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BINDING OF CONCANAVALIN A TO CALF BRAIN SYNAPTIC VESICLES

Paul E. March and Edward R. Thornton

University of Pennsylvania
Department of Chemistry
Philadelphia, Pennsylvania 19104

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Concanavalin A was employed as a tool to investigate the organization of synaptic vesicle glycoproteins. The lectin was incubated in the presence of both intact and Triton X-100 treated calf brain synaptic vesicles. Electrophoresis of treated membranes clearly demonstrated that the majority of Concanavalin A binding sites was not exposed in intact synaptic vesicles. The vesicles were isolated by the procedure of DeLorenzo and Freedman (DeLorenzo, R. J., and Freedman, S. D. (1978) Biochem. Biophys. Res. Commun. 80, 183-192). However, an extra centrifugation step at 55,000 g was required to obtain plain vesicles nearly free from coated vesicles and membrane fragments.

A central problem in understanding neural function is determining the mechanism of neurotransmission on a molecular scale. Synaptic vesicles appear to have an important role in neurotransmission because they are concentrated at the nerve terminus, are known to contain neurotransmitters and seem to be involved in exocytosis at sites where neurotransmitter release occurs (1,2). Mammalian brain synaptic vesicles have been isolated in a variety of laboratories (3-10).

Recently, evidence has accumulated that glycoproteins are present in synaptic vesicles (10,11). Since it is widely believed that plasma membrane glycoproteins face the exterior of cells (12-14) it would be interesting to determine whether this asymmetry is preserved in isolated secretory vesicles. Lectins were employed to demonstrate that most, if not all, glycoproteins face the lumen of chromaffin granules (15). Further, it has been observed that the lectin Concanavalin A (Con A)[1] will bind solubilized synaptic vesicle glycoproteins (11 and 16). However, these studies did not permit assignment of glycoprotein orientation in the membrane. We have isola-

[1] Abbreviations: Con A, Concanavalin A; αMM, α-methyl-mannoside; SDS, sodium dodecyl sulfate; PAS, periodic acid-Schiff.

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ted synaptic vesicles essentially according to the procedure of DeLorenzo and Freedman (7) and determined that most of these Con A binding proteins are not exposed in intact synaptic vesicles.

METHODS AND MATERIALS

Isolation of Synaptic Vesicles: Calf brains, weighing between 200 and 300 g, were obtained from the local slaughterhouse. Brains were placed in a solution of 0.32 M sucrose with 0.3 mM phenylmethylsulfonyl fluoride (pH = 7.0) at 0°C within 15 min after decapitation. Thirty g of cerebral cortex was scraped from these brains and used as starting material to purify synaptic vesicles. Synaptic vesicles were isolated according to the procedure of DeLorenzo and Freedman (7). However, based on the discussion of Tamir and Gershon (18) one extra centrifugation step at 55,000 g was included to remove large membrane and coated vesicle contamination. Figure 1 demonstrates the difference in vesicular fractions when either one or two 55,000 g centrifugation steps were employed. When two were employed coated vesicles and large membrane vesicles were rarely observed. Careful examination of the pellet showed that it consisted almost entirely of plain vesicles 300-450 Å in diameter which compares favorably to the morphological appearance of other vesicular fractions (5, 9, 10, 17, 18, 26). These vesicles were used in all subsequent experiments.

Electron Microscopy: Synaptic vesicles (100 µg of total protein) were resuspended in 300 µl of 2.5% glutaraldehyde in 0.1 M Na_2HPO_4 (pH = 7.4). This suspension was pelleted in bottleneck BEEM capsules (Polysciences Inc.) as described by Cotman and Flansburg (19). This procedure was designed to make uniformly shaped pellets that can be assayed for particulate content in a

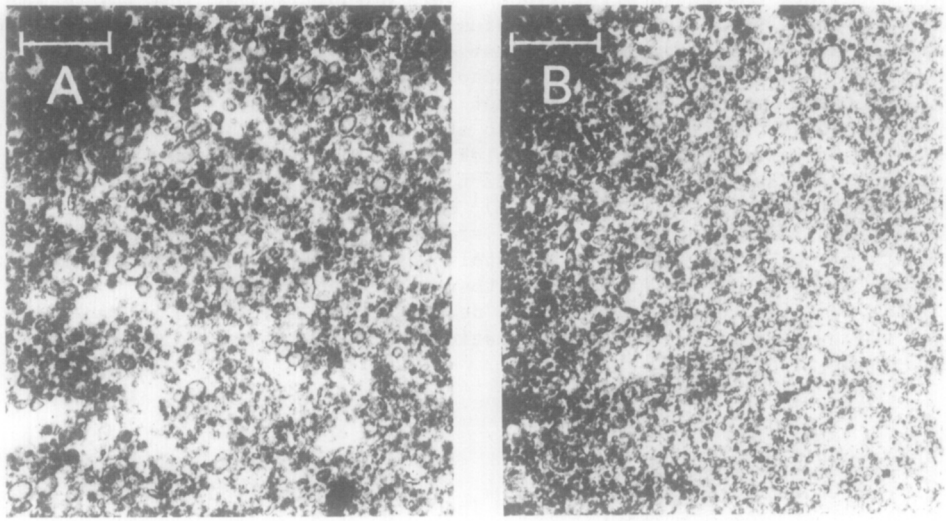

Figure 1. Electron micrographs of isolated synaptic vesicle fractions. A, prepared according to DeLorenzo and Freedman(7). The next to last step of this procedure employs centrifugation for 1 h at 55,000 g. B, prepared according to the same procedure except an extra 1 h centrifugation at 55,000 g was invoked. Note the dramatic morphological differences between A and B. When continous centrifugation for 2 h at 55,000 g was employed, instead of two 1 h steps, a fraction that appeared to be identical to B was observed. However, the protein yield was greatly reduced (data not shown). Based on these results, two 1 h centrifugation steps at 55,000 g were employed in synaptic vesicle isolation from calf brain.

reproducible fashion. The resultant pellet was postfixed with 1% OsO_4 in 0.1 M Na_2HPO_4. The sample was dehydrated through a series of ethanols and embedded in flat molds in Mollenhauer medium (Polysciences Inc.). Sectioning was performed on an LKB ultramicrotome. Sections, 800 Å thick, were collected on 400 mesh nude copper grids. Sections were stained with uranyl acetate and lead citrate and allowed to dry at least 24 h. Grids were viewed in a Philips 201 electron microscope.

Con A-Synaptic Vesicle Binding Experiments: Con A, obtained from Sigma Chem. Co. (Sigma Type IV), required special preparation to maximize specific binding to synaptic vesicles. Two mg of Con A was dissolved in 1 ml of 0.1 M Na_2HPO_4 (pH = 7.8) and 0.02% NaN_3. This sample was passed through an Ultrogel AcA 44 column (LKB) equilibrated with 0.1 M Na_2HPO_4 (pH = 7.8) and 0.02% NaN_3. The column bed volume was 56 ml (diameter = 1.15 cm), and a flow rate of 0.13 ml/min was used. The elution profile (at 280 nm) consisted of a small shoulder followed by the main peak. The shoulder was discarded. The main Con A peak was collected and concentrated to 1 ml in an Amicon minicon concentrator. Purified, concentrated Con A could be stored at -20°C for at least 3.5 months with no apparent effect on its ability to bind synaptic vesicles. The concentrated Con A sample was passed through a buffer exchange column (Sephadex G-25, Pharmacia) equilibrated with a buffer containing 160 mM KCl, 5 mM NaCl, 260 µM pargyline and 10 mM Tris-maleate, pH = 7.5 (SV buffer). The resultant Con A peak was concentrated to 1 ml as above. Ten µl of both 0.1 M $MnCl_2$ and 0.1 M $CaCl_2$ was then added to the Con A fraction. This purified, metallized Con A fraction was employed in Con A binding experiments.

One hundred µl of freshly prepared synaptic vesicles in SV buffer (at a protein concentration of 1.6 mg/ml) was mixed with 100 µl of the Con A fraction described above. This mixture was incubated at 22°C for 15 min, then diluted to 5 ml and centrifuged at 243,000 g for 35 min to pellet synaptic vesicle membranes. In some experiments 0.2 M α-methylmannoside (α MM)[1] was included in the binding mixture. In some cases the vesicles were preincubated at 4°C for 2 min in the presence of 0.2% Triton X-100 prior to exposure to Con A. The membrane pellets collected after binding were solubilized at 37°C for 30 min in 50 µl of a buffer containing 5% sodium dodecyl sulfate (SDS)[1], 10% glycerol, 30 mM Tris-HCl (pH = 7.5), 3 mM ethylene diamine tetraacetate, 0.0025% bromophenol blue and 30% 2-mercaptoethanol. This mixture was stored at -20°C until electrophoresis was performed (not longer than 48 h). Electrophoresis was performed as described by DeLorenzo et al. (20) with the following exceptions: the separating gel was 1.5 mm thick, contained 10% acrylamide, and was run at 8 W constant power for 7 h at 22°C. Gels were stained with Coomassie blue (21) or periodic acid-Schiff (PAS)[1] stain (22) according to established methods. Bio-Rad low molecular weight standard protein mixture was used to calculate molecular weights. Schiff stain was purchased from Sigma Chem. Co. Gels were scanned with an EC 910 densitometer (EC Apparatus).

Total synaptic vesicle protein was determined by the method of Markwell et al. (23). This is a simple modification of the Lowry method designed to detect membrane proteins.

RESULTS AND DISCUSSION

Protein Content of Synaptic Vesicles: The total protein content of the plain vesicular fraction was 0.047 ± 0.006 mg protein/g fresh tissue. Thirteen major Coomassie blue stained bands were observed upon SDS polyacrylamide gel electrophoresis (Figure 2 and Table 1). The molecular weights of synaptic vesicle polypeptides have been reported in a variety of laboratories (5, 8, 9, 10). Polypeptides commonly observed had the following molecular weights: 34,000-

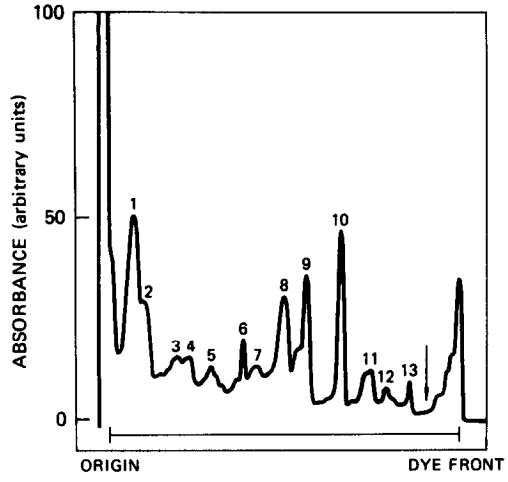

Figure 2. Densitometric scan of Coomassie blue stained synaptic vesicle proteins separated by SDS polyacrylamide gel electrophoresis. Molecular weights of peaks are calculated in Table 1. Note that there is no staining material in the region of the gel where Con A migrates (arrow).

38,000; 42,000-46,000; 55,000-56,000; 58,000-60,000; 64,000-66,000; 95,000-98,000 and 110,000-120,000. The results of PAS staining experiments are also presented in Table 1. A simple PAS staining pattern was demonstrated, in agreement with previous observations (11, 24). Electrophoresis results and careful electron microscopic analysis indicate that this plain synaptic vesicle fraction was quite pure.

Interaction of Con A and Synaptic Vesicles: Con A is a well characterized lectin which is known to have a specificity for mannose and glucose (25). Initial studies were instituted to determine the orientation of synaptic

Table 1. Molecular weight of polypeptides observed in the synaptic vesicle fraction as determined by SDS polyacrylamide gel electrophoresis.

BAND No.*	STAINED WITH COOMASSIE BLUE	STAINED WITH PAS STAIN
1	124†	
2	115	115 † (medium)**
3	98	
4	91	97-91(weak)
5	83	
6	70	73(weak)
7	65	67-63(strong)
8	56	
9	50	52-48(medium)
10	44	45(strong)
11	36	38(strong)
12	33	
13	30	

*Band No. refers to figure 2.
**Strong, medium and weak refer to observed relative intensities of PAS band.
†Observed molecular weights x 10^{-3}.

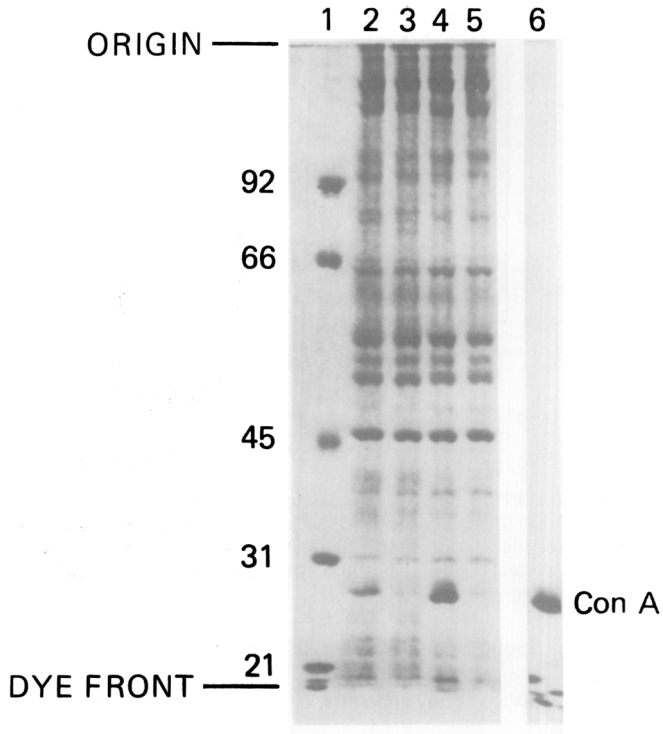

Figure 3. Electrophoresis of membrane pellets derived from Con A-synaptic vesicle binding experiments. Polypeptides were separated by SDS polyacrylamide gel electrophoresis and stained with Coomassie blue. Lane 1, Bio-Rad low molecular weight standard protein mix; lane 2, synaptic vesicles incubated with Con A (see Methods and Materials); lane 3, same as lane 2, but in the presence of 0.2 M αMM; lane 4, same as lane 2, but vesicles were preincubated with 0.2% Triton X-100; lane 5, same as lane 4, but in the presence of 0.2 M αMM; lane 6, Con A alone. Electrophoresis is described in the Methods and Materials.

vesicle glycoproteins using this lectin. We have observed that Con A will bind to intact freshly prepared synaptic vesicles (Figure 3, lane 2). The finding that αMM inhibits this interaction (Figure 3, lane 3) indicated that Con A was binding to a saccharide site on the vesicle surface. These sites were probably glycoproteins since there is little or no glycolipid in synaptic vesicles (27, 28). When synaptic vesicles were briefly preincubated with Triton X-100 Con A binding was increased almost five-fold (Figure 3 and Table 2). Revealing cryptic Con A binding sites in this manner might result from vesicle lysis, or unmasking of external sites by massive disruption of the vesicle structure. The latter possibility seems unlikely in view of the mild detergent treatment employed. In fact it can be seen that there was very

Table 2. Determination of binding of Con A to synaptic vesicle membranes. Relative binding was quantitated by integrating the area of the Con A peak*. For comparison purposes it was assumed that binding to Triton treated vesicles in the absence of αMM represented total binding.

EXPERIMENT	% TOTAL BINDING
Triton treated synaptic vesicles	
-0.2 M αMM	100%
+0.2 M αMM	6.2
Intact synaptic vesicles	
-0.2 M αMM	22
+0.2 M αMM	6.2

*Integration was performed by cutting and weighing peaks, in triplicate, derived from densitometric scans of polyacrylamide gels stained with Coomassie blue.

little protein solubilization upon detergent treatment (in Figure 3 compare lanes 2 and 3 to lanes 4 and 5).

The presence of Con A binding proteins has been demonstrated in solubilized synaptic vesicles (16). The molecular weights of the major Con A binding proteins were determined to be 48,000 and 65,000 (11). We have observed glycoproteins of similar molecular weights (Table 1). Using glyco-specific labeling procedures Smith and Loh demonstrated that two classes of synaptic vesicle glycoproteins exist: one of high molecular weight and externally oriented and one of low molecular weight, internally oriented (10). Taken together these results indicate that the major Con A binding glycoproteins in synaptic vesicles have molecular weights in the range of 48,000 and 65,000. At least 80% of these glycoproteins are not exposed in intact vesicles and apparently face the lumen. In principle Con A affinity chromatography could be employed to determine the origin of non-cryptic Con A binding sites.

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