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Solubilization and Partial Characterization of [^{3}H]Choline Mustard-Labeled High-Affinity Choline Carrier from Presynaptic Plasma Membrane of *Torpedo* Electric Organ

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Abstract. The objective of the present study was to characterize the hydrodynamic properties of the detergent-solubilized high-affinity choline carrier from presynaptic plasma membranes of electric organ of *Torpedo marmorata* following radioaffinity labelling with [^{3}H]choline mustard aziridinium ion ([^{3}H]ChM Az). Membrane proteins were solubilized with 0.8% CHAPS, then analyzed by gel permeation chromatography and equilibrium density sedimentation in sucrose gradients made in H_2O and D_2O. The radiolabeled protein eluted from the gel filtration column with a distribution coefficient of 0.36 allowing calculation of a Stokes radius of 4.4 nm. Distribution of the [^{3}H]ChM Az-labeled proteins differed in the H_2O and D_2O sucrose gradients, with apparent sedimentation coefficients of 5.2 and 4.7, respectively, indicating that detergent may be bound to hydrophobic regions of the protein. The calculated partial specific volume for the protein complex was 0.76. The estimate of molecular mass for the complex was 115,000 Da, with the protein portion having a molecular mass of about 83,000 Da. Analysis of the peak fractions from the gel filtration column and sucrose gradients by sodium dodecyl sulfate-polyacrylamide gel electrophoresis revealed the presence of a ^{3}H-labeled polypeptide at),000–42,000 Da. The choline carrier may exist as a dimer of this polypeptide.

The sodium-coupled, high-affinity choline carrier localized at cholinergic presynaptic nerve terminals transports choline used as substrate in the synthesis of the neurotransmitter acetylcholine (ACh) (Kuhar and Murrin, 1978). Accumulation of choline into the nerve ending by this carrier appears to be the rate-limiting step in the biosynthetic reaction for ACh, but little is known about the physiological mechanisms that regulate the activity of the carrier or about the molecular events involved in the translocation process. The carrier protein has not been purified or characterized biochemically.

We have demonstrated that the nitrogen mustard analog of choline, choline mustard aziridinium ion (ChM Az), binds irreversibly to the choline carrier in rat brain synaptosomes, thereby inhibiting transport of choline (Rylett and Colhoun, 1980, 1984). Under some circumstances, [^{3}H]ChM Az acts as a transportable substrate on the high-affinity choline carrier and is accumulated into the nerve terminal (Rylett and Walters, 1990). As it has been demonstrated that ChM Az has selectivity for the choline transport protein relative to some other neurotransmitter carriers in synaptosomes and is a more potent inhibitor of the high-affinity carrier compared to the sodium-independent low-affinity carrier at low micromolar concentrations (Rylett and Colhoun, 1980; Rylett, 1986; Uney and Marchbanks, 1987), we used [^{3}H]ChM Az as an affinity ligand to radiolabel and identify the choline transport protein in presynaptic plasma membranes from electric organ of *Torpedo marmorata* (Rylett, 1988). These studies revealed that the [^{3}H]ChM Az bound to two polypeptides with apparent molecular masses of about 42,000 and 58,000 and that radiolabeling of these molecular species was dependent upon the presence of sodium ion and blocked by the choline uptake inhibitor hemicholinium-3 (HC-3). The polypeptide with molecular mass of about 42,000 Da was labeled predominantly.

The objective of the present study was to solubilize the [^{3}H]ChM Az-labeled protein from presynaptic plasma membranes of *Torpedo* electric organ and to characterize the hydrodynamic properties of the native protein complex by gel permeation chro-

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matography and equilibrium sucrose density gradient centrifugation.

Materials and Methods

Materials

2-[(N-2′-Chloroethyl)-N-[*methyl*-^{3}H]methylamino]ethyl acetate ([^{3}H]acetylcholine mustard; [^{3}H]AChM) was obtained by custom synthesis from Amersham Radiochemical Corp. (specific activity, 40 Ci/mmol). Ficoll-400 was purchased from Pharmacia, and phenylmethanesulfonyl fluoride (PMSF), imidazole, CHAPS, and hemicholinium-3 (HC-3) were obtained from Sigma Chemical Co. High- and low-molecular-weight standards were obtained from Bio-Rad and Pharmacia, respectively. Gel filtration protein standards were purchased from Bio-Rad. Ultrogel ACA44 and ACA34 was purchased from LKB. BCA protein assay reagent was from Pierce Chemical Co. All other reagents were obtained at the highest purity available.

[^{3}H]ChM Az was prepared from [^{3}H]AChM as follows: [^{3}H]AChM was dissolved in distilled water to allow generation of the aziridinium species, and the pH was adjusted to 11 with NaOH for 20 minutes to hydrolyze the ester bond, liberating acetate and producing the choline moiety. Purity of the [^{3}H]AChM was monitored by thin-layer chromatography on silica gel plates developed in chloroform–acetonitrile–triethylamine (85:15:1), and generation of [^{3}H]ChM Az was monitored by thin-layer chromatography on cellulose plates developed in butanol–ethanol–water–acetic acid (8:2:1:3).

Preparation, Labeling, and Solubilization of Presynaptic Plasma Membranes

Revesiculated presynaptic plasma membranes were prepared from synaptosomes of electromotor neurons of *Torpedo marmorata* according to the method of Ducis and Whittaker (1985), as described by Rylett (1988). Osmotically lysed synaptosomes were resuspended in a small volume of 20 mM imidazole HCl buffer, pH 7.4, containing 300 mM KCl and 0.1 mM $MgCl_2$, and dialyzed for 16 hours against the same buffer to load the revesiculated membranes with potassium. All solutions contained the serine protease inhibitor PMSF (0.1 mM), and procedures were carried out at 4°C.

Choline carriers were radiolabeled by incubating revesiculated presynaptic plasma membranes with [^{3}H]ChM Az (0.2–2 μM) at 30°C for 30 minutes in the presence of an imposed inward sodium gradient. The extracellular medium was comprised of 20 mM imidazole HCl buffer, pH 7.4, containing 300 mM NaCl and 0.1 mM $MgCl_2$. At the end of the incubation, membranes were recovered by centrifugation (20,000*g* for 15 minutes) and washed twice with extracellular medium to remove excess tritium. Parallel samples contained 10 μM HC-3, a specific blocker of choline transport, to determine nonspecific labeling by the affinity ligand.

Membranes were washed three times in 20 mM imidazole HCl buffer, pH 7.4, containing 150 mM NaCl, 0.1 mM $MgCl_2$ and 0.1 mM PMSF (buffer A) with the addition of 5 mM Na_2EDTA to remove peripheral membrane proteins, then resuspended in buffer A. Membranes (4 mg protein/ml) were added to an equal volume of buffer A containing 1.6% CHAPS (w/v) and incubated for 30 minutes at 4°C. Samples were centrifuged at 100,000 *g* for 1 hour to remove insoluble material. In some cases, the supernatant was concentrated by ultrafiltration over an Amicon YM30 membrane. Protein content of samples was measured by the method of Lowry et al. (1951) or by the micro assay of the BCA protein assay method of Pierce Chemical Co., with bovine serum albumin used as a standard.

Gel Permeation Chromatography

Solubilized membrane proteins were fractionated on a calibrated column of Ultrogel ACA44 (40 × 1 cm) at 4°C equilibrated in buffer A containing 0.3% CHAPS. The sample was applied in a 1-ml volume and fractions of 0.5 ml were collected. The flow rate was 5 cm/hr. Calibration proteins were thyroglobulin (8.5 nm), catalase (5.22 nm), aldolase (4.81 nm), ovalbumin (3.05 nm), chymotrypsinogen (2.09 nm), and ribonuclease A (1.69 nm). The total elution volume of the column was marked with [^{3}H]choline.

Sucrose Density Centrifugation

Linear gradients were prepared from 5% and 20% sucrose (w/v) made up in H_2O or D_2O containing 20 mM imidazole HCl, pH 7.4, 150 mM NaCl, 0.1 mM $MgCl_2$ and 0.3% CHAPS. Samples were applied in 0.5 ml and were centrifuged at 4°C in a Beckman SW41 rotor for 16 hours at 35,000 rpm for H_2O and 19 hours at 39,000 rpm for D_2O gradients. After centrifugation, the bottoms of the tubes were punctured and the gradients were run out by a peristaltic pump. Fractions of 0.4 ml were collected. Gradients were routinely checked for linearity of the sucrose concentration by refractometry and calibrated with thyroglobulin (19.2S), catalase (11.2S), gamma-globulin (6.6S), and hemoglobin (4.4S).

Polyacrylamide Gel Electrophoresis

One dimensional sodium dodecyl sulfate–polyacrylamide slab gel electrophoresis (SDS-PAGE)

Table 1. Molecular Parameters for Solubilized [^{3}H]ChM Az-labeled Choline Carrier[1]

Parameter	
Stokes radius, a (nm)	4.4 ± 0.5 (7)
Partial specific volume, $\bar{V}$ (ml/g)	0.76 (5 × 3)
Apparent sedimentation coefficient (S)	
H_2O	5.2 ± 0.1 (5)
D_2O	4.7 ± 0.5 (3)
Sedimentation coefficient, $S_{20,w}$ (S)	5.4 (5 × 3)
Molecular weight, M_r	115,000
Molecular weight of protein	83,000

[1] *Torpedo* electric organ presynaptic plasma membranes were labeled with [^{3}H]ChM Az and solubilized as described under Materials and Methods. The results shown are mean ± SEM of (N) determinations.

was performed by the method of Laemmli (1970) using 11% resolving and 5% stacking gels. Samples were solubilized in sample buffer (pH 6.8: SDS 0.08 M, dithiothreitol 0.1 M, Tris HCl 0.06 M, glycerol 10%, bromophenol blue 0.005%) for 30 minutes at room temperature before electrophoresis. Gels were stained with Coomassie brilliant blue R-250 and photographed, then lanes were cut at 2-mm intervals and digested in 30% H_2O_2 for quantitation of tritium.

Results and Discussion

Incubation of presynaptic plasma membranes with 0.2–2 μM [^{3}H]ChM Az at 30°C for 30 minutes in the presence or absence of 10 μM HC-3 yielded about 60–70% specific binding of the ligand. Subsequent incubation of [^{3}H]ChM Az-labeled membranes with 0.8% CHAPS resulted in solubilization of about 70% of the trichloroacetic acid (TCA) precipitable tritium and about 70% of the membrane proteins. Resuspension of the insoluble material produced after the first solubilization step did not greatly increase the yield of solubilized protein. As reported previously, polypeptides with *Mr* about 58,000 and 40,000–42,000 Da were radiolabeled in a hemicholinium-sensitive manner, with the lower molecular mass polypeptide being predominantly labeled (Rylett, 1988). Analysis of the proteins present in the CHAPS-solubilized and detergent-insoluble fractions revealed that the [^{3}H]ChM Az-labeled polypeptide with apparent molecular mass of about 42,000 Da was solubilized to a greater extent than the 58,000-Da polypeptide.

The solubilized preparation of [^{3}H]ChM Az-labeled protein was chromatographed at 4°C on Ultrogel ACA44 (exclusion limit 130,000 Da) equilibrated with buffer A containing 0.3% CHAPS. The elution profile of a typical separation is shown in Fig. 1A and B. Three peaks of tritium were eluted, two of which could be precipitated by TCA. TCA-precipitable tritium-labeled protein was recovered in the void volume fractions, while nonprecipitable radioactivity eluting in the total column volume appeared to be free tritium. Chromatography of the material eluting in the void volume on a column of Ultrogel ACA34, which has a higher exclusion limit (350,000 Da), did not alter the profile, indicating that this material most likely comprised high-molecular-weight aggregates (data not shown). A major peak of tritium-labeled protein eluted with a distribution coefficient (K_D) of 0.36 ± 0.01. Recovery of [^{3}H]ChM Az-labeled proteins in this peak was largely abolished in samples incubated with the affinity ligand in the presence of HC-3. A plot of the distribution coefficient (K_D) versus the Stokes radius for the calibrating proteins is shown in Fig. 1C. From this relationship, a Stokes radius of 4.4 ± 0.5 nm (N = 7) was obtained for the detergent-solubilized [^{3}H]ChM Az-labeled protein.

The peak fractions eluted from the gel filtration column were combined, the volume reduced by ultrafiltration, and applied to the top of the sucrose gradients. Radiolabeled proteins migrated as a major peak in the gradients as shown in Fig. 2A. Linear relationships were observed between the sedimentation coefficients of the calibration proteins and the distance traveled in both H_2O and D_2O–sucrose gradients as demonstrated in Fig. 2B. The relative positions of the [^{3}H]ChM Az-labeled protein varied in the two types of gradients with estimates for the apparent sedimentation coefficients of 4.7 ± 0.5 S and 5.2 ± 0.1 S in D_2O and H_2O, respectively. The higher value in H_2O than in D_2O indicates that the protein–[^{3}H]ChM Az complex has a higher partial specific volume ($\bar{V}$) (0.76 ml/g) than the calibrating proteins (0.74 ml/g on average) when calculated according to the method of Clark (1975) as described by Davis (1984). This deviation in $\bar{V}$ could be explained by detergent binding to the complex at hydrophobic regions of the protein. Calculation of the molecular mass of the detergent–lipid–protein complex yielded an estimate of about 115,000 Da. This value was corrected for the partial specific volume of the protein using a value of 0.81 ml/g for the partial specific volume of the detergent (Hjelmeland et al., 1983). This gave an estimated molecular mass of about 83,000 Da for the protein component, as shown in Table 1.

Analysis of this protein fraction by SDS-PAGE revealed a tritium-labeled polypeptide with apparent molecular mass of 40,000–42,000 Da (Fig. 3). In parallel samples that were incubated with the ligand in the presence of HC-3, tritium incorporation into this region of the gel was greatly reduced.

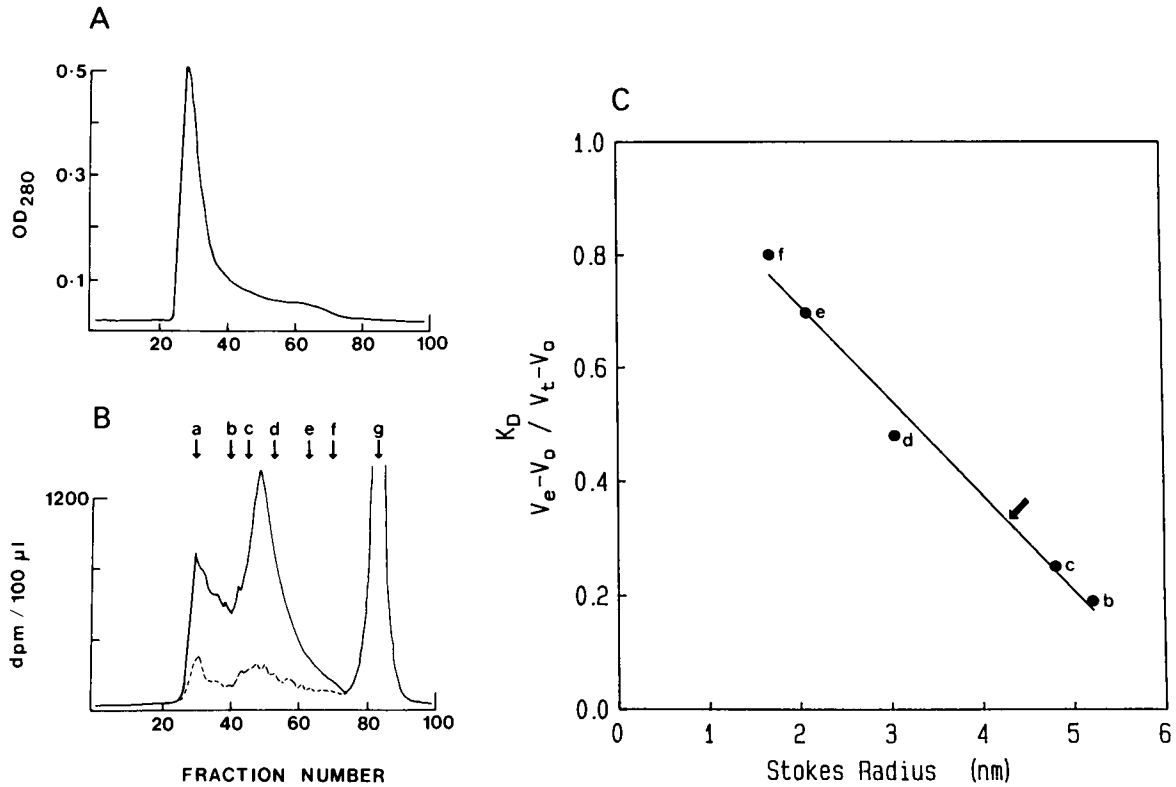
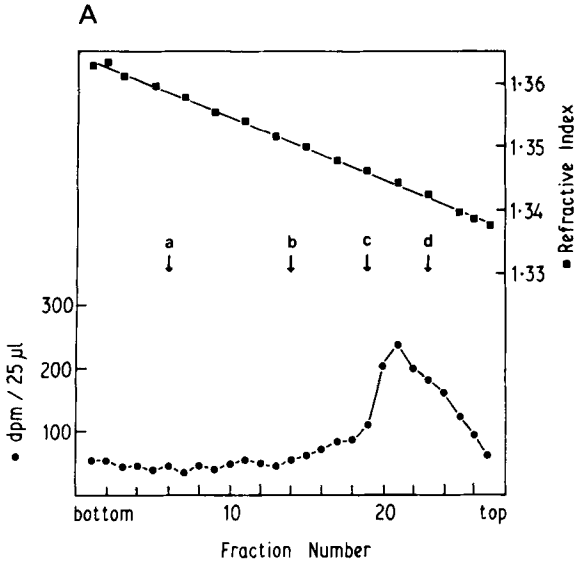


Fig. 1. Distribution of [^{3}H]ChM Az-labeled proteins on gel permeation chromatography. CHAPS-solubilized membrane proteins were fractionated on a calibrated column of Ultrogel ACA44 and protein (**A**) and tritium (**B**) distribution were monitored. (**B**) The solid line represents incubation of membranes with [^{3}H]ChM Az in the absence of HC-3 compared to the presence of HC-3 (dashed line). The relationship between the distribution coefficient (K_D) of calibration proteins and their Stokes radii is given in (**C**). Calibrating proteins are a, thyroglobulin; b, catalase; c, aldolase; d, ovalbumin; e, chymotrypsinogen; f, ribonuclease A; g, [^{3}H]choline. Thyroglobulin marked the void volume of the column, while tritium marked the total elution volume. The arrow indicates elution peak for [^{3}H]ChM Az-labeled protein.

The relationship between the 42,000-Da polypeptide identified on denaturing polyacrylamide gels and the 83,000-Da native protein estimated from the hydrodynamic behaviour of the solubilized radiolabeled proteins remains to be determined, but it is probable that the 42,000-Da polypeptide is a subunit. Based upon the apparent molecular mass of the native protein, one possible interpretation is that the 42,000-Da polypeptide exists as a dimer.

Recently, Knipper et al. (1989) identified a polypeptide with molecular mass of about 80,000 Da from synaptic membranes of locust using [^{3}H]HC-3 as a photoaffinity ligand that they believe to be a constituent of the choline carrier. The relationship between the protein labeled in our studies with [^{3}H]ChM Az and by Knipper et al. (1989) using [^{3}H]HC-3 is not readily apparent. While species variation could account for the observed differences, it is also possible that the ligands bind to different sites on or components of the transport protein. It is predicted that ChM Az binds to the substrate binding site of the transporter because of its structural similarity to choline (Rylett, 1988). As the specific binding of [^{3}H]HC-3 in brain sections and membrane preparations possesses many of the ionic and pharmacological selectivies of sodium-dependent, high-affinity choline transport (Vickroy et al., 1984; Sandberg and Coyle, 1985), it has been proposed that HC-3 binding sites are either colocalized with the high-affinity choline transporters or are an integral part of the transport system (Chatterjee et al., 1987). Furthermore, it has been demonstrated that HC-3 is a potent competitive inhibitor of high-affinity choline transport (Yamamura and Snyder, 1973), but it apparently does not serve as a substrate for the carrier and is not transported into the nerve terminal (Collier, 1973). While the nature of the binding of HC-3 to the high-affinity choline carrier has yet to be determined, the competitive characteristics of its inhibition of choline transport indicate the potential for interaction at the choline binding site.

In summary, [^{3}H]ChM Az binds to presynaptic plasma membranes from *Torpedo* electric organ in a HC-sensitive, sodium-dependent manner, both of which are characteristic of the high-affinity choline



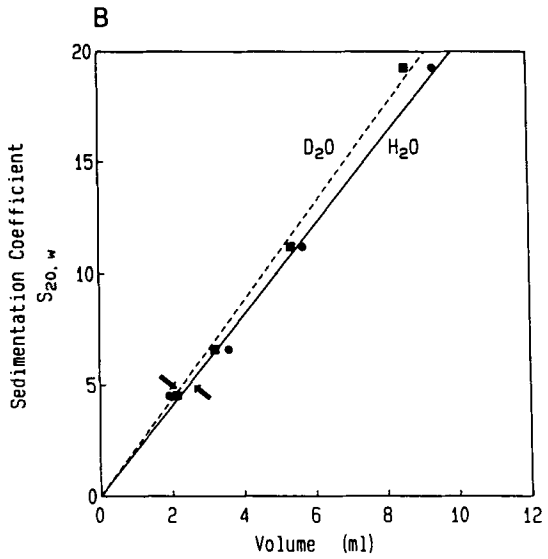


Fig. 2. Sucrose density centrifugation of solubilized [^{3}H]ChM Az-labeled membrane proteins. Samples were prepared and centrifuged as described in Materials and Methods. (**A**, bottom) Distribution of radiolabeled proteins in a H_2O–sucrose gradient relative to marker proteins shown by arrows: a, thyroglobulin; b, catalase; c, gamma globulin; d, hemoglobin. (**A**, top) Refractive index of sucrose solution. (**B**) Relationship between distribution of marker proteins and their sedimentation coefficients in H_2O– and D_2O–sucrose gradients. Distribution of [^{3}H]ChM Az-labeled proteins is indicated by arrows.

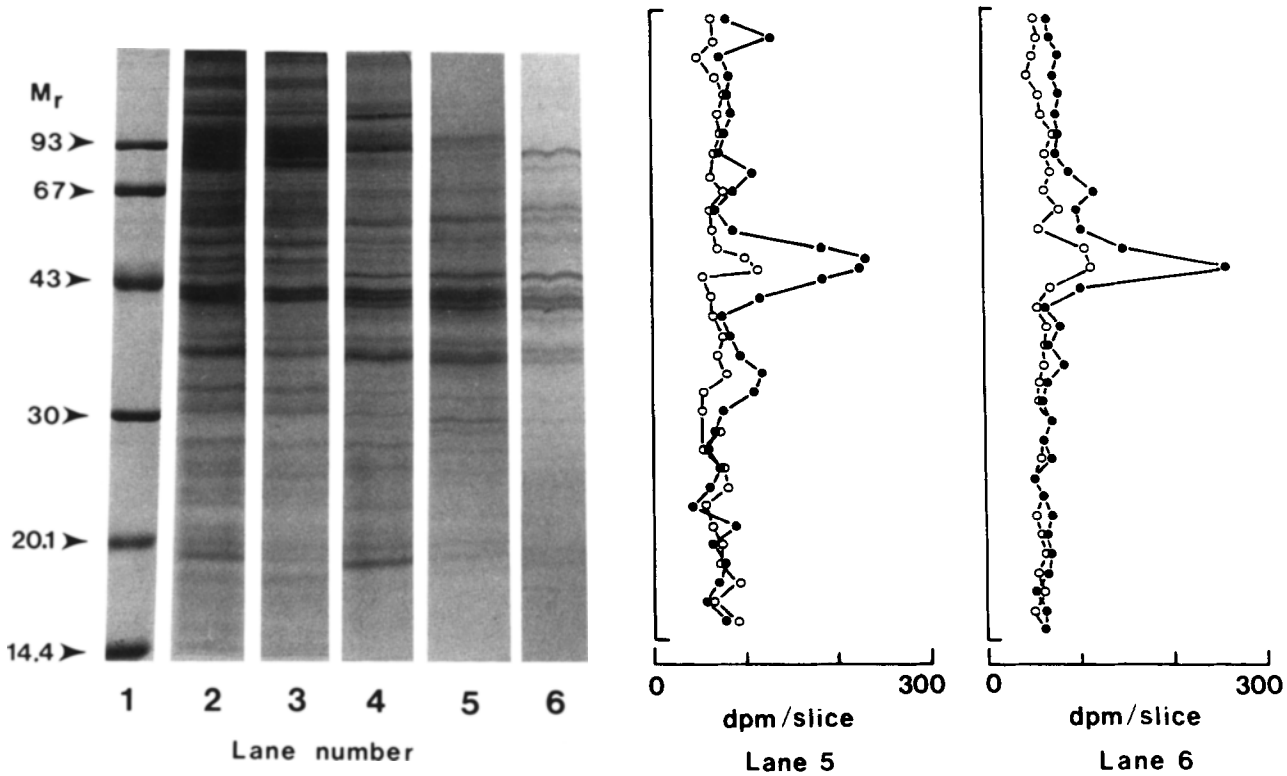


Fig. 3. Polypeptide profiles and distribution of [^{3}H]ChM Az-labeled proteins in total and solubilized presynaptic plasma membrane preparations. Left, polypeptide profile on Coomassie blue R-250-stained SDS–polyacrylamide gels for: lane 1, molecular weight markers in kilodaltons; 2, total membrane fraction; 3, pellet following CHAPS solubilization; 4, supernatant following CHAPS solubilization; 5, peak fractions eluting from gel filtration column at fraction 45–55; 6, peak fractions from sucrose gradient at fraction 20–22. Right, profile of tritium distribution in 2-mm segments of SDS–polyacrylamide gels from lanes 5 and 6. Solid circles are from membranes incubated with [^{3}H]ChM Az in the absence of HC-3 and open circles are in the presence of HC-3.

carrier. Following detergent solubilization, the [^{3}H]ChM Az-labeled protein was found to have an apparent molecular mass of about 83,000 Da, of which the 42,000-Da polypeptide may be a subunit.

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