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Purification and reconstitution of the high affinity choline transporter

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The high-affinity choline transporter has been solubilized from synaptosomal membranes by various detergents. The solubilized carrier protein has been incorporated into liposomes after removal of the detergent by dialysis. Using the reconstitution of choline transport activity as an assay, the components catalyzing choline translocation were purified from the detergent extract by ion-exchange chromatography on a Mono-Q column followed by immunoaffinity chromatography. Monitoring the active fractions by sodium dodecylsulfate polyacrylamide gel electrophoresis and isoelectrofocussing gave one major protein with an apparent molecular weight of about 90000 and an isoelectric point of pH 4.7. The isolated protein appeared to be heavily glycosylated as shown by lectin binding; upon treatment with endoglycosidase F the polypeptide was degraded to an apparent molecular weight of about 65000. Accumulation of choline into liposomes reconstituted with the purified protein was driven by artificially imposed sodium gradients and inhibited by hemicholinium-3.

Introduction

Since nerve cells do not appear to synthesize choline to any significant extent [1], acetylcholine (ACh) synthesis of cholinergic nerve terminals depends on their ability to transport choline (Ch) from extracellular sources into the nerve ending cytosol, the site of ACh synthesis [2]. There is now considerable evidence that the synthesis is supported by accumulation of choline via specific carrier systems with high affinity for choline [3,4]. The specific high affinity transport system for choline was found to be unique for cholinergic neurons [5], and distribution studies showed that functional transporters were confined to terminal regions of neurons [6]. The accumulation of choline via a specific carrier has been proposed to be the rate-limiting regulatory step for the synthesis of ACh [7]. As the choline uptake system plays such a pivotal role in maintaining cholinergic activity, a detailed knowledge of these unique and strategically localized transporter molecules is necessary for understanding the mechanisms of cholinergic synapses. Solubilization and purification of the choline carrier is a prerequisite for a complete molecular description of the function and regulation of the choline transport system. Choline transport activity has been solubilized from brain and electric tissue and detected after reincorporation into liposomes [8–10]. This reconstituted choline uptake activity exhibited many characteristics similar to those observed in native synaptosomal membranes. To date, however, purification of the choline carrier has not been achieved. This may primarily be due to the lack of any specific method for identifying the carrier protein once it is removed from the membrane. Recently, hemicholinium-3, the competitive blocker of choline uptake [11], has been exploited as a specific ligand in binding studies [12–14], and in addition monoclonal antibodies have been isolated which selectively block the high-affinity uptake of choline [15]. Using these two probes for the high-affinity choline uptake system, in conjunction with appropriate experimental approaches (photoaffinity labeling; Western blots), a membrane protein ($M_r \approx 90000$) was identified as a putative carrier constituent [14,15]. However, it is conceivable that such ligands only bind to a single subunit and other components may be overlooked; thus binding and labelling assays cannot provide unequivocal information on the complete subunit composition of the functional carrier protein. The pre-

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sent study describes the purification of a 90 kDa protein from solubilized synaptosomal membranes using reconstitution of choline transport activity as the functional assay.

Materials and Methods

Materials

[*N-Me*-^{3}H]Choline, 80 Ci/mmol, was purchased from New England Nuclear. Locusts (*Locusta migratoria*) were obtained from Dr. Frieshammer, Jaderberg. Sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) reagents were from Bio-Rad. Nitrocellulose sheets were obtained from Schleicher and Schüll. Goat anti-mouse IgG antibody and its phosphatase conjugate were from Promega. *n*-Octyl β-D-glucopyranoside (*n*-octyl glucoside) was purchased from Boehringer. The following Sigma reagents were employed: soybean phosphatidylcholine Type IV-S, peroxidase-labelled concanavalin A, sodium deoxycholate and 3-[(3-cholamidopropyl)dimethylammoniol]-1-propanesulfonate (CHAPS). Sodium ethylmercurithiosalicylate was supplied by Fluka. Endoglycosidase F (*Flavobacterium meningosepticum*) was obtained from Boehringer, Mannheim. All other reagents were of analytical grade. Monoclonal antibodies were prepared as described previously [15].

Purification of the choline transport protein

Synaptosomal membranes were prepared from locust head and thoracic ganglia as described previously [16]. Proteins were solubilized from 10–50 mg of membranes by incubating the membranes at 0°C for 30 min in 2.5 ml of buffer A containing 20 mM Tris-HCl (pH 8.0), 400 mM NaCl and 1.0% of sodium deoxycholate. The solubilized material was isolated by centrifugation at 20000 × *g* for 15 min. Deoxycholate was exchanged for CHAPS (0.05%) using PD-10 columns (Pharmacia). Samples were injected onto a Mono-Q HR 5/5 column (Pharmacia) through a Model 7125 syringe loading sample injector and eluted with a concentration gradient (0–2 M NaCl, resp. 0–2 M KCl in 50 mM Tris-HCl (pH 7.4) containing 0.05% CHAPS) at a flow rate of 1 ml/min. Absorbance at 280 nm was monitored with a Pharmacia single path UV 1 monitor. Fractions from the Mono-Q column were analyzed by SDS-PAGE to identify polypeptide bands and dot blots for immunoreactive polypeptides.

Immunoaffinity purification of transporter proteins

10 mg of purified monoclonal antibody proteins were immobilized on Tresyl-activated Sepharose 4B (Pharmacia) as described by the manufactures. The affinity gel was washed with 0.1 M Na-acetate (pH 5.0) containing 0.5 M NaCl and with 0.1 M $NaHCO_3$ (pH 8.0) containing 0.5 M NaCl; subsequently the immunoglobulin-Sepharose was incubated with 0.2 M Tris-HCl (pH 7.4). Immediately before application of a sample, the column was equilibrated with buffer composed of 150 mM NaCl in 10 mM phosphate-buffer (pH 7.4) [PBS], containing 0.05% CHAPS, 5 mM EDTA, 2 mM EGTA, 0.002% sodium ethylmercurithiosalicylate. In detergent-extracts deoxycholate was exchanged for CHAPS. Positive fractions from the FPLC-column were incubated with the affinity gel for 1 h at 4°C; subsequently, the column was washed with buffer B until the A_{280} of the eluate reached the value of buffer B. The adsorbed antigen was eluted by switching to buffer B with a pH 9.0 adjusted with ethanolamine. The eluate was immediately neutralized by adding an appropriate volume 60 mM sodium acetate (pH 4.0). Fractions containing the antigen were analysed by SDS-PAGE and used for reconstituting the transport activity.

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and isoelectrofocussing (IEF)

Electrophoresis was carried out as described by [17]. Samples were separated on slab gels containing 12.5% acrylamide separating gel and a 6% stacking gel. Molecular weights were determined graphically by plotting the log molecular weights of known protein standards versus the R_f (relative migration) of these proteins. Isoelectrofocussing was performed using the Phast-System (Pharmacia). The IEF gels were pretreated as described by the manufactures and the pH gradients formed were monitored with the aid of carbamyloyte and protein standards (Pharmacia). IEF separation was run for 10 min at 200 volt followed by 15 min at 2000 volt. Subsequently, the polypeptides were visualized by silver staining.

Deglycosylation

The immunoaffinity purified polypeptides were treated with endoglycosidase F [18]. The 90 kDa polypeptide (about 1 μg protein) in 20 μl buffer (100 mM Na^+-phosphate, 10 mM EDTA, 0.2% Triton X100, 0.2% SDS, 0.5% octylglucoside) was incubated for 18 h at 37°C with 2 units of endoglycosidase F. Deglycosylation was stopped with 15 μl sample buffer [18] and the treated samples were analysed by SDS-PAGE acrylamide gels (12.5%) and stained with Coomassie blue.

Lectin-, Western- and dot-blot analysis

Samples were resolved by SDS-PAGE using a 12.5% acrylamide running gel and a 6% stacking gel. Proteins were electrotransferred onto nitrocellulose sheets for Western blot analysis by the methods of Towbin et al. [19]. Polypeptide bands as well as molecular weight markers were localized on the filter by a transient staining with Ponceau S. For lectin assays, the filters

were blocked with 1% bovine serum albumin and then incubated with the lectin conjugate in Tris-buffered saline. The reactive bands were visualized by enzyme reaction with 3,3'-diaminobenzidine and H_2O_2. For Western-blot analysis, the filters were incubated with the indicated monoclonal antibody at room temperature for 1 h, washed, and then incubated with phosphatase-conjugated goat anti-mouse-antibody at a 1:7500 dilution at room temperature for 1 h. Filters were washed and then phosphatase acitivity was visualized using nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate as substrates.

For dot blot analysis, aliquots (50 μl) of each FPLC-fraction were placed onto nitrocellulose filters using a dot-blot device (Schleicher and Schüll). For visualizing the immunoreactive fraction, the filter sheets were processed as described above.

Reconstitution

Soybean phosphatidylcholine Type VI-S (partially purified asolectin) was first prepared as follows. 5 mg of lipid was dissolved in 30 ml chloroform, and subsequently a 6-fold excess of water-free acetone containing 0.1% (w/v) butyrated hydroxytoluene was added. After centrifugation at 3000 × *g* for 20 min at −20°C the pellet was resuspended in chloroform and the procedure repeated. After the second centrifugation, the pellet was dissolved in 50 ml diethyl ether containing 0.2% (w/v) α-tocopherol and recentrifuged. The ether extract was dried and stored until required under liquid nitrogen.

Solubilized extracts, or fractions from FPLC- or immunoaffinity chromatography, were mixed with 0.1 volumes of lipids equilibrated in 100 mM Hepes (pH 7.4) containing 46 mM octyl glycoside and 1 M KCl to give a final lipid concentration of 10 mg/ml in 10 mM Tris, 4.6 mM octyl glucoside, 100 mM KCl. The mixture was placed in presoaked dialysis bags (Visking 8/32, Serva) and then dialyzed against a 250-fold volume excess of dialysis buffer (100 mM KCl, 25 mM sucrose, 0.5 mM DTT, 0.02% deoxycholate in 5 mM Tris-HCl (pH 7.4) for 48 h with three changes of dialysis buffer. Samples were then assayed for choline transport acivity.

Measurement of choline transport activity

Aliquots (50 μl) of proteoliposomes containing about 1–10 μg of protein were allowed to equilibrate at room temperature before being added to the transport medium containing 150 mM NaCl, 3 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, and 0.5 μM tritiated choline buffered with 10 mM Tris-HCl (pH 7.4). In parallel experiments samples were coincubated with hemicholinium-3 (100 μM). Samples were either filtered immediately to obtain blank values or allowed to incubate for 5 min at room temperature and then filtered through Whatman GF/C filters.

Results

In order to isolate and purify functional high-affinity carrier polypeptides appropriate detergents had to be found. Therefore various candidates were assayed for their capacity to solubilize synaptosomal membrane

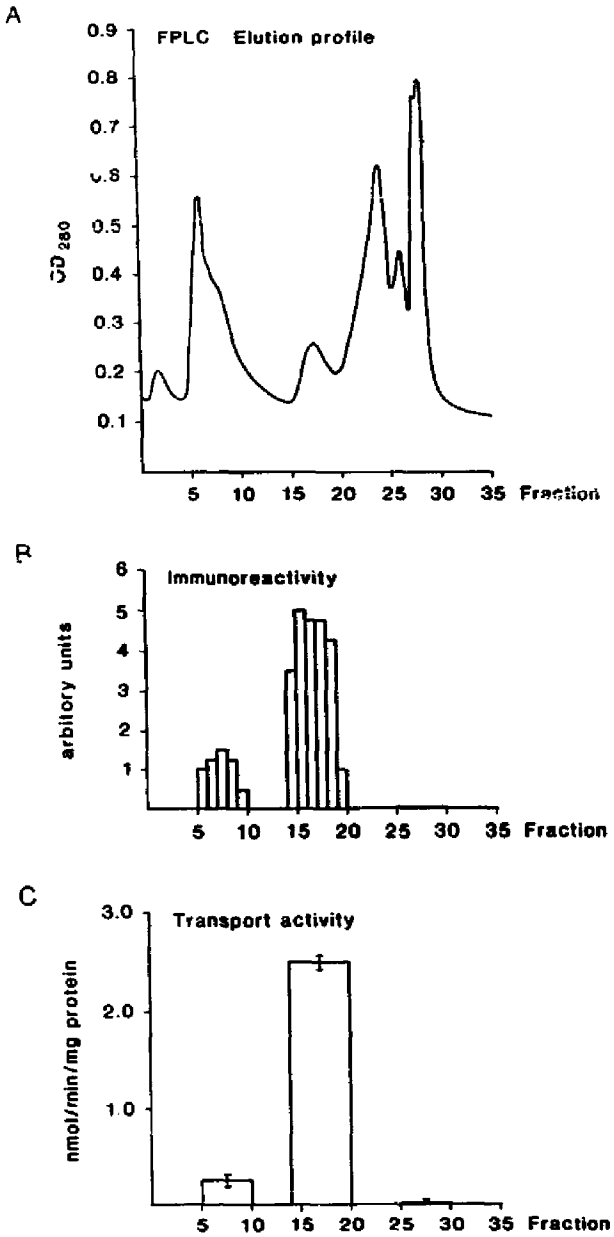

Fig. 1. FPLC-ion-exchange chromatography on a Mono-Q column. Samples of a detergent-extract from synaptosomal membranes were applied to the column and eluted by using a 0–2 M NaCl gradient; the absorbance was monitored at 280 nm (A). Each fraction was assayed for immunoreactivity with the monoclonal antibody VIB6F5 as described in the methods section (B). The fractions 5–10, 15–20 and 25–30 were pooled and the polypeptides incorporated into liposomes using the dialysis technique as described in Methods. The resulting proteoliposomes were assayed for there capablity to accumulate choline (C).

proteins; the carrier proteins were monitored by hemicholinium-3 binding and immunoreactivity using the VIB6F5 monoclonal antibody [15]. Low concentrations of Triton X-100 (0.1%) or CHAPS (0.1%) were found to be ineffective in solubilizing the HC-3 binding sites. Sodium deoxycholate (1.0%) in the presence of 400 mM NaCl solubilized about 75% of the carrier protein. These results confirmed the observations of Yamada [20] on solubilization of a HC-3 binding site from rat brain. Prior to HC-3 binding assays and immunreactivity tests the detergent was exchanged for 0.05% CHAPS after solubilisation.

For purification the detergent extract from synaptosomal membranes was subjected to ion exchange chromatography and the fractions were assayed (a) for immunoreactivity in dot blots and (b) for transport activity after reconstituting the separated polypeptides into liposomes. Fig. 1A illustrates the resolution of solubilized membrane proteins by the FPLC technique using a Mono-Q column and elution by a NaCl gradient. Nonabsorbed proteins appearing in the flow-through gave only a very low immunoreactivity signal. Most of the immunoreactivity as well as carrier activity was recovered in peak II (Fig. 1B). The relative specific activity of the Na^+-dependent choline accumulation was increased by 10-fold when compared with that of the detergent solubilized fraction (deoxycholate supernatant). Western blot analysis of the various fractions

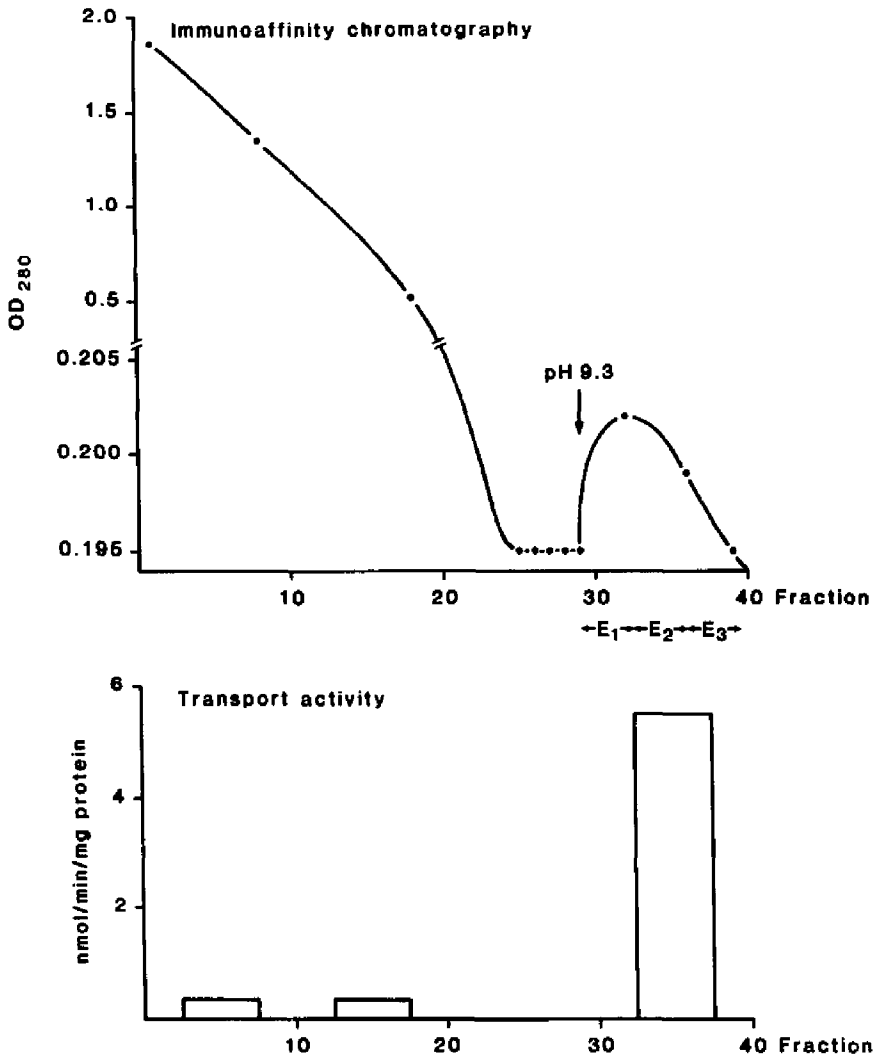

Fig. 2. The fractions 15–20 of several FPLC runs were collected and concentrated by pressure filtration; the detergent (deoxycholate) was exchanged for CHAPS. The material was subsequently incubated with an immunoaffinity gel prepared by coupling the antibody VIB6F5 onto tresyl-activated Sepharose 4B. Unspecifically retained protein was eluted by washing with buffer, the bound antigen was eluted by switching to an alkaline buffer (pH 9.0). Protein elution was monitored by determining the absorbance at 280 nm for each fraction. Polypeptides in fractions 3–7, 13–17 and 33–37 were incorporated into liposomes and the proteoliposomes assayed for choline uptake.

resulted in labelling of a single polypeptide band (M_r around 90000) in fraction II; none of the other fractions contained reactive polypeptides.

For a subsequent purification step, fraction II was applied to immunoaffinity chromatography using the purified antibody VIB6F5 coupled to tresyl-activated Sepharose (Pharmacia). During the washing steps most of the proteins were separated; the antigen-antibody complexes were dissociated by switching to an elution buffer with pH 9.0. Elution of the antigens could be monitored by a slight increase in the optical density (Fig. 2A). The pH of the eluate was immediately neutralized. After concentrating the sample and exchanging the solution using Centricons (Amicon) aliquots were assayed for transport activity after reconstitution in liposomes. As shown in Fig. 2B the transport activity was almost exclusively recovered in the pH 9.0 eluate; very little activity was detected in the washing fractions. The polypeptide composition of the eluate was analysed by SDS-polyacrylamide gel electrophoresis. Fig. 3 demonstrates that only a single polypeptide band in the range of about 90000 could be detected. The purified 90 kDa protein was recognized by the monoclonal antibody VIB6F5 in Western blot analysis. Occasionally, the preparations were slightly contaminated with immunoglobulins obviously bleeding from the columns.

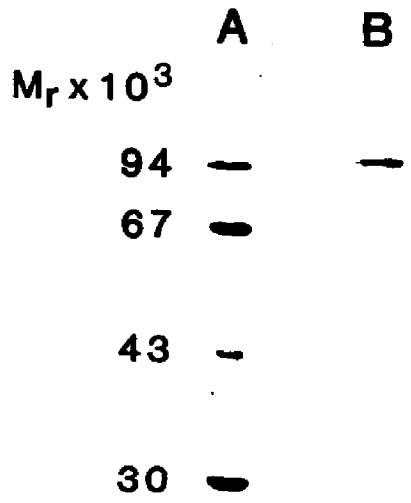

Fig. 3. SDS-PAGE analysis of polypeptides immunoaffinity purified on tresyl-activated Sepharose 4B coupled with VIB6F5 antibodies. Polypeptides from detergent extracts from synaptosomal membranes by 1% sodium deoxycholate were separated by ion-exchange FPLC on a Mono-Q column. The antigen in the active fractions were further enriched by immunoaffinity purification. Fractions eluted with elevated pH were collected and concentrated. Polypeptides were separated on 12.5% SDS polyacrylamide gels and stained with Coomassie blue.

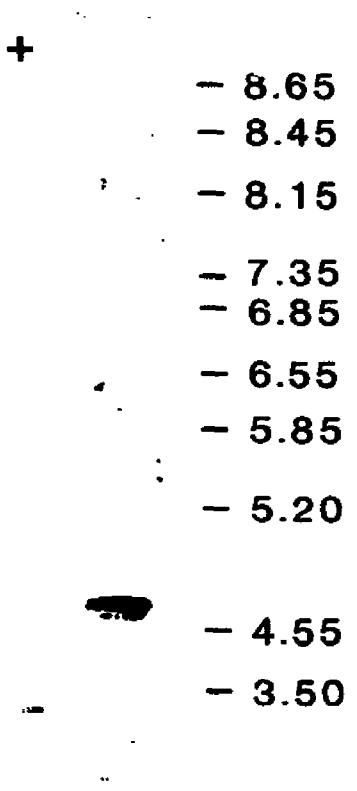

Fig. 4. Isoelectrofocussing of immunoaffinity purified polypeptides using the Phast Gel IEF system as described in Methods. The pH gradient formed was monitored with the aid of protein standards. After separation the polypeptides were visualized by silver staining.

Thus, the 90 kDa polypeptide appear to mediate the high-affinity choline translocation after reconstitution in liposomes.

In electrofocussing experiments, the p*I* value of the purified polypeptide was determined at pH 4.7 (Fig. 4) characterizing the choline transporter as a rather acidic protein.

In order to explore, if the isolated transporter protein was glycosylated, lectin-blotting experiments were performed. Immunoaffinity-purified protein was electrotransferred onto nitrocellulose and assayed for carbohydrate chains using peroxidase-labelled concanavalin A. As can be seen in Fig. 5a the purified transporter protein gave a strong reaction (lane B). In the presence of 0.2 M α-methylmannoside this signal disappeared (lane C). Immunopurified transporter protein was treated with endoglycosidase F, which cleaves N-glycans 'high mannose' as well as 'complex' carbohydrate chains linked through asparagine to the protein backbone [18]. Upon this treatment (Fig. 5b) the apparent molecular mass of the 90 kDa native protein (lane A) was reduced to about 65 kDa (lane B). The polypeptide could not be further fragmented, even on prolonged incubation with the enzyme.

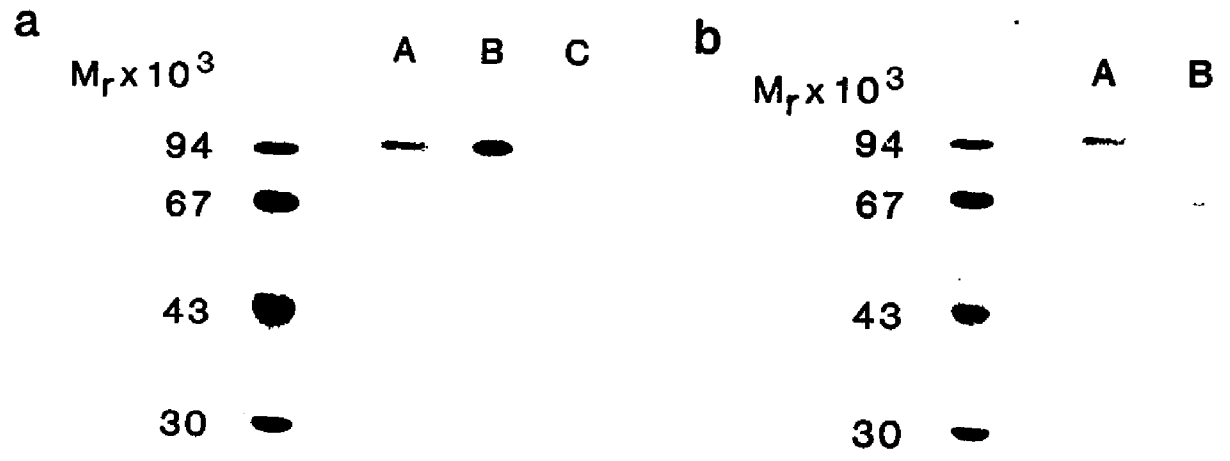

Fig. 5. (a) Reactivity of purified transporter protein with concanavalin A (ConA). Transporter protein was purified by immunoaffinity chromatography and assayed by SDS-polyacrylamide gelelectrophoresis followed by Coomassie blue staining (lane A) or electrotransfer onto nitrocellulose followed by incubation with peroxidase-labelled ConA (lane B). No reaction with the lectin was seen in the presence of 0.2 M α-methylmannoside (lane C). (b) Enzymatic deglycosylation of the purified choline transporter protein. The purified polypeptide (1 μg protein) was incubated for 18 h in the absence (A) or presence (B) of 2 units of endoglycosidase F. The reaction was stopped by adding sample buffer and the samples were subjected to SDS-PAGE using 12.5% polyacrylamide gel.

The functional properties of the purified and reconstituted carrier protein were subsequently analyzed in more detail. As shown in Fig. 6, liposomes containing affinity-purified proteins rapidly accumulated choline in the presence of sodium and an inwardly directed Na^+-gradient. In the presence of hemicholinium-3 only a very small amount of choline was found in the proteoliposomes. Concentration-dependent experiments indicated that the reconstituted transporter has about the same affinity (1 μM) as the carrier in the native membrane [3]. This successful reconstitution of the functional choline transporter indicates that the rapid accumulation of choline in nerve terminals may be due to the purified 90 kDa protein.

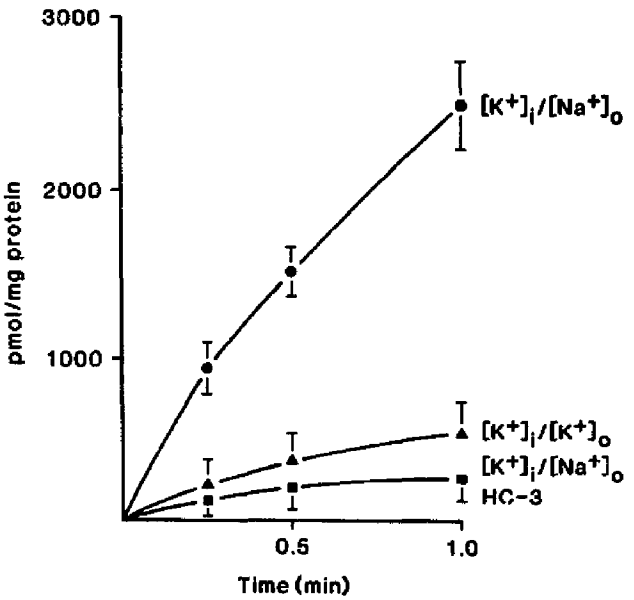

Fig. 6. Time course of choline uptake by reconstituted proteoliposomes containing the immunoaffinity purified 90 kDa protein. Reconstitution of purified transporter protein was performed by the dialysis procedure. Accumulation of [^{3}H]choline by proteoliposomes was determined in filter assays.

Discussion

The unequivocal identification of a membrane transport complex which may be composed of different, related, polypeptide subunits, and may even require additional protein molecules in order to form a functional unit can only be achieved after purification and reconstitution. Thus, solubilization of membrane proteins and their incorporation into liposomes for assay purposes has become a frequently used tool for identifying active transport proteins [21]. Using FPLC ion exchange chromatography in combination with immunoadsorbence techniques, the high-affinity carrier for choline from synaptic plasma membranes has been purified to apparent homogeneity. The isolation of the choline transporter is of special interest since it appears to be central to the maintenance of transmitter levels in cholinergic nerve terminals. The purified component which functions as a choline carrier gives essentially one polypeptide band with an apparent molecular mass around 90 000 dalton, it appears to retain all the properties of the *in vivo* choline transport activity. The molecular weight is close to the component recently identified as constituent of the choline carrier by labelling with

tritiated hemicholinium-3 [14], and in Western-blot analysis using monoclonal antibodies which block choline uptake [15]. The width of the band may be accounted for by the carbohydrate moiety of the protein [18]. This notion was given support by specific binding of Con A as well as by experiments in which application of endoglycosidase F reduced the apparent molecular mass to about 65 kDa, indicating that the choline transporter is a N-glycosylated glycoprotein. The M_r of its peptide core appears to be 65000 or less, if the protein contains glycopeptidase F-resistant sugar chains.

The characteristic properties of the choline carrier, as measured in native membrane vesicles [22,23], are also observed for the liposome-reconstituted purified transporter protein. These include, the high-affinity for the choline, the dependence on external sodium and the sensitivity to hemicholinium-3. Thus, the simplest hypothesis to account for these findings is that the 90 kDa polypeptide is the only component of the transporter. It is interesting to note, that a carrier for the neurotansmitter, γ-aminobutric acid [24,25] as well as of a functional transporter for biogenic amines [26] have recently been identified as glycosylated polypeptides of similar size and an acidic p*I*. Whether the monomer itself or a multisubunit complex constitutes the functional carrier remains to be determined, and may be approached by radiation-inactivation techniques which have successfully been applied to the nucleoside and glucose transporters of erythrocytes [27].

The purification of a functional choline transport protein advances our understanding of the high-affinity choline uptake mechanism in cholinergic synapses; it opens the way for studies on the structure-function relationship, further exploration of the stoichiometry of the sodium-choline symport, and ultimately, deciphering the complete amino acid sequence of the transporter protein molecule.

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References

1 Ansell, G.B. and Spanner, S. (1978) in Cholinergic Mechanisms and Psychopharmacology (Jenden, D.J., ed.), pp. 431–445, Plenum Press, New York.
2 Fonnum, G.A. (1975) J. Neurochem. 24, 407–409.
3 Jope, R.S. (1979) Brain Res. Rev. 1, 313–344.
4 Tucek, S. (1985) J. Neurochem. 44, 11–24.
5 Kuhar, M.J. and Murrin, L.C. (1978) J. Neurochem. 30, 15–21.
6 Suszkiw, J.B. and Pilar, G. (1976) J. Neurochem. 26, 1133–1138.
7 Kuhar M.J., Sethy, V.H., Roth, R.H. and Aghajanian, G.K. (1973) J. Neurochem. 20, 581–593.
8 King, R.G. and Marchbanks, R.M. (1980) Nature 287, 64–65.
9 Meyer, E.M. and Cooper, J.R. (1982) Science 217, 843–845.
10 Ducis, I. and Whittaker, V.P. (1985) Biochim. Biophys. Acta 815, 109–127.
11 Guyenet, P., Lefresne, P., Rossier, J., Beaujofan, J.C. and Flowinski, J.C. (1973) Mol. Pharmac. 9, 630–639.
12 Sandberg, K. and Coyle, J.T. (1985) Brain Res. 348, 321–330.
13 O'Regan, S. (1988) J. Neurochem. 51, 1682–1688.
14 Knipper, M., Krieger, J. and Breer, H. (1989) Neurochem. Int. 14, 211–215.
15 Knipper, M., Strotmann, J., Mädler, U., Kahle, C. and Breer, H. (1989) Neurochem. Int. 14, 217–222.
16 Breer, H. and Knipper, M. (1985) Cell. Mol. Neurobiol. 5, 285–296.
17 Laemmli, U.K. (1970) Nature, Lond. 227, 680–685.
18 Elder, J.H. and Alexander, S. (1982) Proc. Natl. Acad. Sci. USA 79, 4540–4544.
19 Towbin, H., Stachelin, T. and Gordon, J. (1976) Proc. Natl. Acad. Sci. USA 76, 4350–4354.
20 Yamada, K., Saltarelli, M.D. and Coyle, J.T. (1988) J. Neurochem. 50, 1759–1763.
21 Koepsell, H. (1986) Rev. Physiol. Biochem. Pharmacol. 104, 65–137.
22 Breer, H. and Lueken, W. (1983) Neurochem. Int. 5, 713–720.
23 Breer, H. and Knipper, M. (1985) Comp. Biochem. Physiol. 81C, 219–222.
24 Radian, R., Bendahan, A. and Kanner, B.I. (1986) J. Biol. Chem. 261, 15437–15441.
25 Kanner, I.B., S. Keynan and Radian, R. (1989) Biochemistry 28, 3722–3728.
26 Stern-Bach, Y., Greenberg-Ofrath, N., Flechner, I. and Schuldiner, S. (1990) J. Biol. Chem. 265, 3961–3966.
27 Jarvis, S.M., Ellory, J.C. and Young, J.D. (1986) Biochim. Biophys. Acta 885, 312–315.