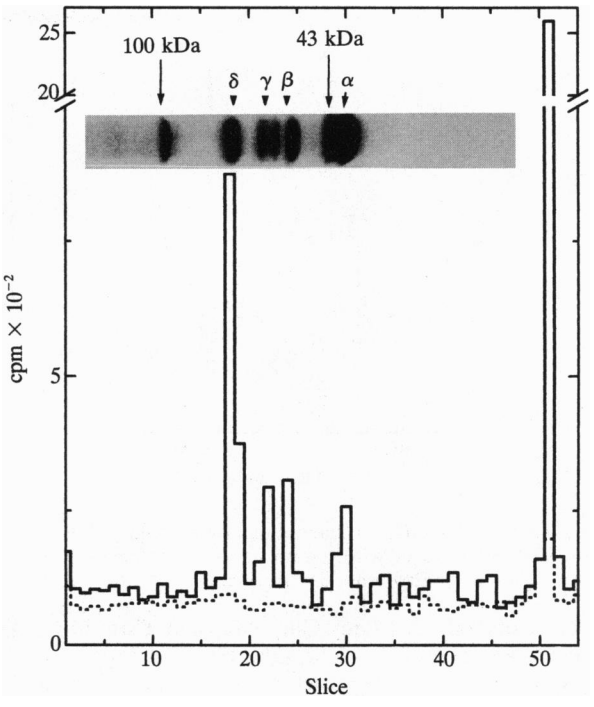

FIG. 2. Radioactivity profile (solid line) of the analytical SDS/PAGE for the AChR membranes labeled with ASED-NT-II. Dashed line represents the same, but the cross-linking was performed in the presence of nonderivatized NT-II. The width of each slice is 2 mm. (*Inset*) Gel stained with Serva Blue R. The band at 100 kDa corresponds to the Na/K-ATPase β subunit.

Tryptic Cleavage of Cross-Linked δ Subunit. Fraction IV was reelectrophoresed on a preparative SDS/polyacrylamide slab gel under reducing conditions. The band containing the labeled (and the excess unlabeled) δ subunit was excised from the gel. This band contained ≈50% of the radioactivity applied to the gel, the other 50% being distributed over the rest of the gel. After tryptic cleavage of the protein in the gel, extraction, and separation of the cleavage products by reversed-phase HPLC (16), two major radioactive peaks were obtained (peaks A and B in Fig. 3). Fractions A and B contained ≈20% (2000 cpm, 34 pmol of iodine) and ≈5% (500 cpm, 9 pmol of iodine), respectively, of the total radioactivity applied. These fractions were further analyzed.

Identification of the Cross-Linking Site in the δ Subunit by MALDI-MS. It is apparent from the mass spectra (Fig. 4) that the respective peptide samples were not homogeneous. While, for example, fractions 127, 128, and 142 exhibited a prominent mass signal at 2278.2 unified atomic mass units (u) that did not match any tryptic fragment of the AChR, fraction 127 had, in addition, a comparably prominent signal at mass 2292.2 u, which was confirmed by PSD fragment ion analysis as a tryptic fragment (amino acids 26–46) of the δ subunit. The averaged intensity of the mass 2278.2 u closely parallels the radioactivity profile, indicating that this peak belongs to the molecule containing the radioactive label.

By subtracting the mass of reduced label (336 u, see Fig. 1) from the recorded mass of the 2278.2 u unknown, one ends up with 1942.2 u for a hypothetical peptide. Since this is not a tryptic peptide, a computer search was performed to determine all potential amino acid stretches within the δ subunit matching this mass. After folding these propositions with the so-called "small fragment ion criteria" filter (Table 1) from which incident or absent residues can be predicted with fairly good confidence, only one proposition was left over [δ-(260–277)] with a full match of the imposed criteria (predicted

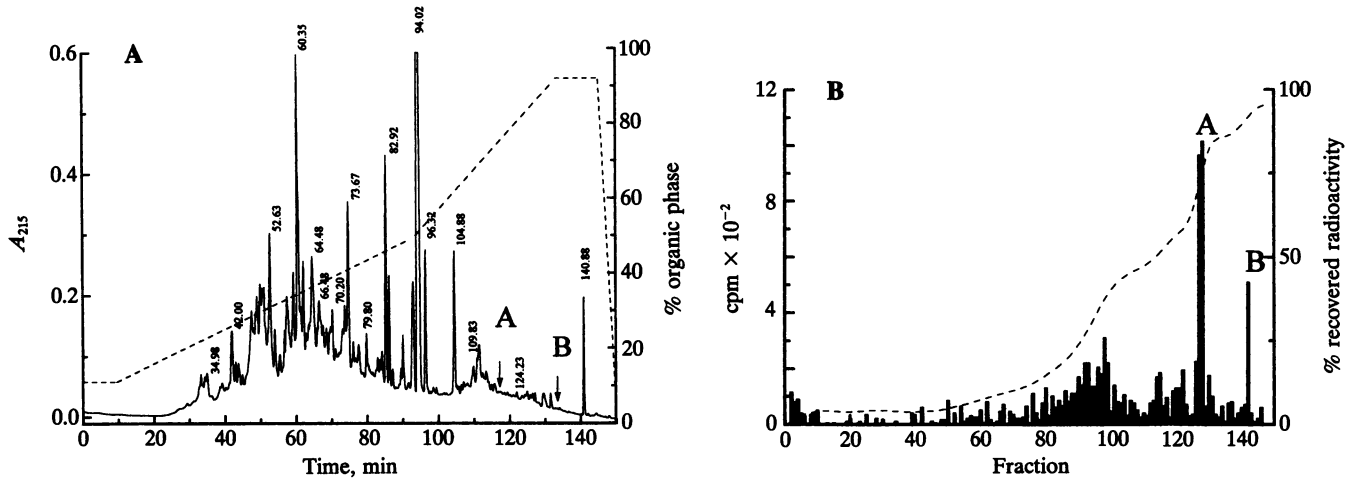

FIG. 3. HPLC isolation of the radioactive cross-linked peptides from the AChR δ subunit labeled with the ASED-NT-II. A Vydac C_{18} column (4.6 × 250 mm) was used with a gradient HPLC system (Waters). Aqueous phase, 0.1% trifluoroacetic acid; organic phase, 0.085% trifluoroacetic acid in acetonitrile. Flow rate, 0.6 ml/min. (*A*) UV profile. (*B*) Radioactivity profile.

presence of Val, Ile, Leu, Glu, Phe, and C-terminal Arg; predicted absence of Pro, Thr, Glu, His, and Trp).

The proposed peptide structure was further confirmed by PSD fragment ion analysis executed on the 2278.2-u precursor in fractions 128 and 142. The list of fragment ions obtained in both cases almost fully matched the fragmentation table of the putative peptide structure. Among the 43 (out of 45) assignable fragments, 7 were found with the photolabel still attached (Table 2). At the same time, 9 of the recorded fragments had apparently lost the label. Within this group of PSD ions, fragment 43 at 1942 u corresponds to the nominal mass of the precursor peptide after cleavage of the label. The unlabeled product ions listed in Table 2 can be traced up to residue 265 (Leu) from the side of the N terminus (b_n type cleavages) and up to residue 269 (Val) from the side of the C terminus (y_n type cleavages). Thus, the putative site of the photolabel attachment could be narrowed down to the 3-residue stretch δ266–268. In the sequence ladder, the first N-terminal fragment bearing the label came as the b_9 fragment (cleaved on the C-terminal side of the δAla-268, fragment 33 in Table 2). The first C-terminal fragment carrying the label was the y_{10} fragment (cleaved at the N-terminal side of δAla-268, fragment 38 in Table 2). Thus, δAla-268 is unequivocally the attachment site for the photo-cross-linked label.

We found that the presence of 10^{-5} to 10^{-4} M tetracaine, a channel blocker, does not affect the binding of ASED-NT-II to AChR. However, it protects the δ subunit from labeling by ≈50% (data not shown).

DISCUSSION

We found that the Lys-25-labeled photoactivatable derivative of NT-II forms a photo-induced cross-link with δAla-268 of the AChR. This residue is located in the upper part of the

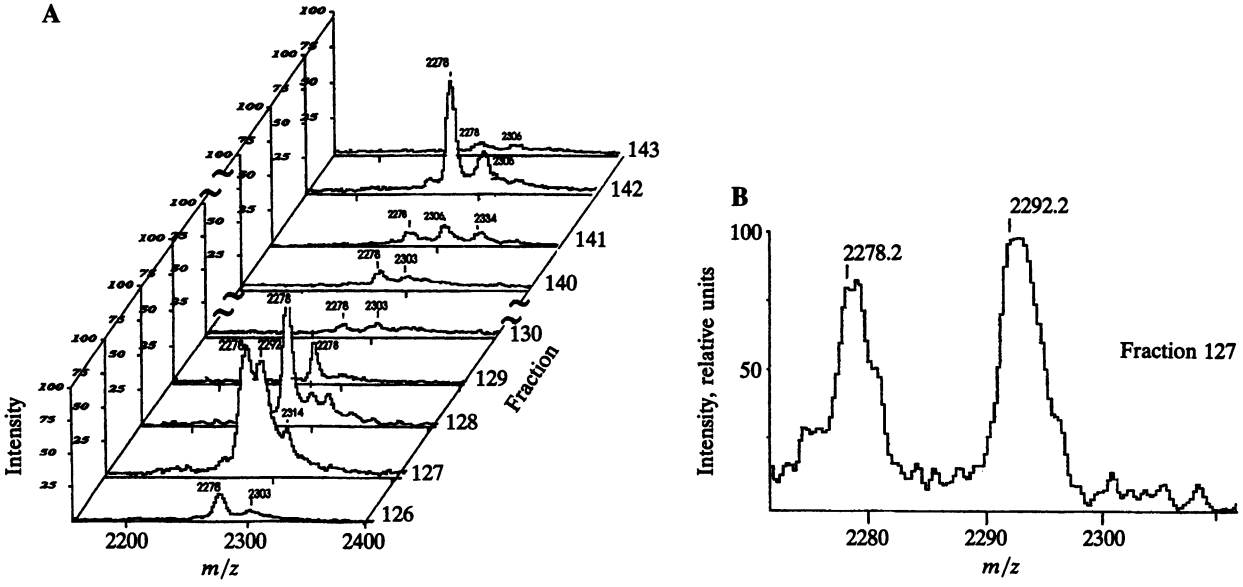

FIG. 4. Mass spectra of HPLC fractions (from Fig. 3) containing cross-linked peptides as indicated by radioactivity and UV absorption. (*A*) In the low-resolution linear time-of-flight (TOF)-MS, radioactive fractions 127, 128, and 142 exhibited a common leading mass signal at 2278 u, the intensity of which paralleled the radioactivity profile (data not shown). In fraction 127, an equally prominent signal at mass 2292 u could be identified as the tryptic fragment δ-(26–46) (nominal monoisotopic mass = 2292.2 u) by PSD fragment ion analysis. It has been taken as an internal standard for the nearby 2278 u unknown in the high-resolution reflectron type time-of-flight (ReTOF)-MS spectra (*B*) for which a monoisotopic mass of 2278.2 ± 0.1 u could be determined.

Table 1. Computer search of all possible amino acid stretches within the δ subunit matching the mass 1943.2 u (1942.2 u + H)

Search tolerance, u	No.	Amino acid positions	M+H(m)	M+H(a)	Sequence	Small fragment ion criteria										
						− P	+ V	+ I/L	− T	− E	− H	+ F	+ Q	− Y	− W	+ R/ct
±1	1	62–76	1942.84	1943.16	WYDHRLTWNASEYSD-OH	*		*								
	2	106–121	1942.98	1944.37	YFCNVLVRPNGYVTWL-OH		*	*		*	*	*				
	3	142–158	1943.98	1944.32	QNCSLKFTALNYDANEI-OH	*		*			*	*	*		*	
	4	214–228	1944.11	1944.41	DVTFYLIIRRKPLFY-OH		*	*		*	*	*			*	
	5	236–253	1944.02	1944.40	CVLISFLASLAFYLPAES-OH		*	*	*		*	*			*	
	6	239–256	1943.02	1943.32	ISFLASLAFYLPAESGEK-OH			*	*		*	*			*	
	7	247–264	1942.98	1943.38	FYLPAESGEKMSTAISVL-OH		*	*			*	*			*	
	8	**260–277**	**1943.17**	**1943.48**	**AISVLLAQAVFLLLTSQR-OH**	*	*	*	*	*	*	*	*	*	*	*
	9	338–353	1942.84	1943.25	LHMSRADESEQPDWQN-OH			*	*				*	*		
	10	342–357	1943.91	1944.24	RADESEQPDWQNDLKL-OH			*	*		*		*	*		
	11	343–358	1943.91	1944.24	ADESEQPDWQNDLKLR-OH			*	*		*		*	*		*
±0.5	6	239–256	1943.02	1943.32	ISFLASLAFYLPAESGEK-OH			*	*		*	*			*	
	8	**260–277**	**1943.17**	**1943.48**	**AISVLLAQAVFLLLTSQR-OH**	*	*	*	*	*	*	*	*	*	*	*
	9	338–353	1942.84	1943.25	LHMSRADESEQPDWQN-OH			*	*				*	*		
±0.2	6	239–256	1943.02	1943.32	ISFLASLAFYLPAESGEK-OH			*	*		*	*			*	
	8	**260–277**	**1943.17**	**1943.48**	**AISVLLAQAVFLLLTSQR-OH**	*	*	*	*	*	*	*	*	*	*	*

Three sets of propositions are obtained with search tolerances ±1 u, ±0.5 u, and ±0.2 u. Even at a tolerance of ±0.2 u, there are still two propositions. Small fragment ion criteria indicates unambiguously which of the two is the peptide fully matching the criteria. A + or − symbol above the amino acid on top of the right column indicates whether an amino acid must or must not be present in the peptide, respectively. ct, C terminus. An asterisk indicates which of the criteria is met in a given peptide of the computer search. Only the peptide δ-(260–277) (in boldface type) meets all criteria.

channel-forming putative transmembrane helix M2 (1–3). The results indicate that the bound neurotoxin is accommodated rather deep in the AChR pore, at least ≈40 Å away from its extracellular entrance.

The specificity of AChR δ-subunit labeling by ASED-NT-II was verified by various controls. The results of the competition experiments with either unlabeled NT-II (Fig. 2) or *d*-tubocurarine indicate that the δ subunit has been labeled at the low-affinity binding site for *d*-tubocurarine (7, 14). On the other hand, the noncompetitive antagonist tetracaine, which blocks the closed channel with a K_d value of 0.3 μM (J. B. Cohen, personal communication), decreases cross-linking without affecting the binding of the NT-II derivative. Also tetracaine was recently shown to react with δAla-268 upon photoactivation (J. B. Cohen, personal communication). This is in full agreement with the observed localization of the photo-cross-linked label imported by the NT-II derivative.

The following interpretation of the results is based on the assumption that the ASED-NT-II derivative finds a preferential sterical orientation within the topology of its binding

Table 2. PSD product ions of 2278-u precursor

Fr.	m_o	m_c	Δ	Fragment assigned	Product ion amino acid stretch
1–21				Not shown	
22	595.9	596.4	−0.5	$(B_{10}Y_{14})_6+1$, $(B_9Y_{15})_6+1$	AISVLLAQAVFLLLTSQR†
23	604.6	604.5	0.1	y_5	AISVLLAQAVFLLLTSQR
24	623.1	623.4	−0.3	$(C_9Y_{12})_3+3$+label	AISVLLAQAVFLLLTSQR*
25	656.1	656.4	−0.3	$(B_{17}Y_7)_6+1$	AISVLLAQAVFLLLTSQR
26	716.9	717.5	−0.6	y_6	AISVLLAQAVFLLLTSQR
27	744.1	743.5	0.6	$(B_{11}Y_{14})_7+1$, $(B_{13}Y_{12})_7+1$	AISVLLAQAVFLLLTSQR†
28	771.3	771.5	−0.2	w_6	AISVLLAQAVFLLLTSQR
29	830.3	830.6	−0.3	y_7	AISVLLAQAVFLLLTSQR
30	977.7	977.7	0.0	y_8	AISVLLAQAVFLLLTSQR
31	1076.0	1076.8	−0.8	b_{10}	AISVLLAQAVFLLLTSQR†
32	1147.1	1147.8	−0.7	y_{10}	AISVLLAQAVFLLLTSQR†
33	1203.8	1202.7	1.1	b_9+label	AISVLLAQAVFLLLTSQR*
34	1301.3	1301.5	−0.2	b_{10}+label	AISVLLAQAVFLLLTSQR*
35	1346.0	1347.0	−1.0	y_{12}	AISVLLAQAVFLLLTSQR†
36	1399.5	1398.9	0.6	$(B_{17}Y_{14})_{13}+1$	AISVLLAQAVFLLLTSQR†
37	1457.9	1456.9	1.0	$(B_{16}Y_{16})_{14}+1$	AISVLLAQAVFLLLTSQR†
38	1482.9	1482.6	0.3	y_{10}+label	AISVLLAQAVFLLLTSQR*
39	1561.9	1561.7	0.2	b_{12}+label	AISVLLAQAVFLLLTSQR*
40	1509.8	1509.7	0.1	$(C_6Y_{13})_{11}+3$+label	AISVLLAQAVFLLLTSQR*
41	1570.8	1570.0	0.8	$(B_{16}Y_{17})_{15}+1$	AISVLLAQAVFLLLTSQR†
42	1673.8	1675.0	−1.2	b_{13}+label	AISVLLAQAVFLLLTSQR*
43	1941.9	1942.2	−0.3	Parent ion minus label	AISVLLAQAVFLLLTSQR†

Partial listing of PSD fragment ions recorded from the 2278-u precursor ion (HPLC fraction 128). m_o, Mass of PSD fragment observed; m_c, mass of PSD fragment calculated according to the sequence of the δ-(260–277) of the AChR δ chain; *, putative fragment bearing the 336-u label on residue 8 (Ala); †, regular peptide after cleavage of the label. Fragment nomenclature according to refs. 18 and 19 for C- and N-terminal ions, and for internal fragments, respectively. The Ala in boldface type is δAla-268; underlined residues represent the peptide for which the mass m_c was calculated.

environment. The alternative of a nonspecifically oriented carrier just "plunged" into the channel "at random" appears rather unlikely. Such a "random" orientation would also be hard to reconcile with the observed high site-specific cross-linking yield.

The maximal extension of the photolabel attached to Lys-25 is 16.5 Å. If we add the length of the Lys side chain (≈7 Å), we conclude that the C^{α} atom, located close to the center of the three-dimensional NT-II structure (13), should be not more than ≈23 Å away from δAla-268.

Electron microscopy describes the AChR in the following dimensions: From its total length of 125 Å, 60 Å protrude into the synaptic cleft (21). This tube-like entrance portion has a constant diameter of 20–25 Å. Within the transmembrane region, it forms a funnel narrowing to 11.5 Å at the binding site of chloropromazine and triphenylmethyl phosphonium (22, 23) and, finally, to 6.5 Å at the location of the ion gate, a part that cannot be resolved by electron microscopy at present resolution (9 Å).

The estimates available so far of the distance between agonist/antagonist binding sites and the membrane surface were obtained from the fluorescence energy transfer measurements (24–26). The photo-cross-linking experiments described here introduce a method for directly measuring the distance between the channel moiety and the ligand-binding domain.

Based on the AChR polypeptide chain topology models (1–3), a neurotoxin center within a distance of ≈23 Å from the δAla-268 translates into a location of this center at a depth of ≈40 Å or deeper from the channel entrance (or ≈20 Å or closer to the membrane bilayer). It also follows that the neurotoxin center should be in proximity to the channel axis. At first glance this seems to contradict evidence from studies on biotinylated toxin (27) and studies with toxins bearing fluorescent labels (24) that localized the toxin at the outer surface of the AChR. However, in both cases a toxin from *Naja naja siamensis* (modified preferentially at Lys-23) was employed. Since this residue protrudes to the opposite side compared to Lys-25 of the NT-II from *Naja naja oxiana* (13, 28), a biotinyl or a fluorescein moiety may well be accessible from the outside in contrast to the Lys-25 side chain, which, in our case, points toward the interior of the channel.

Besides the implications for the topology of the functional domains in the AChR, this investigation has clearly shown the potential of MALDI–PSD-MS for structural investigations following a photoaffinity cross-linking experiment. The classic approach to identifying a cross-linked amino acid by Edman sequencing may often be spoiled by the nonhomogeneous character of the HPLC samples in which the labeled target peptide may be among the least abundant peptides. Such ambiguity can be overcome by MALDI-MS, provided a precursor ion selector and, thereby, a PSD option is available.

The peptide δ-(260–277) is not a true tryptic peptide at its N terminus. "Nontryptic" peptides in a tryptic digest are not rare (for example, see refs. 29 and 30). They may occur with trypsin not treated with L-tosylamido-2-phenylethyl chloromethyl ketone to eliminate chymotryptic activity. Such preparations have been also shown to cleave the peptide bonds after Thr (31).

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