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Molecular Weight in Detergent Solution of Acetylcholine Receptor from *Torpedo californica*†

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ABSTRACT: Acetylcholine receptor extracted in detergent solution from the electric tissue of *Torpedo californica* and purified by affinity chromatography contains predominantly two molecular weight species. These have been separated by sedimentation in a sucrose density gradient, and their molecular properties have been determined by sedimentation equilibrium and sedimentation velocity measurements in the analytical ultracentrifuge. The molecular weights of these species have been determined, without prior determination of the extent of detergent bound to them, by the adjustment of solvent density with D_2O so as to blank out the contribution of bound detergent to the sedimentation potential. The molecular weights of the protein moieties are 250 000 and 500 000. Since these species are identical in specific activity and polypeptide composition they are related as monomer and dimer. The hydrodynamic properties of the detergent complexes of monomer and dimer were derived from combined measurements of sedimentation equilibrium and sedimentation velocity. The $s_{20,w}$'s are 8.6 S and 12.8 S and the Stokes radii are 7.3 nm and 9.5 nm. For both monomer and dimer, the ratio of the Stokes radius to the minimum possible radius for the protein–detergent complex falls outside the range of values for globular proteins.

Nicotinic acetylcholine receptors are multichain, integral membrane proteins. Receptors from electric tissue (reviewed in Changeux, 1975; Karlin, 1977) and from skeletal muscle (Froehner et al., 1977b) have been solubilized, purified, and partially characterized in detergent solution. In nonionic detergent extracts (Raftery et al., 1971; Meunier et al., 1972; Klett et al., 1973) and purified preparations (Biesecker, 1973) from *Electrophorus electricus* electric tissue, receptor has a Stokes radius determined by gel filtration of about 7 nm. A similar value is obtained for receptor extracted from rat diaphragm (Chiu et al., 1973; Brockes & Hall, 1975). The sedimentation coefficients of receptor from *Electrophorus* electric tissue (Lindstrom & Patrick, 1974; Meunier et al., 1972) and skeletal muscle (Berg et al., 1972; Brockes & Hall, 1975) have been estimated by sedimentation in a sucrose density gradient to be about 9 S. Receptors from electric tissue of *Torpedo* species appear heterogeneous in hydrodynamic properties. Two forms are present with sedimentation coefficients of about 9 S and 13 S (Raftery et al., 1972; Potter, 1973; Carroll et al., 1973; McNamee et al., 1975) and Stokes radii of 7 nm (Raftery et al., 1972; Potter, 1973) and 8.5 nm (Potter, 1973). These two forms of *Torpedo* receptor are stable since they can be isolated and subjected to resedimentation and nondenaturing gel electrophoresis without interconversion (McNamee et al., 1975; Karlin et al., 1975); reduction with dithiothreitol, however, converts the larger to the smaller species (Hamilton et al., 1977; Chang & Bock, 1977).

The determination by sedimentation analysis of the protein molecular weight of receptor, as of other hydrophobic proteins in detergent solution, requires either that the quantity of bound detergent be known or that the contribution of detergent to the sedimentation potential be blanked out by adjusting the density of the solvent to equal the inverse of the specific volume of the detergent (Tanford et al., 1974; Reynolds & Tanford, 1976). Methods for obtaining the molecular weight which utilize the comparison of receptor apparent sedimentation velocity in a sucrose density gradient with the sedimentation velocities of characterized, globular, nondetergent-binding proteins are questionable on theoretical grounds (Tanford et al., 1974). Such methods have been applied nevertheless to *Electrophorus* receptor, yielding an apparent molecular weight in the range of 320 000 to 360 000 (Meunier et al., 1972), and to *Torpedo californica* receptor, yielding apparent molecular weights for the predominant species present of 190 000 and 330 000 (Gibson et al., 1976). Sedimentation equilibrium measurements have been made on receptor from *T. californica* and *T. marmorata* which, together with estimations of Triton X-100 binding, yielded estimates of molecular weight of 330 000 and

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TABLE I: Molecular Weights and Hydrodynamic Properties of Receptor Monomer and Dimer.

Receptor form	Preparation	M_p[a]	$s_{20,w}$[b] (S)	R_s[c] (Å)	R_s/R_{min}[d]	δ_D[e]
Monomer	1	238 000 ± 15 000	8.6 ± 0.4	73 ± 3		0.33 ± 0.32
	2	256 000 ± 8 000				0.16 ± 0.13
	Weighted mean	251 000 ± 7 000			1.6 ± 0.1[f]	0.18 ± 0.12
Dimer	1	497 000 ± 7 000	12.8 ± 0.6	95 ± 5		0.08 ± 0.06
	2	508 000 ± 13 000				−0.10 ± 0.11
	Weighted mean	499 000 ± 6 000			1.8 ± 0.1[g]	0.04 ± 0.05

[a] The protein molecular weight from the least-square fit of the data shown in Figures 1 and 2, assuming that at $\rho = 1/\bar{v}_D$, $M(1 - \phi'\rho) = M(1 - \bar{v}_p\rho)$ in eq 1 from Reynolds & Tanford (1976). $\bar{v}_D$ for Brij 58 is 0.919 cm^3/g (Tanford et al., 1974). $\bar{v}_p$ is calculated to be 0.730 cm^3/g from the amino acid composition (Karlin et al., 1975) and from the estimate of 3% hexose content (Raftery et al., 1975) taking $\bar{v}$ for hexose as 0.613 cm^3/g (Gibbons, 1972). Standard errors are given throughout. [b] The sedimentation velocity extrapolated to water at 20 °C was calculated by dividing the value of $M_p(1 - \phi'\rho)$ extrapolated to $\rho(20,w)$ by $6\pi N\eta(20,w)R_s$, where R_s is the mean Stokes radius. [c] The Stokes radius from sedimentation equilibrium and velocity measurements at the same solvent density. These paired measurements were made at three densities in the case of monomer and four densities in the case of dimer. [d] Stokes radius, R_s; minimum radius, R_{min} (eq 10, Tanford et al., 1974). [e] The mass of detergent bound per mass of protein, δ_D, was calculated from the slope of the appropriate linear regression function (eq 2 in Reynolds & Tanford, 1976). [f] The values for molecular weight of 251 000 and for δ_D of 0.18 were used to calculate R (minimum) according to eq 10 of Tanford et al. (1974). [g] The values for molecular weight of 499 000 and for δ_D of 0.04 were used to calculate R (minimum).

660 000 (*T. californica*), and 330 000 and 1 300 000 (*T. marmorata*) (Edelstein et al., 1975). This analysis was complicated by the presence of multiple sedimenting species.

Molecular weights have been estimated by gel electrophoresis in sodium dodecyl sulfate of cross-linked receptors: for *Electrophorus* receptor, molecular weights of 260 000 (Biesecker, 1973) and 230 000 (Hucho & Changeux, 1973), and for *T. californica* receptor, 400 000 (Raftery et al., 1975) and 200 000 (Hucho et al., 1975). A molecular weight of 270 000 has been estimated by membrane osmometry for *T. californica* receptor (Martjnez-Carrion et al., 1975). *T. californica* receptor is, however, a mixture of two molecular weight species. In the present work, we have isolated the two predominant forms of receptor from *T. californica* and have determined their molecular weights by a rigorous method (Reynolds & Tanford, 1976).

Methods

Receptor was extracted from the electric tissue of *Torpedo californica* in Triton X-100 solution and purified as before (Karlin et al., 1976), except that 0.1 mM phenylmethanesulfonyl fluoride was present during the extraction and bromoacetylcholine bromide was used instead of *p*-nitrophenol ester of *p*-carboxyphenyltrimethylammonium iodide to derivatize Affi-Gel 401 (Bio-Rad). The affinity gel contained finally 4 to 5 μmol of choline carboxymethyl groups per g of packed gel. Monomeric and dimeric forms of receptor were separated and isolated as follows: 4 mg of purified receptor, in 2 mL of 0.2% Triton X-100, 50 mM NaCl, 10 mM $NaPO_4$, 1 mM EDTA, 3 mM NaN_3 (pH 7.0), was labeled with [^{3}H]methyl-α-neurotoxin (Karlin et al., 1976) to the extent of 3% of saturation and was concentrated about tenfold in a collodion bag-ultrafiltration apparatus (Schleicher and Schuell). The concentrated receptor was layered over 17 mL of a 5–20% linear gradient of sucrose in the same buffer as above except that 0.2% Brij 58 (I.C.I. United States, Inc.) replaced Triton X-100 and was centrifuged in a SW 27.1 rotor (Beckman) at 27 000 rpm and 5 °C for 19 h. The gradient was collected in 0.3-mL fractions, 5 μL of which was counted to locate the peaks of α-neurotoxin binding activity (McNamee et al., 1975; Hamilton et al., 1977). The peak fractions were pooled and dialyzed for 5 h at 4 °C against the buffer containing Brij 58. Samples were stored in liquid nitrogen. The specific activities of the different preparations of monomer and dimer ranged from 6 to 8 nmol of α-neurotoxin binding sites per mg protein, as determined by a DEAE filter method (Damle & Karlin, 1978) modified after Klett et al. (1973). The final protein concentrations (Lowry et al., 1951) ranged from 0.14 to 0.20 mg per mL.

Receptor-bound phosphate was assayed by the micromethod of Bartlett (1959). Receptor in phosphate buffer as above was dialyzed to remove unbound phosphate and duplicate 1-mg samples in 0.5 mL of 0.2% Triton, 10 mM NaCl, 10 mM 3-(*N*-morpholino)propanesulfonic acid (pH 7.4) were assayed.

Molecular weights were determined by a procedure described in detail elsewhere (Reynolds & Tanford, 1976) using sedimentation equilibrium in a Beckman Model E analytical ultracentrifuge equipped with a photoelectric scanner. The concentration of protein as a function of radial distance was determined by scanning the cell at 280 nm. Initial protein concentration ranged from 0.08 to 0.2 mg/mL. The temperature was maintained at less than 10 °C for all equilibrium measurements.

D_2O was obtained from Bio-Rad Laboratories and its density was checked periodically to guard against possible contamination by atmospheric H_2O.

Solvent densities and viscosities were calculated from the appropriate tabulated data (Landolt-Bornstein Tabellen, 1969; Jones & Fornwalt, 1936).

Results

The individual sedimentation equilibrium runs yielded linear plots of the logarithm of protein concentration (log A_{280}) against the square of the radial displacement (r^2) to as close to the bottom of the cell as could be read, indicating that a single sedimenting particle was being monitored. A small amount of nonsedimentable absorbance was observed which varied for the four preparations between 4 and 20% of the *initial* absorbance. At equilibrium this baseline correction had a negligible effect on the accuracy of the absorbance determinations since at the bottom of the cell the optical density was between 0.8 and 1.5. The data in Table I and Figures 1 and 2 demonstrate the reproducibility between different preparations. The values for the effective mass of the sedimenting particles, $M_p(1 - \phi'\rho)$, obtained from the slopes of the linear plots of log A_{280} against r^2 (Tanford et al., 1974), vary linearly with ρ (Figures 1 and 2). According to eq 1 of Reynolds & Tanford (1976), $M_p(1 - \phi'\rho) = M_p(1 - \bar{v}_p\rho)$ at $\rho = 1/\bar{v}_D$, if the contribution of bound species other than detergent is

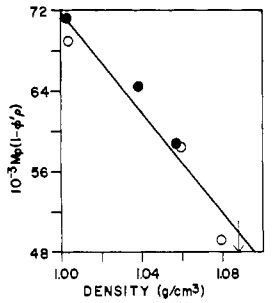

FIGURE 1: Sedimentation analysis of receptor monomer in buffer containing 0.2% Brij 58, 50 mM NaCl, 10 mM $NaPO_4$, 1 mM EDTA (pH 7.0), and varying concentrations of D_2O to give the solvent densities indicated on the abscissa. The arrow indicates the value of the solvent density equal to the inverse of the specific volume of Brij 58. The values of $M_p(1 - \phi'\rho)$ are derived from sedimentation equilibrium measurements. The filled and unfilled circles represent results with two independent preparations of receptor. The line is the least-squares fit to all the data.

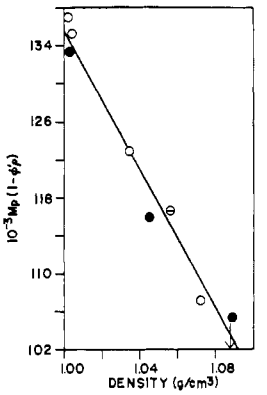

FIGURE 2: Sedimentation analysis of receptor dimer as in Figure 1. $M(1 - \phi'\rho)$ is calculated from sedimentation equilibrium, except that one point (unfilled circle with line through it) is calculated (eq 7 in Tanford et al., 1974) from the sedimentation velocity at the indicated density and the mean Stokes radius determined by paired sedimentation equilibrium and sedimentation velocity measurements at other densities (Table I).

negligible (see below). The values of $M_p(1 - \phi'\rho)$ at $\rho = 1/\bar{v}_D$ are calculated for two independent preparations individually from the appropriate linear regression function, and from these values are calculated the protein molecular weights, M_p (Table I).

Purified receptor (a mixture of monomer and dimer) contains approximately 5 phosphorus atoms per α-neurotoxin binding site or 10 phosphorus atoms per 250 000 daltons. If the phosphorus were due to bound phospholipid, then the protein molecular weights calculated according to eq 1 of Reynolds & Tanford (1976) would be 1% greater than those shown in Table I.

The quantity of detergent bound to monomer and dimer is derivable, albeit imprecisely, from the slope (also from the intercept at $\rho = 0$) of the linear regression function relating $M_p(1 - \phi'\rho)$ to ρ, according to eq 2 of Reynolds & Tanford (1976). It appears that dimer binds less Brij 58 per g of protein than does monomer (Table I), and both species appear to bind less than one detergent micelle. Since $M_p(1 - \bar{v}_p\rho)$ is greater than 70% of the experimental values of $M_p(1 - \phi'\rho)$, the error in determining δ_d for this specific system is very large. The standard deviation in M_p, on the other hand, is <7%.

The hydrodynamic characteristics of the detergent complexes of monomer and dimer were determined by paired sedimentation equilibrium and sedimentation velocity measurements at a number of values of ρ. The values of the ratio, R_s/R_{min} (Table I), are outside the range of this ratio for globular proteins and indicate that the shapes of monomer and dimer deviate considerably from the spherical. For example, a prolate elipsoid with $R_s/R_{min} = 1.6$ has an axial ratio of 11.4.

Discussion

The predominant species of receptor from *Torpedo californica* have molecular weights of about 250 000 and 500 000.[1] These two species have been previously shown to have identical specific activities and polypeptide compositions (McNamee et al., 1975; Karlin et al., 1975), and thus we are justified in calling them monomer and dimer. The specific α-toxin binding activities of these species, when measured by a relatively precise method of gel filtration (Karlin et al., 1976), are 8 nmol of sites per mg of protein (McNamee et al., 1975; Karlin et al., 1975). There are thus 125 000 daltons of protein per α-toxin binding site or 2 sites per 250 000 dalton monomer. Only one site per 250 000 daltons, however, is labeled by the affinity label, 4-(*N*-maleimido)benzyltrimethylammonium iodide (McNamee et al., 1975; Damle & Karlin, 1978), which supports our present result that 250 000 daltons is the minimum molecular weight of intact receptor.

According to present evidence, four different polypeptide chains of apparent molecular weights of 39 000 (α), 48 000 (β), 58 000 (γ), and 64 000 (δ) are present in receptor from *T. californica* (Weill et al., 1974; Raftery et al., 1975). Of these, only the α chain is affinity labeled by 4-(*N*-maleimido)benzyltrimethylammonium iodide, which identifies this chain as a probable site for acetylcholine and α-neurotoxin binding (Weill et al., 1974). A chain of similar apparent molecular weight is also uniquely affinity labeled in *Electrophorus* receptor (Reiter et al., 1972; Karlin & Cowburn, 1973; Karlin et al., 1975). In contrast, two chains in receptor from rat skeletal muscle are affinity labeled (Froehner et al., 1977a). If in *Torpedo* receptor both α-neurotoxin binding sites are on α chains, even though only one of the two sites can be affinity labeled (Damle & Karlin, 1978), then the numbers of chains in monomer compatible with a molecular weight of 250 000 are two α and one each of β, γ, and δ, summing nominally to 248 000. This stoichiometry is roughly consistent with the intensity of the Coomassie Brilliant Blue staining of the chains after separation by electrophoresis on sodium dodecyl sulfate-polyacrylamide gels (Karlin et al., 1975). Raftery et al. (1975), however, have estimated the stoichiometry to be four α, two β, and one each of γ and δ. This leads to a nominal molecular weight for receptor monomer of 390 000, which is not consistent with our estimate of the molecular weight.

Dimer has recently been shown to be cross-linked by disulfide bonds between δ chains (Hamilton et al., 1977; Chang & Bock, 1977). The species formed by reduction of dimer cosediments in a sucrose density gradient with naturally occurring monomer (Hamilton et al., 1977), and our preliminary results suggest that the Stokes radii and molecular weights of naturally occurring monomer and that formed by reduction are similar.

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[1] A small fraction of the total α-neurotoxin binding activity in receptor is present in species of a molecular weight greater than 500 000 (see Figures 1 and 2 in Hamilton et al., 1977).

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