DISULFIDE BOND CROSS-LINKED DIMER IN ACETYLCHOLINE RECEPTOR FROM TORPEDO CALIFORNICA

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SUMMARY Acetylcholine receptor from Torpedo californica electric tissue occurs in membrane, and is purified, as a mixture of monomer and dimer. Dimer is cross-linked by disulfide bonds involving one of the four polypeptide components of receptor, namely the one of apparent molecular weight of 64,000.

INTRODUCTION The acetylcholine receptor from electric tissue of Torpedo species, as isolated in non-ionic detergent solution, is heterogeneous in molecular weight (1-8). In Torpedo californica, two forms of receptor predominate, which by sedimentation analysis have molecular weights of 250,000 and 500,000 (Reynolds and Karlin, in preparation). These species have identical numbers of α-neurotoxin binding sites per mass of protein, namely 2 per 250,000 daltons, and of sites capable of reacting with the affinity label, 4-(N-maleimido)benzyltrimethylammonium iodide, namely 1 per 250,000 daltons (4,5). In addition, these species yield apparently identical polypeptide components when reduced and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (4,5). These species may therefore be considered monomeric (I*) and dimeric (II) forms of receptor.

I and II are apparently stable forms since they can be isolated and subjected to other procedures, such as resedimentation and non-denaturing gel electrophoresis, without interconversion or other obvious changes in hydrodynamic properties (4,5). We present evi-

*Receptor monomer, I; receptor dimer, II.

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dence that receptor in Torpedo electric tissue membrane, as well as in detergent solution, is a mixture of monomer and dimer and that monomers are linked in dimer by disulfide bonds.

MATERIALS AND METHODS Receptor was purified as previously described (9) except that the affinity gel was derivatized with bromoacetylcholine. Receptor partially labeled with [^{3}H]α-neurotoxin was subjected to sucrose density gradient centrifugation to separate monomer and dimer (see Figure 1). The pooled fractions from each peak were concentrated by ultrafiltration in a collodion bag apparatus (Schleicher & Schuell). Membrane was isolated from T. californica electric tissue following ref. 10. The principal α-neurotoxin of Naja naja siamensis (11) was methylated with formaldehyde and [^{3}H]$NaBH_4$ to a specific activity of about 10 Ci per mmole (9).

RESULTS AND DISCUSSION The distribution of bound [^{3}H]α-neurotoxin after sedimentation in a sucrose density gradient of purified receptor labeled with [^{3}H]α-neurotoxin is shown in Fig. 1A. Approximately equal numbers of α-neurotoxin binding sites are associated with monomer (I) and dimer (II). The ratio of the numbers of binding sites, II:I, is in the range 1 to 1.2 (3 preparations). Since there are two sites per monomer and four sites per dimer, purified receptor contains about two molecules of monomer per dimer. This ratio is independent of the degree of saturation of the binding sites with α-neurotoxin (3% to 100%), of the ionic strength of the medium (60 mM to 460 mM), and of the concentration of receptor applied to the gradient, in the range of 0.1 mg per ml to 1 mg per ml. Furthermore, the ratio is the same in three different detergents, Triton X-100, Brij 58, and Ammonyx LO.

A dramatic change in the ratio of binding sites, II:I, occurs following the treatment of receptor with dithiothreitol; e.g., in Figure 1A, the ratio is 1.1 in the control and 0.24 in the sample incubated with dithiothreitol (cf. ref. 3,8). This result suggests that reduction of one or more disulfide bonds in II favors its dissociation to I. In fact, reduction with dithiothreitol converts isolated II almost completely into a species with a sedimentation

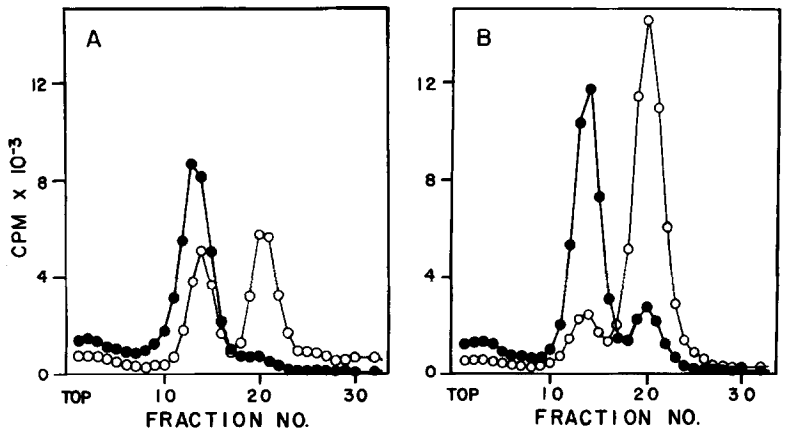

Figure 1. Effect of dithiothreitol on the sedimentation velocities of receptor (A) and of receptor dimer (B). (A) Receptor purified by affinity chromatography (5 µg and 31 pmoles of toxin binding sites), in 100 µl of buffer containing 0.2% Triton X-100, 100 mM NaCl, 5 mM $NaPO_4$, 10 mM TrisCl, 1 mM EDTA, 3 mM NaN_3 (pH 8.0), was incubated for 30 min at 25° either with (●) or without (○) 5 mM dithiothreitol. The pH was brought to 7.0 with 10 µl of 0.56 M $NaPO_4$ (pH 6.7), and 25 pmoles of [^{3}H]α-neurotoxin in 10 µl were added. After 10 min at 25°, 100 µl-aliquots were layered over 17 ml of a linear, 5% to 20% sucrose gradient in a buffer containing 0.2% Triton, 50 mM NaCl, 10 mM $NaPO_4$, 1 mM EDTA, 3 mM NaN_3 (pH 7.0). Centrifugation was in a SW 27.1 rotor at 27,000 rpm, 5°, for 19 hr. Fractions of 0.3 ml (0.6 ml after fraction no. 30) were collected, mixed with 5 ml of Scintisol Complete (Isolab) and counted. Fraction no. 1 is from the top of the tube; the fractions beyond no. 31 were featureless and are not shown. (B) Dimer was isolated by sucrose density gradient centrifugation of purified receptor as in (A); the 12.9 S peak fractions (no. 18 to 23) were pooled and concentrated by ultrafiltration. Dimer (5 µg and 40 pmoles toxin sites) was incubated either with (●), or without, (○), 5 mM dithiothreitol and analyzed as in (A).

velocity identical to that of I (Fig. 1B).

The dimeric form of receptor is also present in membrane isolated from electric tissue of Torpedo californica (Fig. 2) and is more prevalent than in purified receptor preparations. The ratio of binding sites, II:I, is 1.5-2.5, or there is about one molecule of dimer per monomer. As with purified receptor, incubation of the membrane fragments with dithiothreitol converts II to I (Fig. 2).

Since solubilization of receptor in detergent solution pre-

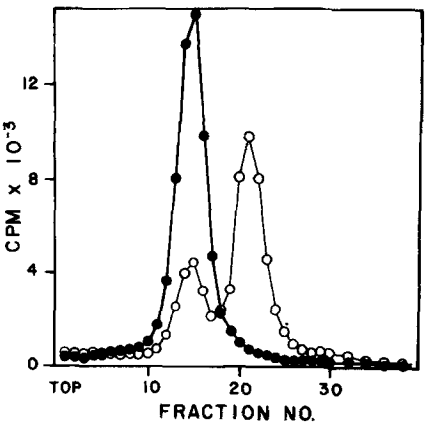

Figure 2. Effect of dithiothreitol on receptor in membrane. Membrane from Torpedo electric tissue (150 µg protein and 135 pmoles of toxin sites) in 100 µl of buffer containing 75 mM NaCl, 10 mM TrisCl, 1 mM EDTA (pH 8.0) was incubated with (●), or without, (○), 10 mM dithiothreitol for 30 min at 25°. The pH was adjusted to 7.0, the volume was increased to 175 µl, and the mixtures were centrifuged in a Beckman Airfuge at 100,000 rpm for 3 min. The supernatants were removed and the pellets extracted in 250 µl of 1% Triton, 50 mM NaCl, 10 mM $NaPO_4$, 1 mM EDTA (pH 7.0). [^{3}H]toxin (45 pmoles) were added and, after 15 min, 100 µl aliquots were centrifuged and fractions collected and counted as in Fig. 1.

cedes analysis for I and II, a possibility to be considered is that disulfide linked dimer is formed by oxidation during solubilization. Three experimental results argue against this possibility. II is converted to I in the membrane by dithiothreitol, which however can be removed prior to solubilization of the membrane: no spontaneous reoxidation of reduced I to II occurs under these conditions (Fig. 2). When membrane is solubilized in the presence of 0.2 mM dithiothreitol at pH 7.0, conditions which do not result in the conversion of II to I, but which should protect against the oxidation of receptor sulfhydryl groups, the ratio of II to I is the same as in the control without dithiothreitol (Fig. 2). Finally, when membrane is solubilized in sodium dodecyl sulfate, which is less likely than solubilization in Triton X-100 to favor disulfide formation, disul-

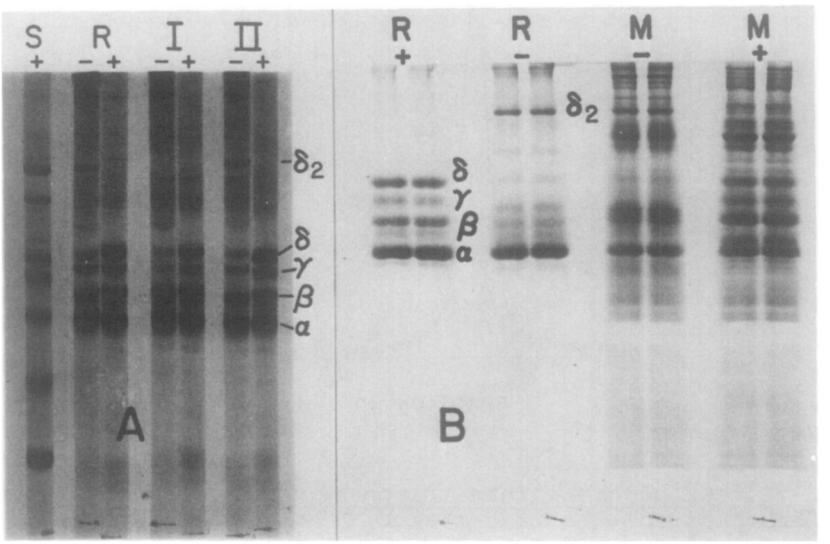

Figure 3. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of reduced (+) and unreduced (-) receptor. (A) Receptor purified by affinity chromatography (R), and monomer (I) and dimer (II), prepared by sedimentation as in Fig. 1, were dialyzed, precipitated with acetone, and redissolved at 50° in sample buffer (2% SDS, 10 mM Tris-acetate, 2 mM EDTA, 10% sucrose, pH 8.0) with (+), or without, (-), 20 mM dithiothreitol (10). About 20 µg of each were applied to cylindrical, 5.6% acrylamide gels, which were run and stained as previously (9). The molecular weight standards (S) were β-galactosidase, phosphorylase, bovine serum albumin, γ-globulin (heavy and light chains), aldolase, and lysozyme. (B) Purified receptor (R) and Torpedo membrane (M) were dissolved in sample buffer (2% SDS, 63 mM TrisCl, 10% glycerol, pH 6.8 with (+), or without (-), 10 mM dithiothreitol. About 9 µg of each sample were layered in wells in a 4% stacking gel over a 10% resolving gel and run as described by Laemmli (16). Staining was as in (A).

fide cross-linked polypeptide chains are obtained (see below). We conclude that II exists in the isolated plasamalemma of the Torpedo electroplax. We cannot however, exclude the possibility that II is formed by oxidation during the isolation of the membrane by subcellular fractionation.

The probable location of the disulfide links between monomers in dimer is revealed by SDS*-polyacrylamide gel electrophoresis.

*Sodium dodecyl sulfate, SDS.

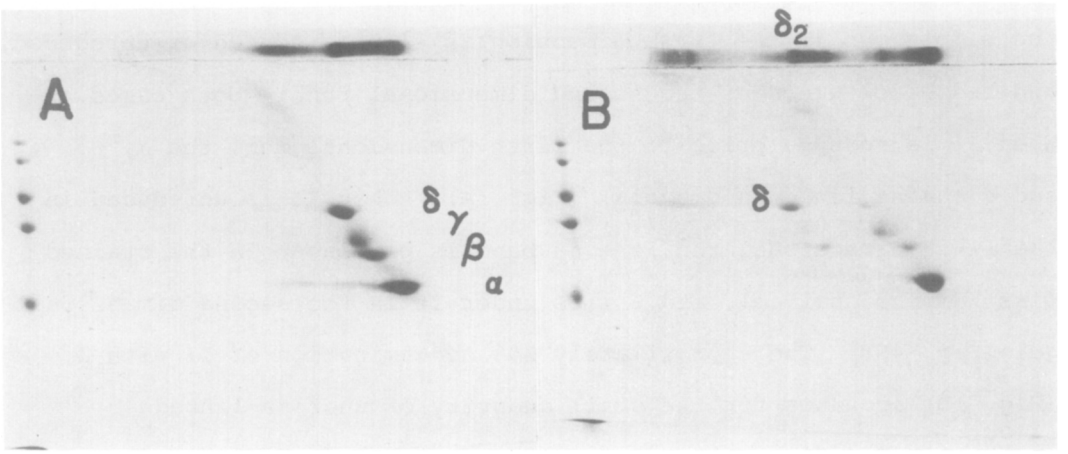

Figure 4. Two-dimensional gel electrophoresis of dimer. About 25 µg of dimer were dissolved in sample buffer with (A), or without (B), 20 mM dithiothreitol and run on 3 mm diameter gels (in duplicate) as in Fig. 3A. One gel of a pair was stained and the other placed in a layer of warm agarose containing Laemmli (16) sample buffer with 20 mM dithiothreitol, over a slab gel prepared, run and stained as in Fig. 3B. The relative mobilities of components in the second dimension may be compared with those of β-galactosidase, phosphorylase, bovine serum albumin, catalase, aldolase, and lysozyme, which were simultaneously electrophoresed at the left of the slab.

Purified receptor, I, and II all yield four major bands when fully reduced and saturated with SDS (Fig. 3A). In a Tris-acetate, SDS-gel electrophoresis system (9), these polypeptide components have apparent molecular weights of 39,000 (α), 48,000 (β), 58,000 (γ), and 64,000 (δ), (4,12,13). In the absence of disulfide reducing agents before and during SDS-gel electrophoresis, the δ band of purified receptor is markedly diminished in staining intensity and a new band of apparent molecular weight of about 130,000 appears. This band which we designate δ_2, is also a major component of unreduced II, but not of I (Fig. 3A). Furthermore, a band corresponding to δ_2 appears in unreduced but not in reduced Torpedo membrane fragments (Fig. 3B and ref. 14).

Evidence that δ_2 is a disulfide cross-linked dimer of δ is

obtained by two-dimensional SDS-gel electrophoresis (15), in which the sample run in the first dimension is either reduced or unreduced, and reduction precedes the second dimensional run in both cases. When II is reduced prior to the first dimensional run, the α, β, γ, and δ chains lie on a diagonal (Fig. 4A). When II is unreduced in the first dimensional run, the δ_2 band is prominent in the stained first dimensional gel, and δ lies under it in the second dimensional gel (Fig. 4B). The approximately 10% contamination of II with I (Fig. 1B) accounts for the small quantity of uncross-linked δ present in II (Fig. 4B). We conclude that all δ chains in II are cross-linked, predominantly as dimers and to a smaller extent as larger oligomers. Two dimensional SDS-gel electrophoresis of I (not shown) indicates that the δ chain in I is also involved in interchain disulfide cross-links (apparent in Fig. 3A), but to a lesser extent than in II, and in combination with a β chain in the same monomer rather than with a second δ chain.

Since δ_2 represents the predominant difference between the components of unreduced II and unreduced I, and the conversion by reduction of δ_2 to δ is concomitant with the conversion of II to I, it is most likely that dimer is cross-linked by disulfide bonds between δ chains. If there were two δ chains per monomer, however, it would be possible that cross-linking between these two would lead to non-covalent association of monomers. Although the stoichiometry of the chains are not well-determined, there are likely to be two α chains per monomer, since these bear the acetylcholine binding site and there are two binding sites per monomer (5). A molecular weight for monomer of 250,000 (Reynolds and Karlin, in preparation) is then compatible with two α and only one each of the β, γ, and δ chains. We conclude that dimer is cross-linked by disulfide bonds between δ chains.

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