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Functional Reconstitution of the Nicotinic Acetylcholine Receptor by CHAPS Dialysis Depends on the Concentrations of Salt, Lipid, and Protein†

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ABSTRACT: The detergent CHAPS was found to be the preferable surfactant for the efficient purification and reconstitution of the *Torpedo californica* nicotinic acetylcholine receptor (AChR). The main result is that the incorporation of the AChR proteins into lipid vesicles by CHAPS dialysis was strongly dependent on the salt and protein concentrations. As monitored by sucrose gradients, by electron microscopy, and by agonist-induced lithium ion flux, the best reconstitution yields were obtained in 0.5 M NaCl at a protein concentration of 0.5 g/L and in 0.84 M NaCl at 0.15 g/L protein. Electron micrographs of receptor molecules, which were incorporated into vesicles, showed single, nonaggregated dimer (M_r = 580000) and monomer (M_r = 290000) species. CHAPS dialysis at NaCl concentrations <0.5 M largely reduced the receptor incorporation concomitant with *protein aggregation*. Electron micrographs of these preparations revealed large protein sheets or ribbons not incorporated into vesicles. The analysis of static and dynamic light scattering demonstrated that the *detergent-solubilized* AChR molecules aggregate at low lipid contents (≤500 phospholipids/AChR dimer), *independent* of the salt concentration. AChR proteins eluted from an affinity column with a solution containing 8 mM CHAPS (but no added lipid) still contained 130 ± 34 tightly bound phospholipids per dimer. The aggregates (about 10 dimers on the average) could be dissociated by readdition of lipid and, interestingly, also by increasing the CHAPS concentration up to 15 mM. This value is much higher than the CMC of CHAPS = 4.0 ± 0.4 mM, which was determined by surface tension measurements. The data clearly suggest protein–*micelle* interactions in addition to the association of *monomeric* detergents with proteins. Furthermore, the concentration of the (free) monomeric CHAPS at the vesicle–micelle transformation in 0.5 M NaCl ($[D_w]^c$ = 3.65 mM) was higher than in 50 mM NaCl ($[D_w]^c$ = 2.8 mM). However, it is suggested that the main effect of high salt concentrations durig the reconstitution process is an increase of the fusion (rate) of the ternary protein/lipid/CHAPS complexes with mixed micelles or with vesicular structures, similar to the salt-dependent fusion of vesicles.

A necessary and essential step in the detailed characterization of intrinsic membrane proteins is the detergent solubilization and the reconstitution of the protein species in artificial lipid membranes. Impressive examples are channel proteins where the channel characteristics of defined protein species may be studied in planar bilayers or in lipid vesicles of defined composition.

It has been recognized that it is the actual solubilization and reconstitution process which determine the protein content and the channel function in the membrane. The choice of detergent for the solubilization of the membrane proteins and the type of lipid to be used for vesicle formation are crucial and often

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require empirical screening. Frequently, the detergent of the detergent–protein micelles is removed by dialysis in the presence of lipids in order to reconstitute the protein into lipid vesicles.

The formation of vesicles from micelles has been studied in detail for lecithins solubilized by cholate or octyl glucoside (Jackson et al., 1982; Lichtenberg, 1985; Almog et al., 1986; Paternostre et al., 1988; Ollivon et al., 1988; Schürholz et al., 1988b). It was found that vesicles and micelles behave as if they were different phases for the incorporation of lipids, detergents, and proteins. The transformation of the vesicle and micelle phases is characteristically dependent on the ratio R of the concentration of detergent bound to vesicles or to micelles $[D_b]$ and the concentration of the total lipid $[L_T]$; $R = [D_b]/[L_T]$. In equilibrium the critical values R^c for the onset of micellization (M) and for the onset of lipid vesicle formation (V) are different; $R^c(M) > R^c(V)$ (Lichtenberg, 1985; Schürholz et al., 1989b).

In order to preserve the structural and functional integrity of a membrane protein, it is often necessary to add lipid to the detergent solutions (Epstein & Racker, 1978; Heidmann et al., 1980; Rivnay & Metzger, 1982).

In general, lipid extracts from natural sources like soybean phospholipids are favored in reconstitution experiments, but frequently the suitable lipid type depends on the type of detergent applied for the solubilization (Rivnay & Metzger, 1982; Dencher et al., 1986). Even the protein-denaturing detergent SDS[1] has been employed to purify channel proteins like the nicotinic acetylcholine receptor (AChR). In this case, the ion transport function of the receptor protein could be retained when SDS was exchanged against a mild detergent before vesicle reconstitution (Hanke et al., 1990).

In certain cases, macroscopic phase separations have to be taken into account, e.g., in the system lecithin–octyl glucoside. The phase separation interferes with the detergent removal and the distribution of the protein in the lipid vesicles (Schürholz et al., 1989b). Furthermore, when polyethers such as Triton X-100, Lubrol-WX, or other alkylpoly(ethylene glycol)s are used as detergents, peroxide formation must be avoided, because membrane proteins often contain sulfhydryl groups (Chang & Bock, 1980).

This is especially important for the AChR of *Torpedo* electrocytes, which can be isolated as a monomeric protein complex (M_r = 290 000) and a disulfide-bridged dimer (M_r = 580 000). For preparations of the monomeric AChR, generally, reducing agents like DTE are added to the membrane fractions before solubilization by detergents (Chang & Bock, 1977).

Even without any lipid added during the purification procedure on an affinity column, about 40–100 residual lipids remain bound at the AChR monomer (Chang & Bock, 1979; Jones et al., 1988; Herlemann, 1989). Spectroscopic data also reveal two types of lipids, protein bound and free (Bhushan & McNamee, 1990). Cholesterol and negatively charged lipids have been found necessary for proper AChR function, especially for the ion channel activity (Criado et al., 1982; Fong & McNamee, 1986, 1987). The incorporation of cholate-solubilized AChR protein into lipid vesicles was reduced when dialysis was performed at high ionic strength ([NaCl] > 0.1 M) or in the absence of negatively charged lipids (Popot et al., 1981). At lipid to protein ratios higher than 16/1 (w/w), the protein is unevenly distributed into vesicles during reconstitution with cholate; protein-rich vesicles are formed besides those which are void of AChR (Anholt et al., 1981).

The identification of the species which causes a particular channel event, however, requires the presence of isolated, individual protein species in the membrane. In this context, it was reported that the conductance of the isolated dimer is twice as large as that of the isolated monomer; receptor proteins aggregated during reconstitution can cause cooperative opening of several channels and formation of less-defined channel events (Schindler et al., 1984; Schürholz et al., 1989a).

According to common practice, we had used cholate as the detergent for the reconstitution of the various AChR protein species into proteolipid vesicles. In attempts to reconstitute AChR *monomers*, we found that only a small fraction of the receptor molecules was incorporated in the liposomes. Most of the protein was aggregated in the form of large tubes or clusters. Although the few incorporated AChR monomers were still functional, as proved by agonist-induced channel activity, the patch clamp measurements were very tedious due to the low receptor concentration. Electron micrographs of AChR *dimers* reconstituted in the same way show that these receptor species, at higher concentrations, have a tendency to form clusters in the membrane, too.

With cholate as detergent, the yield of purified AChR protein eluted from the affinity column material dicaproyl-MP/Sepharose 4B (Chang & Bock, 1977) is very low. The highest yield of purified receptor is usually obtained when the electrocyte membranes are solubilized by Lubrol-WX. However, when Lubrol-WX (CMC ≤ 0.06 mM) was used for AChR reconstitution into vesicles, no channel activity was measured. When CHAPS was applied for the purification, the protein yield was similarly high as with Lubrol, provided that lipid was added to the detergent-containing solutions. As CHAPS has a relatively high critical micellization concentration (CMC, as below), it was also used for the AChR reconstitution into lipid vesicles. Initially, the same conditions as for the cholate dialysis procedure were applied. The functional analysis of the reconstituted vesicles by Li^+ flux measurements revealed only a relatively low channel activity. Again, the electron micrographs showed the formation of large protein aggregates. To optimize solubilization and reconstitution of AChR proteins with CHAPS, we have analyzed the influence of salt, lipid, detergent, and receptor concentration, respectively, by structural and functional assays. To get a better understanding of the reconstitution process, it was necessary to determine at first the CMC of CHAPS.

Materials and Methods

Materials. Electric organ tissue from *Torpedo californica* was received on dry ice from Pacific Bio-Marine Laboratories, Inc., Venica, CA, and was stored in liquid nitrogen. ^{125}I-α-Bungarotoxin (^{125}I-BTX) was obtained from Du Pont, NEN Research Products. Benzamidine hydrochloride, phenylmethanesulfonyl fluoride (PMSF), iodoacetamide (IAA), dithioerythritol (DTE), *N*-ethylmaleimide (NEM), carbamoylcholine, and gallamine triethiodide (Flaxedil) were purchased from Sigma. The soybean phosphatide extract [≈20% (w/w) phosphatidylcholine] and phosphatidylinositol (soybean) were from Avanti Polar Lipids. The affinity gel methyl[*N*-(6-aminocaproyl-6′-aminocaproyl)-3-amino]pyridinium bromide (dicaproyl-MP)/Sepharose 4B was a generous gift from Prof. H. W. Chang, New York. The detergents CHAPS

[1] Abbreviations: ^{125}I-α-BTX, ^{125}I-α-bungarotoxin; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; CMC, critical micellization concentration; dicaproyl-MP, methyl[*N*-(6-aminocaproyl-6′-aminocaproyl)-3-amino]pyridinium bromide; DTE, dithioerythritol; IAA, iodoacetamide; AChR, nicotinic acetylcholine receptor; NEM, *N*-ethylmaleimide; PI, phosphatidylinositol; PMSF, phenylmethanesulfonyl fluoride; SBL, soybean phospholipid; SDS, sodium dodecyl sulfate.

[3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate], Lubrol-WX, and sodium dodecyl sulfate were from Sigma; sodium cholate was purchased from Serva, Lubrol-WX was purified according to Chang and Bock (1980).

Preparation of AChR (Dimer). All procedures except the first homogenization step were carried out at 4 °C. The buffers were adjusted to pH 7.4, Millipore filtrated, and deaerated. The purity of the AChR protein was analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). Protein concentrations were estimated by absorbance at 280 nm ($A_{280}^{0.1\%} = 1.8$) (Chang & Bock, 1979) or measured with the BCA protein assay (Pierce).

Method A. Frozen *Torpedo* electric organ (100–200 g; all buffer volumes refer to 100 g of tissue) was homogenized in 300 mL of 10 mM Hepes, 5 mM EDTA, 5 mM EGTA, 3 mM NaN_3, 5 mM IAA, 3 mM benzamidine hydrochloride, and 0.2 mM PMSF and centrifuged for 1 h at 25000*g*. The pellet then was homogenized in 200 mL of 10 mM Hepes, 1 M NaCl, 1 mM EDTA, and 3 mM NaN_3 and was centrifuged for 1 h at 25000*g*. The pellet was resuspended in 150 mL of 10 mM Hepes, 1 mM EDTA, and 3 mM NaN_3, followed by centrifugation for 1 h at 25000*g*. Membrane proteins were extracted by shaking the resuspended pellet for 1.5 h with 100 mL of 10 mM Hepes, 1 mM EDTA, 3 mM NaN_3, 1% (w/w) CHAPS, and 5 g/L soybean phospholipids (SBL). Then the nonsolubilized material was separated by 1-h centrifugation at 45000*g*. The supernatant was immediately applied to the affinity column. The gel was washed successively with 100 mL of buffer W1 (10 mM Hepes, 0.1 mM EDTA, 0.5% CHAPS, and 5 g/L SBL), 50 mL of buffer W2 (buffer W1 and 80 mM NaCl), and 50 mL of buffer W3 where the [NaCl] was reduced to 60 mM. The gel-bound receptor protein was eluted with 150 mM Flaxedil in buffer W3 and stored at −80 °C.

Method B. Extraction and purification of the receptor protein were carried out as described in method A, but without lipid in the last washing step, W3 (80 mL instead of 50 mL), and in the elution buffer.

Preparation of AChR Monomers. After the first centrifugation of method A, the pellet was resuspended in 50 mL of 50 mM Hepes, 1 mM EDTA, 10 mM carbamoylcholine chloride, and 3 mM NaN_3. DTE was added to yield 7.5 mM, and the homogenate was shaken for 30 min at 4 °C. After the reductive splitting of the S–S bond of the dimer, sulfhydryl group alkylation was performed with 18 mM IAA, again for 30 min, to stabilize the monomeric form. The suspension was diluted with 100 mL of 50 mM Hepes, 1 mM EDTA, 1.5 M NaCl, and 3 mM NaN_3 and centrifuged for 1 h at 45000*g*. The following steps were performed as described above.

Reconstitution of AChR into Vesicles. (a) CHAPS Dialysis. In all reconstitution assays the initial bound detergent/total lipid ratio was $R = [D_b]/[L_T] = 3/1$ (mol/mol). The solutions contained additional CHAPS to provide a concentration of free, monomeric detergent $[D_w] = 8$ mM. The lipid/protein ratio was 20/1 (w/w). Lipid was added to the purified receptor solution from a concentrated suspension (100 g/L); CHAPS was added from a 10% solution. The final solution contained 2.7% CHAPS, 10 g/L lipid [soybean phospholipids and cholesterol, 9/1 (w/w)], 0.5 g/L AChR, and 60 mM NaCl in buffer A (10 mM Hepes, pH 7.4, 3 mM NaN_3). When the AChR concentration was reduced to 0.15 g/L, the detergent and lipid concentrations were reduced in the same ratio. The samples were thoroughly mixed and incubated for 5 min at 20 °C, followed by shaking for 2 h at 4 °C. For detergent removal the preparation was divided into several samples and dialyzed against 5 × 200 volumes of buffer A containing different salt concentrations (10 mM–1 M NaCl) in Visking dialysis tubing (molecular mass cutoff of 12 000) at 4 °C. The buffer was changed five times during 72-h dialysis.

(b) Cholate Dialysis. For reconstitution of AChR in lipid vesicles, detergent-solubilized receptor was suspended in a lipid/cholate mixture to yield a final concentration of 0.5–1 g/L protein, 1.2% cholate, and 25 g/L lipid. The soybean phospholipids were dissolved in chloroform together with 20% cholesterol (w/w), and the solvent was removed before storage. The homogeneous suspension was dialyzed for 2 days with three changes against 500 volumes of 10 mM Hepes, 100 mM NaCl, 3 mM NaN_3, and 0.1 mM $CaCl_2$, at 4 °C. Samples analyzed by electron microscopy had been frozen in liquid nitrogen and stored at −80 °C.

Sucrose Density Gradient Centrifugation. The incorporation of AChR into vesicles was monitored by discontinuous flotation sucrose gradients. After dialytic detergent removal, the samples were dialyzed against 500 volumes (two changes) of buffer A containing 20 mM NaCl for 12 h at 4 °C to get identical ionic strengths. Aliquots of 0.5 mL were carefully mixed with 1.5 mL of sucrose (50% w/w) in buffer A (final concentration 37% sucrose) in a 12-mL centrifuge tube. Volumes, each of 2 mL, with 30%, 20%, 10%, and 5% sucrose in buffer A were successively layered, followed by 1 mL of buffer A on the top. The sucrose gradients were centrifuged at 30 000 rpm (70000*g*) for 3 h in a Kontron TST 41 rotor at 4 °C. Fractions of 0.8 mL were collected from the bottom of the tube and analyzed for protein and lipid content. To receptor samples, reconstituted at 10 mg/mL lipid, was added 0.001% Lubrol-WX in the gradient to make the vesicles more leaky for sucrose.

Intrinsic AChR Fluorescence. The intrinsic AChR fluorescence of the sucrose gradient fractions was measured in 1-mL quartz cuvettes with a Hitachi F 4010 fluorescence spectrophotometer at an excitation wavelength of 280 nm, an emission wavelength of 340 nm, and slit widths of 10 nm.

Radioactive Binding Assay. The AChR content in the sucrose gradient fractions was also assayed by binding of the radioactive ligand ^{125}I-α-bungarotoxin (α-BTX). Aliquots of 0.2 mL were diluted with wash buffer (10 mM Pipes, 30 mM NaCl, 1% Triton X-100, pH 7.4) to a final volume of 1 mL. Samples of 100 μL were incubated overnight with 10 μL of ^{125}I-α-BTX (stock solution ≈ 3 μM) at 4 °C. For blank values, wash buffer was incubated with radioactive toxin under identical conditions. Measurements of bound toxin were conducted with samples of 50 μL which were applied homogeneously on Whatman DE-81-2.3 CMS filter paper and washed with 500 volumes of wash buffer to remove unbound toxin molecules. For standard samples, 5 μL of toxin solution was applied on the filter paper and the content directly measured. Radioactivity was measured in a Berthold γ-counter. The specific activity in the presence of detergent was 6.9 nmol of α-BTX/mg of protein or 2 mol of α-BTX bound to 1 mol of AChR monomer (M_r = 290 000).

Assay of AChR Function (Lithium Flux). Functionally active ion channels were analyzed by agonist-induced uptake of Li^+ into reconstituted vesicles. After detergent removal the vesicles were dialyzed against 2 × 500 volumes of 100 mM NaCl in buffer A, followed by dialysis for the same period against 2 × 500 volumes of 145 mM sucrose in buffer A at 4 °C. For the flux assay, samples of 115 μL were mixed with 5 μL of 2 M LiCl and 2.5 μL of 5 mM carbamoylcholine chloride. The control values were determined by substituting

distilled water for the agonist solution in the assay. Entrapped Li^+ was separated from external Li^+ by passage through a 1.5-mL Dowex 50 WX-8-100 cation-exchange column preequilibrated with 3 mL of sucrose solution (170 mM, 3.3 mg/mL BSA). The column was immediately eluted with 1.6 mL of sucrose solution (175 mM), and fractions of 200 μL were analyzed by atomic absorption (AAS) at 670 nm. All assays were performed in duplicate.

Electron Microscopy. For negative staining, vesicle samples were diluted to about 1 g/L of lipid. A carbon-shadowed Formvar-filmed grid was applied to a sample drop of 50 μL, which was blotted and stained with 1% aqueous uranyl acetate. The samples were viewed at 50 kV in a Zeiss TEM-109 electron microscope.

Light Scattering. (a) The process of gradual vesicle solubilization or protein aggregation was measured by light scattering at 90° with a Hitachi F 4010 fluorescence spectrophotometer. (b) The distribution of particle sizes of detergent-solubilized AChR was determined by quasi-elastic light scattering at the laser wavelength 488 nm. The scattering signal was analyzed with a Brookhaven Correlator BI-2030. The apparent diffusion constant and the Stokes radius were calculated according to Hallet et al. (1989). All measurements were done at 10 °C.

Determination of Lipid. (a) Extraction of the lipid was performed according to Hara and Radin (1978), followed by an additional acidic extraction (Varsanyi et al., 1983). In detail, 2 mL of detergent-solubilized AChR (≈0.4 mg) was mixed with an equal volume of hexane/2-propanol (3:2 v/v) and sonicated for 10 min in a bath cooled by ice. Phase separation was achieved by centrifugation of the mixture in a table centrifuge at medium speed. The upper, organic phase was collected with a pipet and the aqueous phase extracted a second time. The combined organic phases were evaporated, and the dry lipid was redissolved in 20 μL of the eluent $CHCl_3$/MeOH/H_2O (65:25:4 v/v) for thin-layer chromatography (TLC). The aqueous phase was extracted two times with $CHCl_3$/MeOH/concentrated HCl (40:20:1) as described above. The lower, organic phases were combined as well, evaporated to dryness, and redissolved in 20 μL of solvent. (b) Densitometric determination of phospholipids (Gustavsson, 1986) was performed by applying 5- and 10-μL samples to a 10 cm × 10 cm silica gel 60 HPTLC plate (E. Merck, Darmstadt). After separation of the lipids the dried TLC plate was bathed for 5 s in the molybdenum blue solution prepared as described (Gustavsson, 1986). The plate was dried again (5 min) and bathed for 25 s in distilled water and finally 10 min in ethanol. The lipid spots on the dried plate were scanned with a LKB 2202 Ultroscan laser densitometer and analyzed by aid of the Kontron Data System 450. Calibration curves were made with PC, PE, and PI (from soybean, Avanti Polar Lipids).

Determination of CHAPS in Lipid Vesicles. Vesicles, which were prepared by dialysis of CHAPS-solubilized lipids, were lyophilized and dissolved in $CHCl_3$/MeOH (2:1 v/v). Reference samples, which contained 1, 0.5, 0.2, and 0.1 mol of CHAPS/mol of lipid [M_r(lipid) = 800], respectively, were treated as above. The unsoluble salts were precipitated, and the supernatant (0.1 mg of lipid) was applied to a silica gel 60 HPTLC plate (E. Merck, Darmstadt). After separation of the surfactants through the use of the eluent $CHCl_3$/MeOH/20% aqueous methylamine (60:36:10 v/v), the dried plates were sprayed with H_2SO_4/acetic acid (1:1) and incubated for 10 min at 100 °C. CHAPS yielded yellow spots, whereas the lipids turned grey-brown.

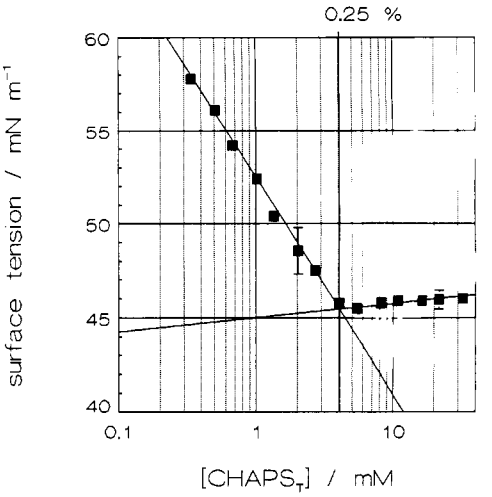

FIGURE 1: Determination of CMC(CHAPS) by surface tension measurements. Buffer: 10 mM Hepes, pH 7.4, and 50 mM NaCl, 20 °C. The values are averages from three preparations and triplicate measurements. The standard deviation was largest below the CMC and lowest above the CMC as indicated. [$CHAPS_T$] is the total CHAPS concentration.

Vesicle Formation. (a) Dialysis vesicles: vesicles were prepared as described for the AChR reconstitution. (b) Lipid suspensions: 10 g/L SBL and buffer A containing 50 mM NaCl were sonicated in a bath until formation of a homogeneous suspension (about 15–20 min). Ice was added to the bath for cooling.

Surface Tension Measurements. The surface tension of the detergent solutions was measured with a Krüss tensiometer (ring method). In the region below the CMC, the surface tension decreased with time after the solution had been poured into the beaker and reached a constant value after about 5–10 min.

Results

The solubilization of the lipid vesicles (see below) was completed much below 8 mM CHAPS, the average value of the CMC given by the commercial distributors. Since the knowledge of the exact CMC value and its salt and temperature dependence was a prerequisite for the reconstitution analysis, we analyzed first the CMC of CHAPS by surface tension measurements.

Determination of the CMC of CHAPS. In Figure 1 the surface tension γ is plotted against the CHAPS concentration (logarithmic scale), in line with the Szyskowski equation for the surface pressure:

$$\pi = \gamma_0 - \gamma = RTT_\infty \ln (1 + K^{-1}[D]) \quad (1)$$

where R is the gas constant, T the absolute temperature, and γ_0 the surface tension of the aqueous solution without the detergent; γ_0(20 °C) = 72.8 mN·m^{-1}. The surface tension decreased linearly over a wide concentration range until a minimum was reached (Figure 1). The point with the highest curvature is indicative for the CMC. At 50 mM NaCl, pH 7.4 and 20 °C, the CMC(CHAPS) = 4 ± 0.4 mM. The CMC values determined at 5 °C and 50 mM NaCl (3.8 mM) or at 20 °C and 500 mM NaCl (4 mM) fall inside the error margin of 4 ± 0.4 mM.

Vesicle Solubilization. In order to analyze the micelle–vesicle transformation, we added defined amounts of CHAPS to preformed pure lipid vesicle suspensions, which had been made by bath sonication. The transformation from vesicles to mixed micelles was accompanied by a marked decrease in the intensity of the light scattering at 90°. The minimum total

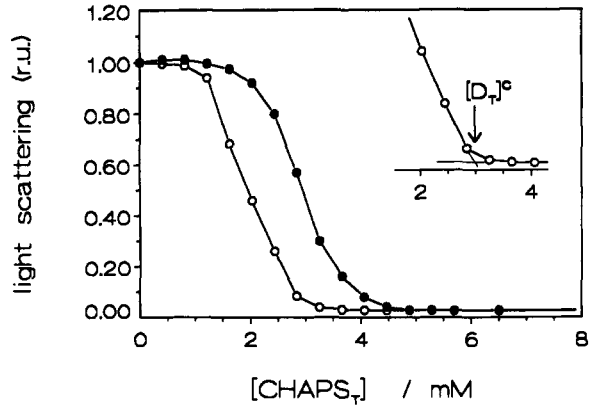

FIGURE 2: Solubilization of lipid vesicles (0.5 g/L) as a function of the total detergent concentration, measured by light scattering at 500 nm and 90° in relative units (ru): (○) 50 mM NaCl; (●) 500 mM NaCl. The 50% solubilization constants are $[D_T]^{0.5}$ = 1.7 mM at 50 mM NaCl and $[D_T]^{0.5}$ = 3 mM at 500 mM NaCl. Inset: the point of complete vesicle solubilization is marked $[D_T]^c = [CHAPS_T]^c$.

detergent concentration, $[D_T]^c$, which was needed for complete solubilization of the vesicles, was determined by linear extrapolation of the data at the transition and the line parallel to the x-axis; see inset of Figure 2. The solubilization of vesicles was measured at 0.1, 0.5, 1.0, 2.5, and 5.0 g/L SBL, respectively. It was found that $[D_T]^c$ depends linearly on the lipid concentration according to the mass conservation (see Appendix):

$$[D_T]^c = [D_w]^c + R^c(M)[L_T] \quad (2)$$

where $[D_w]^c$ is the critical free detergent concentration in the transition range; here $[D_w]^c = [CHAPS]^c = 3.2 \pm 0.4$ mM. The slope, $R^c(M) = d[D_T]^c/d[L_T] = [D_b]/[L_T]$, gives the critical molar ratio of CHAPS to total lipid in the micelles at the onset of the transition (Paternostre et al., 1988; Ollivon et al., 1988). Using the average relative molar mass M_r(lipid) = 800, we obtain $R^c(M) = 1.5 \pm 0.2$.

To investigate the influence of the ionic strength on the vesicle–micelle transformation, vesicles were prepared by dialysis of CHAPS-solubilized lipids. Since the light scattering signal of the (small) vesicles, which were prepared at 50 mM NaCl, was not sufficient for the analysis of the transformation to micelles, part of the vesicles made at 500 mM NaCl were dialyzed against 50 mM NaCl before solubilization. After the dialytic reduction of the salt concentration, the intensity of the light scattering signal was only slightly lower (≤10%). However, the concentration of CHAPS, which was needed for complete solubilization, was considerably reduced. Specifically, at 50 mM NaCl, $[CHAPS_w]^c = 2.8 \pm 0.2$ mM, and at 500 mM NaCl, $[CHAPS_w]^c = 3.65 \pm 0.2$ mM. To exclude that the effect was due to changes in the lipid concentration, the content of phosphorus and the volume of the samples before and after dialysis were measured, and no change was found. The slope, $R^c(M)$, was obtained using eq 2 and found to be only slightly different for the two salt concentrations. Specifically, $R^c(M) = 0.46 \pm 0.05$ in 50 mM NaCl and 0.54 ± 0.05 in 0.5 M NaCl, respectively. These values are relatively low compared to the value $R^c(M) = 1.5$ for the solubilization of pure lipid vesicles. The difference suggested that considerable amounts of CHAPS had remained in the dialysis vesicles. Therefore, we analyzed the content of CHAPS in the vesicles by thin-layer chromatography. We found 0.1–0.2 mol of CHAPS/mol of lipid. Extending the CHAPS dialysis to 7 days (one change every day) had no influence on these results. The main components of the soybean phospholipids were unchanged by the dialysis. However, the lipids with a

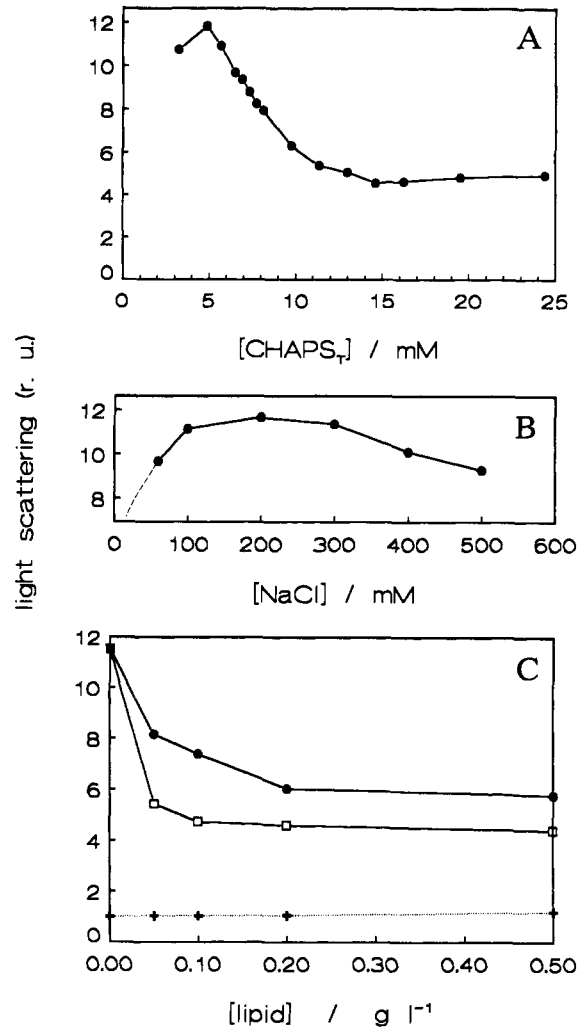

FIGURE 3: Aggregation of the AChR dimer in detergent solution measured by light scattering (90°, 500 nm). (A) Aggregation of partially delipidated AChR (0.2 g/L) as a function of [CHAPS] in buffer A and 50 mM NaCl. (B) AChR aggregation as a function of salt concentration. Samples were prepared as in (A) with 8 mM CHAPS. (C) AChR aggregation as a function of the lipid content. Samples were prepared as in (A); [CHAPS] = 4.9 mM; [NaCl] = 50 mM. Lipid: (●) SBL; (□) PI. The lower curve without protein (+) is identical for both lipids. The decrease of the light scattering signal indicates the disintegration of aggregates.

R_f value < 0.2 (lysophospholipids) were completely lost.

Protein Solubilization. Stimulated by the biochemical analysis and the electron microscopy, we also analyzed the aggregation of AChR in detergent solution. In order to mimic the different stages of the reconstitution process, the concentration of CHAPS was varied from 3.25 to 24.4 mM, and the changes of the aggregation were measured by light scattering. The protein concentration was constant (0.2 g/L) in all experiments. To minimize the contribution of vesicular and micellar lipid structures to light scattering, no lipid was added in the last step of the protein purification (see preparation method B). Attempts to purify the AChR without added lipid throughout the whole purification process resulted in the loss of about 50%–90% of the normal protein yield.

Although the CHAPS concentration was always above the CMC (see below), the light scattering signal decreased from its highest value at 5 mM CHAPS to one-third of that at 14.5 mM CHAPS (Figure 3A). At 16 mM CHAPS the correlation functions of the quasi-elastic light scattering profile could be well fitted by two exponentials, indicating at least two components. The calculated diffusion coefficients and re-

Table I: Dynamic Light Scattering Data of CHAPS-Solubilized AChR at 10 °C[a]

[CHAPS] (mM)	component 1		component 2	
	D (10^{-7} cm^2 s^{-1})	r_s (nm)	D (10^{-7} cm^2 s^{-1})	r_s (nm)
16	2.05	10.4	0.41	50.4
8	0.97	16.3	0.15	107

[a] AChR dimers were prepared by method B. The diffusion constant $D_{20,w}$ and the hydrodynamic radius r_s were calculated for water at 293 K (20 °C) according to eq A4. In each case the two observed exponential components contribute approximately equally to the intensity of the scattered light.

spective hydrodynamic radii are given in Table I. The smallest Stokes radius, r_s = 10.4 nm, is consistent with the AChR dimer/lipid species (see Appendix). The Stokes radius of the second component, 50.5 nm at 16 mM CHAPS, is already in the range of the radii of small vesicles. The values given at 8 mM CHAPS can only be taken as a rough estimation of the aggregate size, because the analysis showed a broad size distribution and an additional "static" component, indicating very large particles like those found by electron microscopy (Figure 6).

The effect of the salt concentration on the aggregational state of the AChR was relatively small (Figure 3B). The light scattering signal increased only about 15% between 60 mM NaCl and its maximum value at 200 mM NaCl. At 500 mM NaCl, the signal returned to its initial value measured at 60 mM NaCl.

At 4.9 mM CHAPS, titration of lipid to the partially delipidated AChRs effectively dissociated the protein aggregates (Figure 3C). Even at lipid concentrations ≤ 0.2 g/L (≈0.25 mM), the reduction of the light scattering intensity was of the same magnitude as achieved by 15 mM CHAPS (Figure 3A). The charged lipid PI (second curve) had a markedly higher efficiency in protein dissociation than SBL (upper curve). The lower curve was measured without protein; the signal intensity was the same for both lipids and hardly exceeded that of the pure detergent solution (zero lipid).

Determination of AChR-Bound Lipid. To interpret the lipid effect, it was necessary to analyze the lipid bound to the detergent-solubilized AChR in the mixed micelles. The lipid extraction method of Hara and Radin (1978) was superior to the more common method of Bligh and Dyer (1959). The aqueous phase was extracted by an additional acidic step, resulting in about 70% of the yield of the first step. Two different preparations, with four independent extractions each, have been analyzed. The eluate from the affinity column (method B) contained 280 ± 30 phospholipids per AChR dimer, as measured densitometrically. Residual unbound lipid was removed by gel permeation chromatography on a Superose 6 column (1.6 cm × 60 cm) using buffer W3. It was found that 130 ± 34 phospholipids remained bound per AChR dimer after this separation.

Analysis of Reconstituted Vesicles in Sucrose Gradients. AChR proteins, which were reconstituted at a concentration of 0.5 g/L, at a lipid to protein ratio of 20/1 (w/w), and at NaCl concentrations between 10 mM and 1 M, were analyzed by discontinuous gradient centrifugation (flotation, 37%–5% sucrose). The peak of the vesicle distribution, as determined by turbidity measurements (OD) at the wavelength 450 nm, appeared in fractions 6–9, where the sucrose density was 20%–10% (Figure 4A). The contents of the AChR protein in the gradient fractions, monitored by intrinsic protein fluorescence and by ^{125}I-α-BTX binding, were identical within 10%. The sucrose gradient profiles (intrinsic fluorescence,

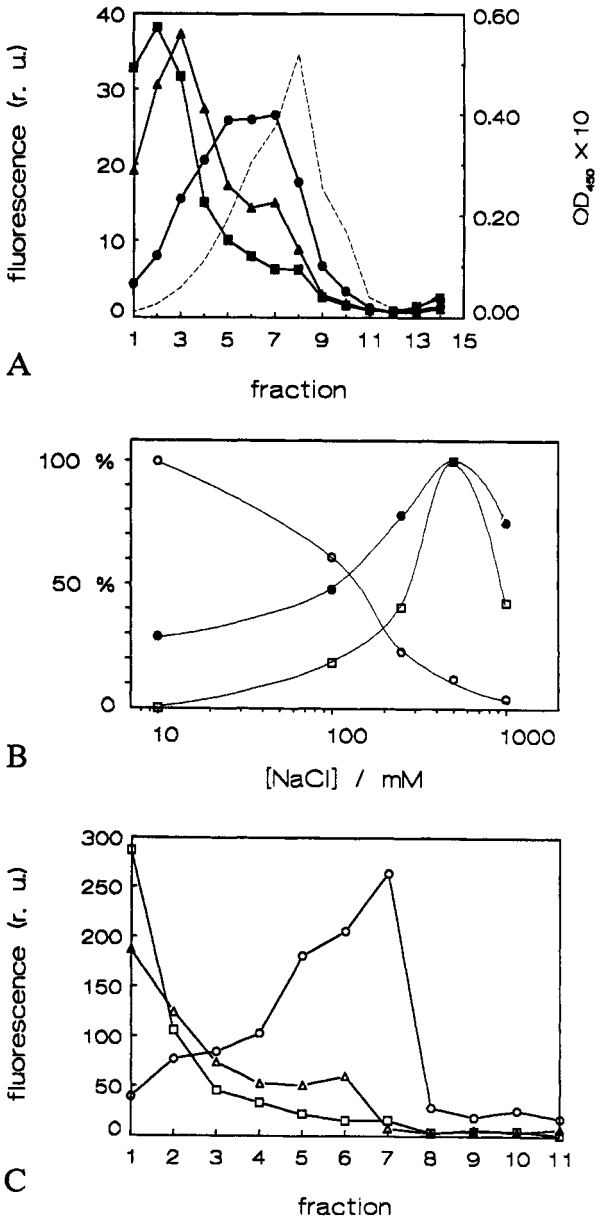

FIGURE 4: Reconstitution of AChR protein. (A) Fluorescence signal (λ_{exc} = 280 nm; λ_{em} = 340 nm) reflecting the concentration of AChR protein, reconstituted at 0.5 g/L in 10 g/L soybean lipid/cholesterol (9:1 w/w), as a function of the gradient fraction number and of the salt concentration. The protein/lipid samples were placed at the bottom of a discontinuous sucrose gradient containing 2 mL of each of 37% (including sample), 30%, 20%, 10%, and 5% sucrose and 1 mL of 0% sucrose. For clarity, only three NaCl concentrations are represented [(■) 10 mM; (▲) 100 mM; (●) 500 mM] together with the turbidity data (OD, dotted line) at 450 nm, reflecting the vesicle density (which is roughly independent of the salt concentration). The relatively high optical density values of fractions 1–4 are partially due to the large protein aggregates. (B) AChR protein contents of fraction 1 (○) and fraction 8 (●), which contains the major portion of vesicles, and carbamoylcholine-induced Li^+ influx (□) into vesicles reconstituted at the different NaCl concentrations, in percent of the highest values, respectively. (C) AChR at the lower protein concentration 0.15 g/L, compared to 0.5 g/L in (A). The NaCl concentration are 10 mM (□), 100 mM (△), and 840 mM (○). The highest protein content of the vesicles is at 840 mM NaCl (fraction 7). All measurements were done in triplicate on two different preparations.

Figure 4A) are shown for three different salt concentrations. At 10 mM NaCl the whole protein was recovered near the bottom of the gradient; no vesicle incorporation had occurred. When the salt concentration was increased to 100 mM NaCl, there was again a large peak near the bottom and, in addition,

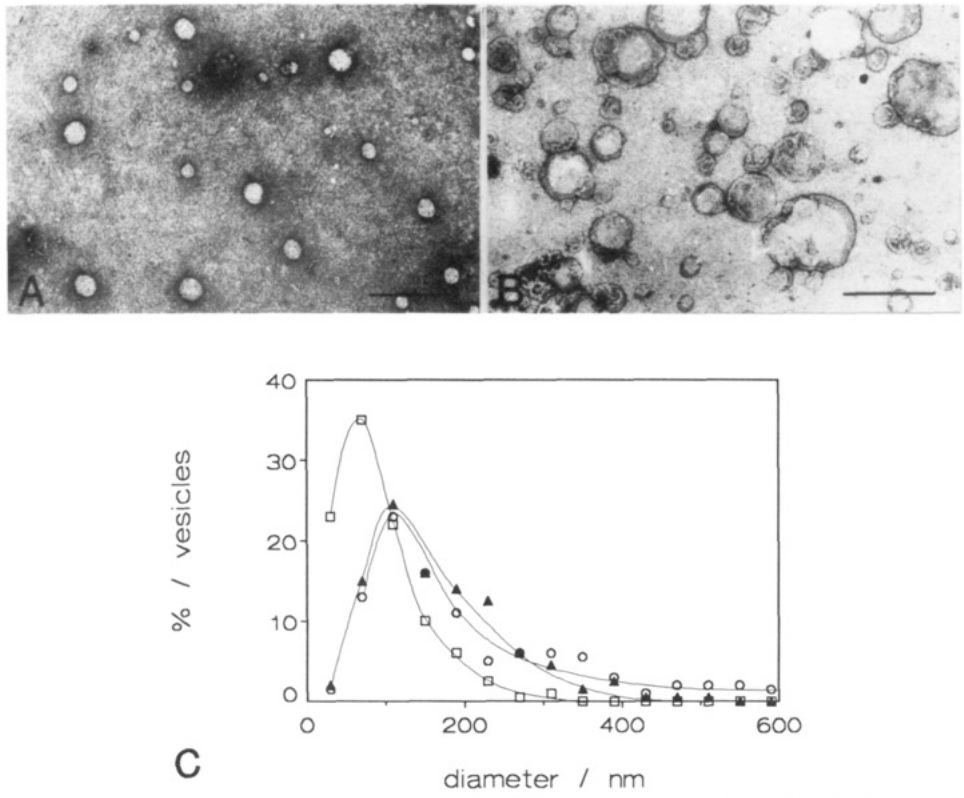

FIGURE 5: Size distribution of AChR-containing vesicles reconstituted at different salt concentrations. (A, B) Electron micrographs of vesicles reconstituted by CHAPS dialysis at 100 mM NaCl and 500 mM NaCl, respectively. (C) Size distribution of vesicles at different NaCl concentrations [(□) 100 mM; (▲) 0.5 M; (○) 1 M]. The diameters of the vesicles are uncorrected values measured from electron micrographs.

a small one coinciding with the vesicle peak, as determined by turbidity measurements. The vesicle peak and the protein curve of the samples reconstituted at 500 mM NaCl had the highest overlap. In Figure 4B the AChR contents of fractions 1 and 8, respectively, normalized to the highest value, are plotted as a function of [NaCl]. Concomitant with the reduction of the protein concentration in fraction 1, the protein was increasingly recovered in fraction 8 together with the vesicles when the salt concentration was raised. It was only at 1 M NaCl that the protein concentration in fraction 8 was obviously too low as compared to its reduction at the bottom of the gradient. It appeared that part of the protein was lost during the detergent dialysis at 1 M NaCl and with a starting protein concentration of 0.5 g/L. This was presumably due to partial precipitation.

At a lower protein concentration, 0.15 g/L, and at respectively lower concentrations of lipid and detergent, the highest reconstitution yield was achieved at 840 mM NaCl. There was apparently no loss of protein at high ionic strengths (Figure 2C). It is clearly seen that the large increase in the protein incorporation happened between 100 and 840 mM NaCl.

Electron Microscopy. The toplogy and distribution of the vesicles and of the AChR molecules in the reconstituted vesicles as analyzed by electron microscopy are shown in Figure 5. The average diameter of the vesicles at 1 M NaCl was about 57% larger than at 100 mM NaCl. Conspicuously, the average size of the vesicles increased with increasing salt concentration.

Most of the receptor protein reconstituted at [NaCl] $\geq$ 500 mM was recovered as single dimers, randomly distributed on the surface of the vesicles (Figure 6A). At a lower salt concentration a considerable amount of receptor was found in large aggregates (Figure 6B). These aggregates were obviously too large to be incorporated in vesicles. The aggregates corresponded to the protein recovered in the lower fractions of the sucrose gradients. Similar aggregates were seen when AChR monomers were reconstituted by the usual cholate dialysis at 100 mM NaCl (see Figure 6C).

Vesicle Flux. The functional activity of AChRs, reconstituted at a protein concentration of 0.5 g/L and at different salt concentrations, was analyzed by Li^+ flux measurements. In Figure 4B the carbamoylcholine-induced Li^+ uptake is compared with the protein incorporation into the vesicles. Apparently, vesicle reconstitution at 10 mM NaCl did not lead to AChR incorporation. Correlating with the increased protein contents, the flux activity also increased with the salt concentration. In correspondence with the lower protein incorporation, the Li^+ flux was lower at 1 M than at 0.5 M NaCl.

When the AChR monomer species were reconstituted at 500 mM NaCl by CHAPS dialysis, the amount of overall Li^+ uptake was in the same range as with dimer preparations at otherwise identical conditions. Compared with the dimer values, the Li^+ uptake of the monomer preparation was 65 $\pm$ 8% at 0.1 mM carbamoylcholine and 103 $\pm$ 10% at 1 mM carbamoylcholine.

DISCUSSION

Comparison of CHAPS with Other Detergents. The results have shown that CHAPS is a good detergent for the reconstitution of AChR proteins into lipid vesicles when appropriate conditions are maintained. The flux data consistently match the results of the biochemical analysis and the reconstitution products as seen by electron microscopy. Especially with respect to the monomeric receptor protein, CHAPS was superior to cholate for quantitative reconstitution. Although the agonist binding constant of the monomer species is apparently smaller, the number of incorporated receptor molecules appears to be equivalent to the dimer preparations. In contrast to cholate, CHAPS can also be used for the purification of AChR

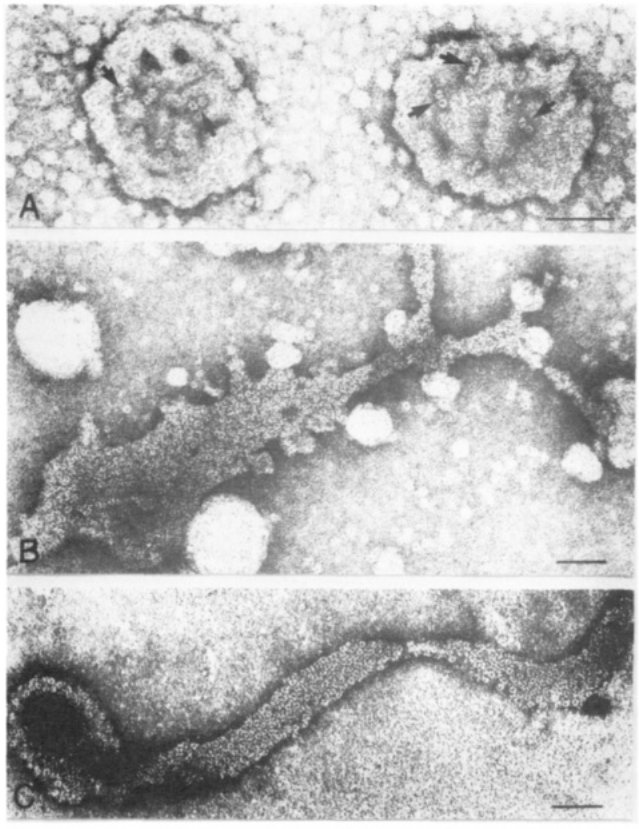

FIGURE 6: (A, B) Electron micrographs of AChRs reconstituted by CHAPS dialysis. (A) Singly distributed AChRs reconstituted in 500 mM NaCl. The number of vesicles containing reconstituted receptors increased with the salt concentration. (B) Large aggregate of non-incorporated AChRs reconstituted in 250 mM NaCl. The number of aggregates was largest at 10 mM NaCl. (C) Aggregate of AChR monomers reconstituted at 100 mM NaCl by cholate dialysis.

proteins on the dicaproyl-MP affinity column. Lubrol-WX has a C_{18}-hydrocarbon chain. Hydrocarbon chains of this length are typical of natural lipids. Other detergents like CHAPS or octyl glucoside, commonly used for the reconstitution of membrane proteins, have considerably shorter hydrocarbon chains. This might be the reason why Lubrol-WX can be used without the need of additional lipid to stabilize the receptor. On the other hand, Lubrol-WX has a very low CMC (0.02–0.06 mM), rendering dialytic removal of detergent impracticable.

CMC of CHAPS. The CMC values reported for CHAPS are 10 mM (Repke & Liebmann, 1987), 8 mM (Sigma, Fluka), 6–10 mM (Calbiochem), 6.5 mM (Lasic et al., 1989), and 4 mM (Boehringer, Mannheim). A possible reason for these differences is the use of different methods of CMC determination (e.g., light scattering or conductivity). The monomeric detergent concentration, $[D_w]^C$ = 2.8–3.2 mM, characterizing the vesicle–micelle transformation, is *below* the CMC = 4 mM, which was determined by surface tension measurements. In the Appendix it is shown that the small temperature dependence of the CMC is in agreement with the thermodynamic estimations. Furthermore, it has been reported that the CMC of CHAPS is not affected by the addition of NaCl, up to 0.6 M (Lasic et al., 1989), which is also in agreement with our data.

Vesicle Solubilization. When *sonicated vesicle suspensions* were solubilized, it was found that the detergent/lipid ratio in the micelles for CHAPS [R^c(M) = 1.5] is larger than for cholate [R^c(M) = 0.9] but considerably smaller than for octyl glucoside [R^c(M) = 3.8; Paternostre et al., 1988]. The value $[D_w]^c$ = 2.8 mM, which was determined from the solubilization of the *dialysis vesicles* at 50 mM NaCl, is only slightly lower than that of the sonicated vesicle suspensions ($[D_w]^c$ = 3.2 mM). In contrast, the value of R^c(M) was only 0.46 when the *dialysis vesicles* were solubilized. Although this low value can be partly explained by the residual CHAPS, which was not removed during the dialysis, additional effects should be considered as well. For example, the structure of the lipid vesicles affects the form of the solubilization curve. However, R^c(M) was the same for multilamellar and unilamellar vesicles solubilized with cholate or octyl glucoside (Paternostre et al., 1988). We presume that the residual CHAPS in the vesicles after extensive dialysis is due to stable and specific CHAPS–lipid interactions. On the contrary, the removal of the lysophospholipids by the dialysis reflects normal detergent behavior. It should be noted that residual CHAPS in the vesicles does not affect the determination of the free detergent concentration $[D_w]^c$.

Protein (Dis)aggregation. The dissociation of protein aggregates by increasing detergent concentrations, between [CHAPS] = 5 and 15 mM, is rather unexpected. The latter value significantly exceeds the CMC of 4 mM (determined here). It therefore appears that it is the detergent micelles as such which associate with the protein aggregates and, in turn, cause aggregate dissociation.

Titration of lipid to the delipidated system showed that even lipid concentrations $\leq$0.2 g/L can induce the separation of the aggregated proteins. As the AChR protein concentration was also 0.2 g/L, one can easily estimate that 150–300 of the added lipid molecules interact with the dimer species. At $[CHAPS_T]$ = 4.9 mM $[D_b]$ is $\approx$1 mM. When the concentration of the lipid in the micelles $[L_m] \leq 0.1$ mM, then the ratio is R(M) = $[D_b]/[L_m] \geq 10$. In the light of this high ratio, the AChR can bind lipids with considerably high affinity, in addition to the tightly bound lipids. In the presence of Lubrol-WX, which is considered a mild detergent, approximately 200 lipid molecules remain bound to the AChR dimer (Herlemann, 1989), compared to 130 tightly bound phospholipids extracted from the CHAPS-solubilized AChR. The stronger delipidation of the protein micelles by CHAPS also explains why additional lipid is needed to stabilize the receptor protein during purification. Jones et al. (1988) found a minimum number of 90 lipids/AChR dimer necessary to prevent irreversible loss of agonist-induced transport function. This value is comparable to our data, 130 lipids/dimer, when it is considered that we extracted one-third of the lipids in an additional acidic step.

About 35% of the boundary lipids are negatively charged (Herlemann, 1989), a value comparable with that of soybean phospholipids. The overall charge of about 35 charged lipids is probably larger than the number of the charged groups at the surface of the protein itself. In addition, the charged lipid PI was more effective in the receptor aggregate dissociation than SBL (Figure 3C). Therefore, charge repulsion seems to be the driving force of aggregate dissociation.

Loss of agonist binding was found at lipid concentrations below 0.5 g/L (Heidmann et al., 1980). Popot et al. (1981) have also shown that negative lipids are necessary for protein incorporation into vesicles when cholate is used as the detergent. Applying solely the uncharged egg phosphatidylcholine, most of the AChR protein was recovered in nonincorporated "caterpillar"-like aggregates, similar to those shown in the electron micrograph (Figure 6).

It is widely accepted that lipid added to detergent solutions can preserve the functional structure of delicate membrane proteins during purification. Our results with the AChR

suggest that the lipid prevents a reversible aggregation, rather than an irreversible denaturation of the proteins. Our interpretation is corroborated by the experiments by Hanke et al. (1990), who have reconstituted insect nAChRs eluted from SDS gels. Recently, we were able to maintain the transport function of AChR species, purified in the presence of Lubrol-WX without added lipid, when CHAPS was used for the reconstitution into protein–lipid vesicles (data not shown).

Salt Effects. Usually, reconstitution of membrane proteins is performed at, or below, physiological salt concentrations (≤0.15 M). It has been shown that incorporation of AChRs into lipid vesicles by cholate dialysis is diminished at higher salt concentrations, e.g., [NaCl] > 100 mM (Popot et al., 1981). The detergent properties of cholate, an anionic surfactant, can be expected to depend critically on the charge screening effect at higher ionic strengths. For acidic lipids, it was found that charge screening by monovalent counterions is dominant at ≥100 mM NaCl (Helm et al., 1986). High ionic strength is known to favor aggregation and fusion of cell membranes and lipid vesicles. The threshold concentration of NaCl for the aggregation of vesicles consisting of negatively charged lipids is in the range of 200–500 mM (Ohki et al., 1982; Yishimura & Aki, 1985). The average size of the vesicles, which were formed by CHAPS dialysis, increased with the salt concentration (Figure 5).

As in vesicle fusion, electrostatic repulsion forces have to be overcome during reconstitution. The ternary protein–lipid–detergent micelles have to aggregate and "fuse" with other micelles or with coexisting vesicular structures in order to become protein–lipid vesicles. Several other observations are consistent with the incorporation of membrane proteins into detergent-saturated vesicles. For instance, bacteriorhodopsin and ATP synthase are incorporated into lipid vesicles at subsolubilizing detergent concentrations (Rigaud et al., 1988; Richard et al., 1990). Similarly, we found that pure lipid vesicles occurred before protein-containing membrane structures were formed during dialytic removal of octyl glucoside from mixed micelles (Schürholz et al., 1989b).

On the other hand, the light scattering experiments have shown that the enhancement of protein incorporation by high ionic strength is not a matter of protein (dis)aggregation.

Another effect of the salt concentration concerns *the solubilization* of vesicles. At 500 mM NaCl, $[D_w]^c$ and $[D_T]^c$ were shifted toward higher detergent concentrations. This implies that, vice versa, vesicles are formed in an earlier phase during *detergent dialysis*. At higher detergent concentrations protein aggregation is reduced and protein insertion into vesicles is facilitated. As $R^c(M)$ did not change significantly with the salt concentration, the increase of the $[D_T]^c$ values in 500 mM NaCl is based on a reduction of the partition coefficient $\Gamma = [D_b]/[D_w]$.

AChR Distribution. The light scattering intensity at 16 mM CHAPS is comparable to the values obtained at 5 mM CHAPS and lipid concentrations ≥0.2 g/L (Figure 3A,C). Thus, the particle sizes measured at 16 mM CHAPS, i.e., dimers and aggregates of about 50-nm mean radius, should be similar to those at the beginning of the CHAPS dialysis. Anholt et al. (1981) found a maximal lipid/protein ratio of 16/1 (w/w), which could not be increased by addition of lipid. In the light of the aggregates that we have found in detergent solution, such results are readily understood. Assuming that an aggregate incorporates into, or fuses with, a preformed lipid vesicle as a whole, the size of the protein aggregate determines the minimal protein/lipid ratio in the vesicles. (Only in the case of nonaggregated single protein species can the minimum of *one* protein per vesicle be achieved.) The broad peaks in the sucrose gradients (Figure 4A) probably reflect a broad distribution of lipid/protein ratios. A distinct lipid vesicle peak void of protein as has been reported for cholate dialysis (Anholt et al., 1981) was not found. Since the electron micrographs mainly show individual monomers or dimers, the aggregates obviously can dissociate after incorporation into vesicles.

In conclusion, these quantitative estimates provide a reliable basis to choose the proper conditions of the concentrations of CHAPS, of the lipids, of the protein, and of the NaCl concentrations not only for the reconstitution into vesicles but also for solution studies on isolated defined protein–lipid particles. So far, high ionic strength does not seem to denature the AChR. Other membrane proteins such as dopamine receptors and G-proteins have been purified at 1 M NaCl (Ohara et al., 1988).

The best results concerning membrane protein reconstitution were also obtained with CHAPS in investigations of the sodium channel (Wondra, 1990) and the F_c-receptor (Rivnay & Metzger, 1982). Thus, CHAPS seems to be one of the most suitable detergents for the reconstitution of membrane proteins.

Acknowledgments

We thank Dr. Wolfgang Eimer, Bielefeld, for the dynamic light scattering measurements and Barbara Rauer and Vera Volz for AChR monomer preparations.

Appendix

Temperature Effect on Micellization. If micelle formation is viewed as a (diffuse) phase transformation, the isobaric (p = constant) temperature depencence of the CMC for the overall process $mD_w \rightleftarrows M$, where m is the mean aggregation number, can be described by

$$\left(\frac{\partial \ln X(\mathrm{CMC})}{\partial T}\right)_{p,\mathrm{coex}} = \frac{\Delta_M H^\ominus}{RT^2} \qquad \text{(A1)}$$

In eq A1 X(CMC) is the critical micellization mole fraction defined by $X(\mathrm{CMC}) = n_w/[n_w + n(H_2O)] = \mathrm{CMC}/(\mathrm{CMC} + 55.56\ \mathrm{M})$, and $\Delta_M H^\ominus = H_m^\ominus - H_w^\ominus$ is the standard molar enthalpy change for the transfer of detergent molecules from the aqueous phase (partial molar standard enthalpy $H_w^\ominus$) into the micellar phase (standard partial enthalpy $H_m^\ominus$, per mole of detergent) under coexistence conditions, i.e., $[D_w] = \mathrm{CMC}$.

In a narrow range of temperature $\delta T \ll T$, where $\Delta H^\ominus$ may be considered constant, eq A1 can be written as

$$\frac{\delta X(\mathrm{CMC})}{X(\mathrm{CMC})} = \frac{\delta \mathrm{CMC}}{\mathrm{CMC}} = \frac{\Delta H^\ominus}{RT}\frac{\delta T}{T} \qquad \text{(A2)}$$

Here we take 293 K (20 °C) as the reference temperature; the respective reference CMC is CMC(CHAPS) = 4 ± 0.4 mM and X(CMC) = 7.2 (±0.7) × 10^{-5}.

For nonionic and zwitterionic detergents like CHAPS the $\Delta_M H^\ominus$ values in the temperature range at 293 K are positive but small ($\Delta_M H^\ominus \approx RT$) as for all detergents (Tanford, 1973; Evans & Ninham, 1986). With $\Delta_M H^\ominus = RT$ (=2.44 kJ mol^{-1}) the decrease of T from 293 to 278 K (5 °C) implies a relative decrease $\delta T/T = -0.05$ resulting in a relative decrease of the CMC(CHAPS) by δCMC/CMC = 5%. The experimental observation that CMC(CHAPS, 5 °C) = 3.8 (±0.4) mM is within the error margin of the value at 20 °C is thus consistent with the thermodynamic estimation.

Vesicle–Micelle Phase Transformation. The solubilization of lipid vesicles by detergents can be described in terms of a transition between two macroscopic phases. Within the ves-

icle–micelle phase transformation region, at $[D_w] = [D_w]^c$ = constant, the ratio $R = [D_b]/[L_T]$ is a mean value **R** of the two contributions: the fraction β_M of mixed lipid–detergent micelles characterized by $R^c(M \rightarrow V)$ and the fraction $(1 - \beta_M)$ of detergent binding vesicles with $R^c(V \rightarrow M)$. Hence

$$\mathbf{R} = \beta_M R^c(M) + (1 - \beta_M) R^c(V) \qquad (A3)$$

The minimum value of $[D_w]^c$ in Figure 2 indicates the complete solubilization of the lipid–detergent vesicles or, vice versa, the onset of vesicle formation. At $[D_w]^c$ we thus have $\beta_M = 1$ and $\mathbf{R} = R^c(M)$. Applying mass conservation $[D_T] = [D_b] + [D_w]$ to this point and denoting the $[D_w]$ value at $[L_T] = 0$ by $[D_w]^c$ yields $[D_T]^c = [D_w]^c + R^c(M)[L_T]$, which is eq 2 of the text. Obviously, $[D_w]^c = [D_T]^c{}_0$. The numerical value of $R^c(M)$ is obtained from the slope according to $R^c(M) = d[D_T]^c/d[L_T]$.

Particle Size. The small component of the scattering intensity profile of the AChR dimer preparation at 16 mM CHAPS is characterized with a translational diffusion coefficient of $D_{20,w} = 2.05 \times 10^{-7}$ cm^2 s^{-1} (see Table I). The hydrodynamic (Stokes) radius r_s of a spherical particle at 20 °C is given by

$$r_s = kT/(6\pi\eta D_{20,w}) \qquad (A4)$$

where k is the Boltzmann constant, η is the viscosity of the buffer solution, and $\eta(20\ °C) = 10^{-3}$ J·s·m^{-3} (=0.01 P). Substitution of $D_{20,w}$ into eq A4 yields the Stokes radius r_s = 10.4 nm.

Electron density maps of the AChR protein (Toyoshima & Unwin, 1988) show that the protein part of a receptor dimer would fit into a spherical cross section of a radius, which is roughly 8–9 nm. It should be noted that the Stokes radius reflects the sphere equivalent to the real, probably not spherical, shape of the receptor species and of the aggregates and that the dimer value of 10.4 nm includes the receptor-associated lipids.

The molar mass of the diffusing protein–lipid–detergent particle can be estimated by

$$M = s_p RT/[D(1 - v\rho)] \qquad (A5)$$

where s_p is the particle's sedimentation coefficient in units of S = 10^{-13} s, v the specific protein volume, and ρ the mass density of the solution. For the dimer species, the so-called H-form of the nAChR, $s_D(20\ °C) = (14 \pm 1) \times 10^{-13}$ s [see, e.g., Rüchel et al., (1981)], $v = 0.745$ cm^3·g^{-1}, and $\rho = 1.02$ g·cm^{-3} (buffer solution). The dimer preparation, component 1 (see Table I), yields M(dimer) = 690 000 g·mol^{-1}. The protein and sugar parts of the dimer add up to M_r = 580 000. If the difference of 110 000 g·mol^{-1} is attributed to bound lipid and detergent, we obtain 110 000/800 = 137.5, where M_r-(lipid) = 800 on the average. This estimate compares well with the value of 130 lipids per dimer from the chemical analysis (see Results).

If the Stokes model is applied to the other receptor species and if the volume of the AChR monomer particle is taken as one-half of that of the dimer, we can estimate the translational diffusion coefficient of the monomer by $D_{20,w}$(monomer) = $(0.5)^{1/3} D_{20,w}$(dimer) = 2.56×10^{-7} cm^2 s^{-1}. This numerical value is similar to the estimate of 2.94×10^{-7} cm^2 s^{-1} given by Rüchel et al. (1981) for a *Torpedo* receptor monomer preparation.

From eq A4 it is obvious that $D \propto (1/V)^{1/3}$. Applying the Stokes model also to the higher components of the intensity scattering profile, the aggregation number N of a receptor aggregate is given by $N = [D_{20,w}(\text{dimer})/D_{20,w}(\text{dimer})_N]^3$. Component 1 at the smaller CHAPS concentration (8 mM) thus yields the average value of N = 9.4 dimers per aggregate particle.

Registry No. CHAPS, 75621-03-3; NaCl, 7647-14-5.

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Characterization of Biotinylated Repair Regions in Reversibly Permeabilized Human Fibroblasts†

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ABSTRACT: We have examined the incorporation of biotinyl-11-deoxyuridine triphosphate (BiodUTP) into excision repair patches of UV-irradiated confluent human fibroblasts. Cells were reversibly permeabilized to BiodUTP with lysolecithin, and biotin was detected in DNA on nylon filters using a streptavidin/alkaline phosphatase colorimetric assay. Following a UV dose of 12 J/m^2, maximum incorporation of BiodUTP occurred at a lysolecithin concentration (80–100 μg/mL) similar to that for incorporation of dTTP. Incorporation of BiodUTP into repair patches increased with UV dose up to 4 and 8 J/m^2 in two normal human fibroblast strains, while no incorporation of BiodUTP was observed in xeroderma pigmentosum (group A) human fibroblasts. The repair-incorporated biotin was not removed from the DNA over a 48-h period, and only slowly disappeared after longer times (~30% in 72 h), while little of the biotin remained in cells induced to divide. Furthermore, the stability of the biotin in repaired DNA was unaffected by a second dose of UV radiation several hours after the biotin-labeling period to induce a "second round" of excision repair. Exonuclease III digestion and gap-filling with DNA polymerase I indicate that the majority of biotin-labeled repair patches (~80%) are rapidly ligated in confluent human cells. However, the remaining patches were not ligated after a 24-h chase period, in contrast to dTTP-labeled repair patches. The BiodUMP repair label in both chromatin and DNA is preferentially digested by staphylococcal nuclease, preventing the use of this enzyme for nucleosome mapping in these regions. However, restriction enzyme and DNase I digestions of the isolated nuclei demonstrate that at least some of the repair-incorporated BiodUMP becomes associated with nucleosome core DNA following nucleosome rearrangement. Therefore, the biotin tag does not appear to prevent the folding of nascent repair patches into native nucleosome structures.

The biotinylated deoxyuridine triphosphate nucleotide (BiodUTP)[1] developed by Langer et al. (1981) has been used extensively to label DNA. Incorporation of biotinylated nucleotides into DNA has been accomplished enzymatically in vitro (Langer et al., 1981), as well as in vivo (Hiriyanna et al., 1988). By exploiting the strong interaction ($K_d = 10^{-15}$) between biotin and streptavidin, a 60-kDa protein isolated from the bacterium *Streptomyces avidinii*, picogram quantities of biotinylated DNA can be detected when streptavidin is coupled to an appropriate indicator molecule such as alkaline phosphatase (Leary et al., 1983). The extremely sensitive streptavidin–biotin affinity system has been used to isolate DNA–protein complexes (LeBlond-Francillard et al., 1987; Vincenz et al., 1991), and was employed to visualize replicated DNA in the electron microscope (Hiriyanna et al., 1988).

Hunting et al. (1985a) have shown that BiodUTP is also incorporated into DNA during repair synthesis in mechanically disrupted human cells. Mammalian cells are not permeable to highly charged molecules, including nucleotide triphosphates, and therefore require permeabilization prior to BiodUTP addition. However, in the studies by Hunting et al. (1985a), cells are irreversibly permeabilized by mechanical disruption, and this method does not allow examination of repaired regions at long times after biotin labeling. Transient permeabilization of cell membranes to small molecules is possible, however, with lysolecithin under conditions where the cells remain viable (Castellot, 1980). Indeed, Lorenz et al. (1988) demonstrated that confluent human fibroblasts exposed

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[1] Abbreviations: dNTPs, 2′-deoxynucleotide 5′-triphosphates; BiodUTP, 5-[*N*-(*N*-biotinyl-ε-aminocaproyl)-3-aminoallyl]-2′-deoxyuridine 5′-triphosphate; BiodUMP, 5-[*N*-(*N*-biotinyl-ε-aminocaproyl)-3-aminoallyl]-2′-deoxyuridine 5′-monophosphate; dThd, thymidine; PBS, phosphate-buffered saline; UV, ultraviolet; dCyd, deoxycytidine; dTTP, thymidine 5′-triphosphate; dTMP, thymidine 5′-monophosphate; dCTP, 2′-deoxycytidine 5′-triphosphate; dCMP, 2′-deoxycytidine 5′-monophosphate.

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