STUDIES ON THE STRUCTURE OF THE ACETYLCHOLINE RECEPTOR FROM *TORPEDO MARMORATA*

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SUMMARY: The purified acetylcholine receptor of *Torpedo marmorata* has been characterized by sedimentation velocity measurements on dilute solutions using an ultracentrifuge and scanner. Several preparations were studied and all exhibited sedimentation coefficients in the vicinity of 24S. In a number of experiments the receptor could be resolved into two sedimenting boundaries of 18S and 26S, corresponding to minimum molecular weights of about 5×10^5 and 10^6, respectively. Additions of sodium dodecyl sulfate or Triton X-100 resulted in marked decreases in sedimentation coefficient, while treatment with Lubrol-WX had only a slight effect on the S values. Small changes in $S_{20,w}$ were produced by guanidine hydrochloride alone, although addition of dithiothreitol with 6 M guanidine hydrochloride resulted in an 8.8S component. Electrophoresis in sodium dodecyl sulfate gave one principal band with a molecular weight of 46,000.

Acetylcholine receptors have recently been isolated in a number of laboratories (see ref. 1 for review) and several reports on the properties of the polypeptide units in SDS gel electrophoresis have appeared (2-4). However, studies on the structure of the intact receptor molecule have been much more limited and involved measurements in the presence of high concentration of detergents (5). With the availability of large amounts of receptor from *Torpedo marmorata* purified by affinity adsorption using cobra toxin (6), it has now been possible to study the structure of the receptor by performing sedimentation measurements in the analytical ultracentrifuge. Sedimentation coefficients have been determined in dilute solutions with the UV scanner (7) and their dependence on detergent concentration for several different detergents investigated. Measurements have also been made on receptor treated with guanidine hydrochloride along with parallel studies on the receptor polypeptide chains by SDS gel electrophoresis.

EXPERIMENTAL PROCEDURE

The ACh-receptor was purified as described (6) and used in these studies without further treatment except where noted. The ACh-receptor was stored at

4°C in 5 mM sodium phosphate (pH 7.4) for a maximum of 7 days and protein concentrations as measured by the Lowry method (8) ranged from 0.05 to 0.15 mg/ml. Sodium dodecyl sulfate (SDS) electrophoresis grade was purchased from Biorad. Guanidine HCl ultrapure was a Schwartz-Mann product. Centrifugations were performed in a Beckman Model E Ultracentrifuge equipped with a scanner and on-line computer system (9). All runs were carried out at 20°C with double sector cells. Treatment of the ACh-receptor with denaturants or detergents was performed at room temperature at least 4 hours prior to the velocity runs. Scans were recorded with light of 280 to 290 nm. For SDS-gel electrophoresis, the procedures of Weber and Osborn (10) were followed with 10% polyacrylamide and 0.27% bisacrylamide (1X cross-linking) gels (7.5 cm long).

RESULTS

Sedimentation coefficients of the ACh-receptor preparations. Sedimentation coefficients for six preparations of ACh-receptor were determined (Table I). Centrifugations were at 20°C and the ACh-receptor preparations were in 5 mM sodium phosphate (pH 7.4). The average S value of these preparations was 24S with a range from 22-26S. Assuming a value of $\bar{v} = 0.74$ and frictional coefficient (f/f_o) of unity, this sedimentation coefficient corresponds to an estimated molecular weight of about 8×10^5.

When receptor samples were subjected to an overnight dialysis at room temperature, in addition to the dialysis routinely employed in the preparation (6), two sedimenting boundaries were observed. Values of $S_{20,w}$ of 18S and 26S were found for the two boundaries, corresponding to minimum molecular weights of about 5×10^5 and 10^6. When observed, the two components were in roughly equal amounts. The S values of the two boundaries are such that the single boundaries observed at other times may have contained the two species unresolved, possibly due to residual Triton X-100. The appearance of the two components with molecular weight ratios of roughly 2 suggests a possible "monomer-dimer" relationship.

TABLE I

Sedimentation Coefficients of the Isolated ACh-Receptor

Preparation Date	Protein Conc. (mg/ml)	RPM	$S_{20,w}$ (Svedbergs)
5/23/73	0.07	30,000	22.5
6/4/73	0.15	30,000	25.8
6/8/73	0.05	36,000	24.0
6/15/73	0.08	30,000	23.8
6/18/73	0.10	36,000	21.8
6/28/73	0.06	30,000	23.1

Treatment of the ACh-receptor with SDS. The sedimentation coefficients obtained in the presence of concentrations of SDS from 0.003 to 0.1% are given in Fig. 1. The sedimentation coefficient of the ACh-receptor is reduced to smaller values with the addition of 0.005 to 0.05% SDS. From 0.05% to 0.08% SDS a plateau value of about 3.6S is maintained. In these experiments only a single boundary was observed.

Treatment of the ACh-receptor with guanidine HCl. Treatment with guanidine hydrochloride, up to 6 M, produced surprisingly small effects on the sedimentation behavior of the receptor (see Table II). Observed values of the sedimentation coefficients were converted to values of $S_{20,w}$ using the correction factors of Kawahara, *et al.* (11). More pronounced decreases in $S_{20,w}$ were observed when dithiothreitol (10 mM) was added, although the value of 8.75 S in 6 M guanidine was still unexpectedly high. Evidently the ACh-receptor is unusually resistant to treatment with guanidine hydrochloride even in the presence of reducing agents which would be expected to break disulfide bonds. Some effect of dithiothreitol is evident, as might be expected from its influence on functional properties (12), but the protein is clearly not broken down to the individual peptide chains with molecular weights around 5×10^4 seen in SDS gels (2-4; see also below).

Treatment of the ACh-receptor with the detergents Triton X-100 and Lubrol-WX. Sedimentation data was obtained in the presence of the detergents Triton X-100 and Lubrol-WX. In several experiments with Triton X-100 two components were ob-

TABLE II

Sedimentation Coefficients of the ACh-Receptor in the Presence of Guanidine HCl

Conc. of Guanidine HCl	RPM	$S_{20,w}$ (Svedbergs)
1 M	48,000	22.8
3 M	48,000	17.8
6 M	48,000	17.8
With 10 mM Dithiothreitol Present		
1 M	48,000	16.3
3 M	48,000	15.7
6 M	48,000	8.8

TABLE III

Sedimentation Coefficients of the ACh-Receptor in the Presence of Lubrol-WX*

Lubrol-WX Conc. (%)	$S_{20,w}$ (Svedbergs)
0.05	19.7
0.10	18.9
0.50	18.9

*Measurements at protein concentrations of 0.05 mg/ml at rotor speeds of 36,000 RPM.

served. Typical data are plotted in Fig. 2. Of the two components, the absorbance of the smaller component contributed approximately one-third of the total absorbance of the sample. This percentage contribution remained the same in the titration range of Triton X-100 from 0.005 to 0.05% even though the sedimentation coefficients decreased from 22.8S to 12.0S for the larger component and from 13.9S to 6.8S for the smaller component. In contrast to the results obtained with Triton X-100, only one sedimenting boundary was observed in solutions with Lubrol WX (Table III). Also, Lubrol was much less effective than Triton in reducing the S values of the ACh-receptor.

SDS-gel electrophoresis of the ACh-receptor. The size of the receptor polypeptide chains was examined by SDS-gel electrophoresis. A scan of a 10% polyacrylamide gel is given in Fig. 3. One major component was observed with a

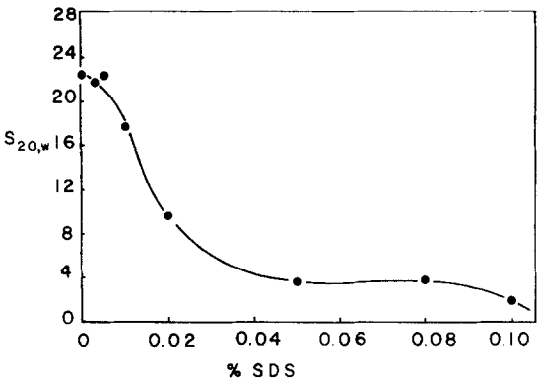

Figure 1. Sedimentation coefficients of ACh-receptor as a function of SDS concentration. Small aliquots of concentrated solutions of SDS (1 to 10%) were added to 400 µl aliquots of receptor and allowed to stand four hours at room temperature before centrifugation.

relative mobility (R_f) of 0.304 which corresponds on the standard curve to a molecular weight value of 46,000. Several minor components were also observed and some material failed to penetrate these gels. Whether the material outside the main peak represents other components of the receptor, chemically altered receptor molecules or contaminating proteins cannot yet be determined. Based on the intensity of the Coomassie blue staining it can be estimated that the 46,000 molecular weight peak represents roughly half of the total stained material.

DISCUSSION

It is quite clear that the molecular size of the purified ACh-receptor is dependent on the kind of detergent present and its concentration (Tables 1, 3; Figs. 1, 2). The major new finding presented here is that in low concentrations of Triton X-100, the ACh-receptor can be resolved into two components (18S and 24S) with estimated molecular weights of about 5×10^5 and 10^6, respectively. These molecular weights are lower limits based on an assumption of a spherical shape for the protein molecules and a $\bar{v}$ with the usual range. With many preparations, the two components were not resolved in sedimentation velocity measurements, and a single broad boundary was observed with an S value intermediate between the values observed for the isolated components. In studies by Meunier, *et al.* (5) with the *Electrophorus* ACh-receptor, high concentrations of Triton X-100 (1%)

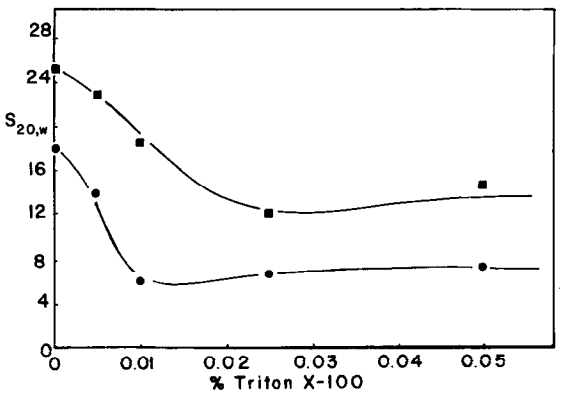

Figure 2. Sedimentation coefficients of ACh-receptor as a function of Triton X-100 concentration. The sedimentation coefficients of the fast and slow components are indicated by (■) and (●), respectively. Other details as in Fig. 1.

were employed and a value of $S_{20,w}$ = 12.5S was observed. However, with high Triton X-100 concentrations a buoyancy effect occurs, reflected by an increase in $\bar{v}$. Meunier, *et al.* (5) reported an increase of $\bar{v}$ from 0.73 to 0.78 in the presence of Triton X-100 and arrived at an apparent molecular weight of 4.7×10^4 for the receptor in 1% Triton X-100 with 160-170 molecules of Triton X-100 bound. Correcting for the bound Triton X-100 gives a molecular weight of 3.6×10^5 for the protein alone (5). This value may be contrasted with a minimum molecular weight of about 5×10^5 for the smaller component (18S) observed in solutions with very low Triton levels. Addition of Triton X-100 resulted in a marked reduction in sedimentation coefficients (Fig. 2), with values leveling off at 7S and 14S observed in the highest concentrations of Triton X-100 employed (0.05%). Only a single peak was reported by Meunier, *et al.* (5), with an S value of 12.5S. Possibly their value reflects an unresolved mixture of the 7S and 14S components reported here. Alternatively, structural differences may exist for the receptor from eel compared to its *Torpedo* counterpart. For example, two affinities for agonists are found with *Torpedo* receptor (13,14) in contrast to one for eel receptor (15,16). Current investigations are directed at determining if the two affinities are related to the two components observed by sedimentation analysis.

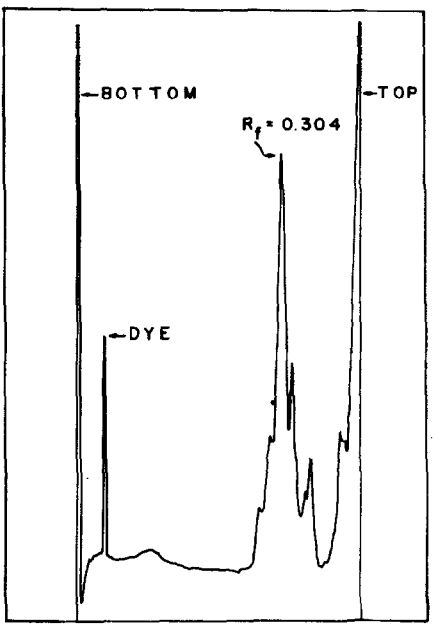

Figure 3. Gel scan of SDS-gel electrophoresis of ACh-receptor. A lyophilized sample was dissolved in 2% SDS and 2% β-mercaptoethanol in 0.01 M sodium phosphate buffer (pH 7.0).

The molecular weight of the major receptor polypeptide chain of 46,000 from SDS-gel electrophoresis agrees with earlier reports (2-4), although the molecular weight corresponding to one acetylcholine binding site was calculated to be about twice this value (6). One may conclude that the ACh-receptor protomer (i.e., the unit that carries a single ACh-binding site) may consist of two 46,000 subunits. In this case, approximately six protomers are required to form one oligomeric ACh-receptor molecule of 5×10^5 daltons. The species observed in ultracentrifuge experiments with receptor in solutions of SDS with an s = 3.6S (Fig. 1) may correspond to the 46,000 unit, but conclusions on this question must await determination of the contributions of bound SDS to the molecular properties (17). It should also be noted that the binding of acetylcholine to the receptor is markedly reduced by SDS (95% reduction with 0.01% SDS for 1 μM acetylcholine); in contrast Triton and Lubrol reduce binding only slightly. Especially surprising was the apparent inability of guanidine hydrochloride to disrupt the structure of the receptor to the level of individual polypeptide chains. The minimum S value observed (8.8S) must correspond to a species with

a molecular weight in excess of 2×10^5, or at least five times greater than the polypeptide size obtained by SDS-gel electrophoresis. Whether the resistance to guanidine hydrochloride is related to detergent extraction, found with other membrane proteins, or a consequence of masked disulfide bonds, warrants further investigation. Also of interest would be experiments to compare the size of the receptor in the membrane with the size of the isolated material, since differences in ligand binding properties exist (16, 18) which suggest a transition to a relaxed structure in the isolated receptor with affinity increases (approximately 30-fold) as predicted by a two state model (19,20).

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