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Structural Organization of Filamentous Proteins in Postsynaptic Density†

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ABSTRACT: Actin is one of the major protein constituents of the postsynaptic density (PSD), a characteristic structural entity subjacent to the postsynaptic membrane in excitatory synapses of the vertebrate central nervous system. In isolated purified PSD preparations, it is present to the extent of 29 ± 2 μg/mg of total protein, 90% of which is in the filamentous (F-actin) form. Iodination by a discriminatory labeling technique demonstrates that actin is located on the surface of the PSD from which it can be stripped by treatment with a mixture of strong anionic detergents, leaving behind an insoluble core held together by disulfide bridges, consisting in part of tubulin and "PSD protein".

Although the function of the postsynaptic density (PSD),[1] the filamentous organelle that underlies the postsynaptic membrane at excitatory synapses in the central nervous system, is unknown, it is becoming increasingly well characterized structurally. In common with many cytoskeletal elements, it may be isolated by virtue of its insolubility in detergents. Organelles isolated in this fashion are enriched in both actin and tubulin (Banker et al., 1974; Walters & Matus, 1975; Cohen et al., 1977; Matus & Taff-Jones, 1978; Kelly & Cotman, 1978a; Carlin et al., 1980) and have been shown to contain microtubule-associated proteins (Matus et al., 1981) and fodrin (Carlin et al., 1983) in lesser amounts. The major constituent of the PSD is a protein with an apparent molecular weight (M_r) of 50 000 ± 2000 called the postsynaptic density protein (PSD protein) (Banker et al., 1974; Blomberg et al., 1977; Kelly & Cotman, 1978a). This protein appears to be a major structural element, since, following treatments that remove many of the approximately 30 proteins composing or associated with the PSD, it is the major protein that remains in a detergent-insoluble residue that retains some of the structural attributes of the intact PSD (Blomberg et al., 1977; Matus & Taff-Jones, 1978).

The presence of filamentous proteins such as actin and tubulin in the PSD have led to speculation that the function of the density might be a structural one; membrane proteins could be anchored at the synapse by linkage to the density. The presence of Ca^{2+} and calmodulin binding proteins (Grab et al., 1979; Wood et al., 1980; Carlin et al., 1982, 1983) and of elements of a protein phosphorylation system (Florendo et al., 1971; Ariano & Appleman, 1979; Ng & Matus, 1979; Carlin et al., 1980; Grab et al., 1981a,b; Mahler et al., 1982) suggests the possibility of dynamic interactions among density proteins, leading to modification in protein–protein interactions perhaps linked to long- or short-term changes in a synapse's response to depolarization.

Freeze–fracture and deep etching (Gulley & Reese, 1981) of synapses in situ and careful observation of thin sections (Grey & Guillery, 1966; LeBeaux, 1973; Hansson & Hyden, 1974; Grey, 1975; Fifkova & Delay, 1982), as well as replicas made from isolated PSDs (Cohen et al., 1977), show filaments emerging from the main, mostly compact, body of the PSD, which itself may consist of particles linked by filaments (Cohen et al., 1977; Blomberg et al., 1977). The particles (Blomberg et al., 1977; Matus & Taff-Jones, 1978) that are observed by ethanolic phosphotungstic acid staining of PSDs in critical point dried preparations appear to be rodlike and may represent bundles of 10–20-nm filaments seen in cross section (Blomberg et al., 1977).

In order to begin to analyze the organization of the PSD and the interaction between it and the overlying postsynaptic

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[1] Abbreviations: ADF, actin depolymerizing factor; DOC, deoxycholate; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); DDT, dithiothreitol; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; M_r, apparent molecular weight; βME, β-mercaptoethanol; NEM, *N*-ethylmaleimide; PSD, postsynaptic density; $NaDodSO_4$, sodium dodecyl sulfate; SLS, sodium lauroyl sarcosinate; Temed, *N*,*N*,*N*′,*N*′-tetramethylethylenediamine, CBB, Coomassie Brilliant Blue; Tris, tris(hydroxymethyl)aminomethane.

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membrane, we have used a surface labeling technique to determine which PSD proteins are exposed at the surface of the density. We have also attempted to open up the density to determine the nature of the structural interactions that might be altered under physiological conditions.

Experimental Procedures

PSD Isolation. Postsynaptic densities were isolated from the cerebral cortex of 30-day-old male Sprague-Dawley rats by the procedure of Carlin et al. (1980). PSDs were resuspended by homogenization in 0.32 M sucrose–1 mM sodium bicarbonate.

Iodination. Surface labeling of PSDs was accomplished by iodination with a solid-phase technique employing 1,3,4,5-tetrachloro-3α,6α-diphenylglycouril (iodogen) as the iodination catalyst (Fraker & Speck, 1978; Markwell & Fox, 1978). Chloroglycouril (Pierce Chemical Co.) was dissolved in dichloroethane at a concentration of 0.05 mg/mL. Unless noted otherwise, 20 μL (1 μg) was placed in 10 × 75 mm borosilicate tubes while being vortexed under a stream of nitrogen. Samples were used immediately or stored up to several weeks in a desiccator. Before labeling, tubes were rinsed in water and air-dried. Iodination of PSDs was accomplished by the addition of 100 μg of postsynaptic densities, 5 μL of 0.04 M Hepes, pH 7, and 10 μCi of ^{125}I (New England Nuclear, carrier free, ~17 Ci/mg) in a final volume of 100 μL. Reactions were carried out for 10 min on ice with shaking and stopped by the addition of an equal volume of Laemmli sample buffer or removal to a catalyst-free vessel. Tubulin, actin, and calmodulin were iodinated as above, but the amount of protein subjected to iodination was 25 μg. For addition of actin to brain tissue at the start of a subcellular fractionation procedure, 3 mg of actin was iodinated with 1 mCi of ^{125}I, followed by desalting over a 10-mL column of Sephadex G-100 in the presence of 1 mg/mL bovine serum albumin. Peak column fractions were collected and allowed to polymerize for 30 min at room temperature in 5 mM Hepes, pH 8, 20 μM Na_2ATP, 1 mM $MgCl_2$, and 0.2 mM $CaCl_2$. Approximately half the actin was sedimentable F-actin as assayed by centrifugation. This value is comparable to the 1:1 F-actin:G-actin ratio in brain (Bray & Thomas, 1976).

Reduction and Alkylation of Postsynaptic Densities. A 100-μg sample of postsynaptic densities was brought to 20 mM dithiothreitol (DDT) (Sigma Chemical Co.) by the addition of 200 mM DTT in 10 mM Hepes, pH 7. After 20 min at 23 °C, 200 mM *N*-ethylmaleimide (NEM) (Sigma Chemical Co.) in 100 mM Hepes, pH 9.6, was added to reduced PSDs to a final concentration of 100 mM (Crestfield et al., 1963). The final volume of the reaction mixture was 100 μL. After an additional 20 min at room temperature, reduced and alkylated postsynaptic densities were iodinated as described above.

Partial Solubilization of Postsynaptic Densities. A total of 100 μg of PSDs, native or previously reduced and alkylated, was boiled for 10 min in 1% sodium lauroyl sarcosinate (SLS) (Sigma Chemical Co.)–1% sodium dodecyl sulfate ($NaDodSO_4$) (Sigma Chemical Co.) at a final protein:detergent ratio of 1:1 or 2:1 and then iodinated.

Isolation of Aggregate. PSDs were fractionated following detergent treatment on a Sephadex CL-4B or CL-6B column (1.6 × 22 cm). The column was equilibrated with 1% $NaDodSO_4$, 1% SLS, 20 mM Hepes, pH 7, and 40 mM NaCl. Approximately 1 mg of PSD was applied to the column, and 0.5-mL fractions were collected. Aliquots (100 μL) of fractions were iodinated in some experiments. Alternatively, PSDs reduced by incubation in 80 mM dithiothreitol (DTT) for 20 min at room temperature followed by solubilization as above were fractionated on a column equilibrated in column buffer containing 80 mM DTT.

Determination of Free –SH Groups. Free –SH groups were determined by the method of Ellman (1959) using 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB). Samples containing 100 μg of protein were diluted to 1 mL in 0.1 M sodium phosphate buffer, pH 8, and 20 μL of 0.1 M DTNB was added and the mixture incubated 30 min at 37 °C. Absorbance was corrected with two blanks, one containing protein and buffer and the other buffer and DTNB. Samples containing native PSD were centrifuged for 5 min in a Beckman microfuge prior to reading in the spectrophotometer cell. Quantitation of free sulfhydryls used either the extinction coefficient of DTNB (ϵ_m = 13 600 at 412 nm) or a comparison with yeast alcohol dehydrogenase, which contains 9 mol of –SH/mol of protein (Steinert et al., 1974). The assay was linear between 100 and 800 μg of PSD per reaction. For analysis of total sulfhydryl content of the PSD fraction, PSD was solubilized as described above in 1% $NaDodSO_4$–1% SLS following reduction in 80 mM DTT and then desalted on an 18-mL Sephadex G-25 column equilibrated with 1% $NaDodSO_4$, 1% SLS, and 100 μM sodium phosphate buffer, pH 8. The void-volume fractions, containing solubilized PSD proteins, were collected and assayed for free –SH.

Peptide Maps. Bands (containing approximately 3000 cpm of bound ^{125}I) from gels stained with CBB were digested with 0.3 μg of *Staphylococcus aureus* V8 protease in the sample wells of a 15% Laemmli gel according to Cleveland et al. (1977). Following electrophoresis, gels were fixed in 10% MeOH–10% acetic acid, dried, and exposed to SB-5 X-ray film (Kodak) for approximately 1 week.

Gel Electrophoresis. $NaDodSO_4$–polyacrylamide gel electrophoresis was carried out with a modification of the technique of Laemmli (1970). Running gels of 10% acrylamide–0.1% bis(acrylamide), 9.5 cm long (0.375 M Tris, pH 8.8, 0.1% $NaDodSO_4$, 0.1% ammonium persulfate, and 0.05% Temed), were polymerized for at least 2 h. Stacking gels contained 4% acrylamide, 0.04% bis(acrylamide), 0.125 M Tris, pH 6.8, 0.1 $NaDodSO_4$, 0.1% ammonium persulfate, and 0.1% Temed (polymerized for 1 h). Running buffer contained 0.025 M Tris, 0.19 M glycine, and 0.1% $NaDodSO_4$, final pH 8.4. Electrophoresis was performed at 30 mA per slab (~3.5 h). Gels were stained in 40% MeOH, 10% acetic acid, and 0.04% Coomassie Brilliant Blue R for 4 h and destained overnight in 25% MeOH–10% acetic acid. Apparent molecular weights (M_r) were determined by parallel electrophoresis of molecular weight standards obtained from Bio-Rad [myosin (200×10^3), β-galactosidase (116.5×10^3), phosphorylase B (94×10^3), BSA (68×10^3), ovalbumin (43×10^3), carbonic anhydrase (30×10^3), soybean trypsin inhibitor (21×10^3)]. Internal standards were provided by certain abundant proteins such as α-tubulin (57×10^3), β-tubulin (55×10^3), and actin (45×10^3). For autoradiography, dried gels were exposed to Kodak SB-5 Medical X-ray film at –20 °C.

Two-Dimensional Analysis. Two-dimensional (isoelectric-focusing and electrophoretic) analysis on polyacrylamide gels were carried out essentially as described by O'Farrell (1975), except that sample solubilization was as follows: 9 μg of iodinated protein in iodination media was added to 10 μg of authentic cold actin (total volume 14 μL) and solubilized in 30 μL of 1% $NaDodSO_4$–2.5 mM DTT by boiling for 2 min, cooled, and then brought to 8 M urea by the addition of solid urea. A total of 30 μL of 10% NP-40–2.5 mM DTT was then added (final volume ~100 μL). Samples of 35–50 μL were

focused on a 4% acrylamide gel in a pH gradient of 5–7 or 3–10. Second dimensions were run as described above. Visualization of unlabeled actin in the stained gels was correlated with labeled PSD proteins by autoradiography.

Isolation of Actin. Actin was prepared by the method of Moring et al. (1975) from whole rat brain. For other experiments, rabbit muscle actin was obtained from Dr. Carl Frieden, Washington University School of Medicine.

Depolymerization of Actin. Actin depolymerizing factor (ADF) was the kind gift of Dr. Joseph Bamburg. PSDs (100 μg) were incubated for 30 min at 23 °C with 3 μL of crude ADF, purified from chick brain by ion-exchange chromatography and gel filtration as described previously (Bamburg et al., 1980). A total of 1 μL of ADF should depolymerize 1.3 μg of F-actin in 5–10 min (Dr. J. Bamburg, personal communication). This procedure makes use of the observation that 100 μg of PSD contains approximately 3 μg of actin (see below).

Isolation of Tubulin. Tubulin was prepared from whole rat brain by the polymerization–depolymerization method of Sloboda et al. (1976).

Isolation of Calmodulin. Calmodulin was prepared from bovine brain as described by Watterson et al. (1980).

Amino Acid Analysis. Amino acid analysis was performed by hydrolysis in 6 N HCl for 24 or 48 h at 110 °C. Cysteine and cystine were oxidized to cysteic acid in 85% performic acid for 2 h at room temperature prior to HCl hydrolysis.

Protein Determination. Protein concentrations were determined by the modification of the Lowry procedure described by Markwell et al. (1978).

Results and Discussion

Actin Is a Major Constituent of Isolated PSDs. In agreement with previous studies (Cohen et al., 1977; Kelly & Cotman, 1978a; Mahler et al., 1982; Ratner & Mahler, 1983), we find actin to be a major constituent of isolated PSDs. In the preparations used in these studies it is present at a level of 29 ± 2 μg/mg of total PSD proteins (Mahler et al., 1982). The other principal filamentous proteins in the PSD are tubulin and PSD protein, in amounts of 119 ± 7 and 116 ± 12 μg/mg of total protein, respectively.

Evidence That Actin Is the Major Surface Protein of Postsynaptic Density. In order to determine whether some proteins are buried in the interior of the PSD and therefore inaccessible to iodination, while others are exposed on the surface and therefore accessible to this treatment, we used the chloroglycouril technique (Fraker & Speck, 1978; Markwell & Fox, 1978). Under controlled conditions (1:10 ratio of chloroglycouril to protein), this procedure has been shown to specifically label the coat rather than the membrane proteins of viruses and to result in the predominant introduction of ^{125}I into tyrosine residues (Dr. John Speck, personal communication). Wang & Mahler (1976) have shown that when PSDs are iodinated by the lactoperoxidase method, which is not as surface specific a reagent as chloroglycouril [see Markwell & Fox (1978)], PSD proteins are labeled in proportion to their concentrations, as determined by their Coomassie Blue staining profile following separation by $NaDodSO_4$–polyacrylamide gel electrophoresis. In initial experiments using iodination in the presence of chloroglycouril, we confirmed the results of Markwell & Fox (1978) with respect to the specificity of the reagent. However, at a ratio of 1:10 or 1:100 (we typically used 1 μg of iodogen to 100 μg of PSD), only one major protein became iodinated. The iodinated protein comigrated with authentic brain or muscle actin on $NaDodSO_4$–polyacrylamide gels (Figure 1). Raising the concentration of catalyst while maintaining a fixed PSD concentration caused iodination of many other stained bands, but neither the major PSD protein nor bands comigrating with tubulin were iodinated to any significant extent. These proteins appear to be inaccessible to surface labeling even at less stringent iodination conditions, suggesting that they are buried within the structure of the PSD. This hypothesis is discussed below.

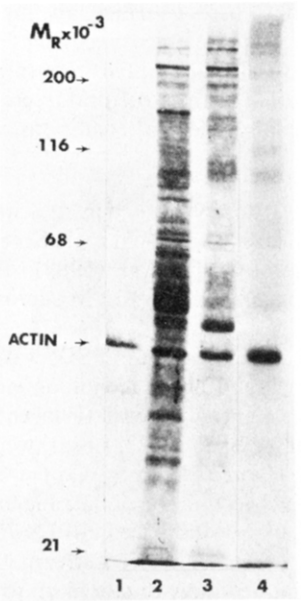


FIGURE 1: Surface labeling of PSD: (1) Coomassie blue staining of authentic action from rabbit skeletal muscle; (2) Coomassie blue staining of synaptosomes from which PSD fraction is derived; (3) Coomassie blue staining of a typical postsynaptic density (PSD) preparation; (4) Radioautograph of the same PSD sample, labeled with iodogen as described in the text.

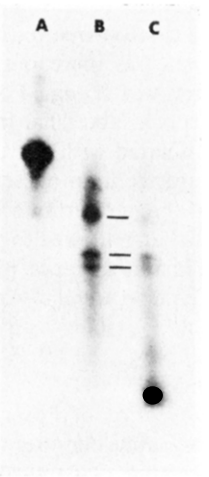


FIGURE 2: Peptide maps of actin and major 45-kdalton band iodinated in isolated PSD: (A) authentic actin isolated from rat brain, ^{125}I labeled and undigested; (B) iodinated brain actin digested with 0.3 μg of protease from *S. aureus* V-8; (C) major 45-kdalton protein iodinated in isolated PSD, digested with 0.3 μg of protease from *S. aureus* V-8. Lines indicate comigrating fragments in (B) and (C).

To determine whether the iodinated protein obtained by surface labeling was indeed actin, we used the Cleveland technique of partial proteolysis. Proteolytic fragments of the labeled polypeptide comigrated with those of authentic brain actin (Figure 2). Additional corroboration of its identity was obtained on two-dimensional gels where it shared isoelectric points with β- and γ-actin (Figure 3).

Cohen et al. (1977) have demonstrated that the protein with M_r 45 000 in this PSD preparation cross-reacts immuno-

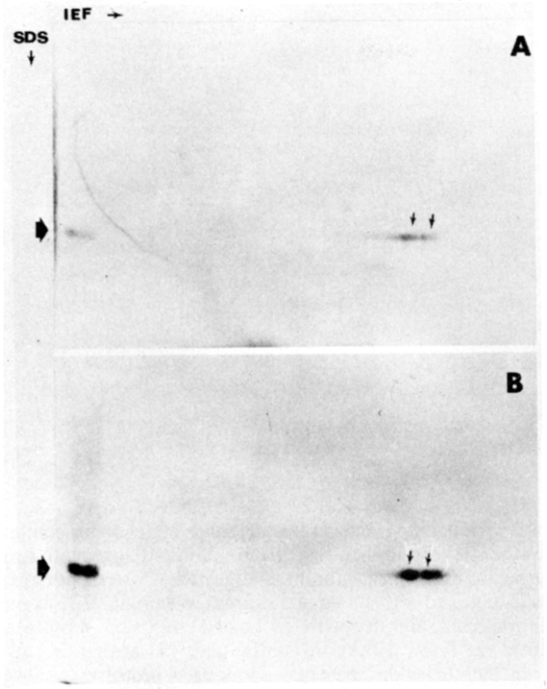


FIGURE 3: Two-dimensional electrophoresis of actin and major 45-kdalton band iodinated in isolated PSD. Coelectrophoresis of 5 μg of authentic actin with 10 μg of surface-labeled PSD fraction on a pH 5–7 isoelectric focusing gel. (A) Coomassie blue staining. The large arrow points to standard actin run in $NaDodSO_4$ dimension only. Small arrows point to β- and γ-actin. (B) Radioautograph of the same gel showing comigration of the major iodinated PSD proteins with the Coomassie blue stained spots.

chemically with muscle actin and that the amino acid composition of this protein agrees well with that of authentic actin, especially with respect to the presence of the unusual amino acid methylhistidine in both proteins. Similarly, Kelly & Cotman (1978a) demonstrated the identity of the M_r 45 000 Coomassie blue stained component with β- and γ-actin using two-dimensional analysis on polyacrylamide gels.

Since Matus and his collaborators (Matus et al., 1980) have shown that PSDs are sticky for brain cytoplasmic proteins, we wanted to confirm that the actin observed on the surface of the PSD was not a contaminant, artifactually adsorbed onto the PSD during isolation from whole cortex. The addition of ^{125}I-labeled actin to the homogenization buffer as described by Matus et al. (1980) for neurofilament proteins showed that only 5% of exogenously added actin remained bound to the PSD at the end of the isolation, indicating that the actin we observe is not adsorbed artificially. A total of 3 mg of actin (4.5×10^8 cpm) was added to the homogenization buffer. On the basis of the results of Pardee & Bamburg (1976), 6% of the total protein in brain is actin. Therefore, approximately 60 mg of actin is present in 10 brains (1 g of protein). The starting specific activity of the actin is therefore 4.5×10^8 cpm/63 mg of actin, which equals 7×10^6 cpm/mg. A total of 2 mg of PSD was isolated from the 10 cortices, and 2.9% or 0.058 mg of this protein is actin (Mahler et al., 1982). The specific activity of the isolated PSD was 2×10^4 cpm/0.058 mg or 5% the specific activity of the starting material.

Evidence That Actin on the Surface of PSD Is Polymerized. Observations of thin sections of PSDS (Grey & Guillery, 1966; LeBeaux, 1973; Hansson & Hyden, 1974; Grey, 1975; Fifkova & Delay, 1982) have shown filaments emerging from the main part of the density in situ that could be decorated with heavy meromyosin (LeBeaux & Willemont, 1975; Fifkova & Delay, 1982). Gulley & Reese (1981) have observed 8–9-nm microfilaments, tentatively identified as actin, concentrated under the postsynaptic density, by rapidly freezing and deeply etching synapses of the cochlear nucleus. Cohen et al. (1977) also observed unidentified filaments extending from PSDs in vitro in critical point dried, rotary shadowed replicas of the PSDs. We therefore attempted to determine biochemically whether the surface actin of the PSD was present in a polymerized (F-actin) or depolymerized (G-actin) state. PSDs were incubated with actin depolymerizing factor (ADF), a protein isolated from chick brain that is capable of depolymerizing F-actin when the two proteins are present in equal amonts (Bamburg et al., 1980). Following incubation of PSD with ADF (see Experimental Procedures), 90% of labeled actin failed to pellet with the PSD and was recovered in the supernatant of a centrifuged reaction mixture. This result indicates that most of the actin initially attached to the PSD can be removed from it by depolymerization and, therefore, that most of the actin associated with the PSD is in the form of F-actin.

Evidence That Actin Is Attached to Other Proteins by Hydrophobic Interactions. To determine how the F-actin covering the PSD is linked to other PSD proteins, PSDs were solubilized in a combination of 1% $NaDodSO_4$–1% SLS at 100 °C. Both detergents have a high negative charge density, and the stringent conditions used should result in the disruption of all noncovalent interactions among PSD proteins. Following solubilization, the almost clear suspension was passed over a Sepharose CL-4B or CL-6B column. A group of proteins eluted at the void volume of the column (see below). Actin was not present in this group of proteins; it eluted with authentic actin in the included volume of the column. Actin therefore appears to be attached to the PSD by strong but noncovalent bonds.

The interpretation of results of earlier studies on the attachment of actin to the PSD differs from those reported here; these differences may reflect the less stringent reaction conditions used previously. For example, Matus & Taff-Jones (1978) found that PSDs isolated by treatment with 3% SLS contain a band comigrating on one-dimensional $NaDodSO_4$–polyacrylamide gels with actin. These PSDs, however, were not exposed to the elevated temperature employed here. Blomberg et al. (1977) found that actin could be extracted from the PSD by an ionic detergent (0.5% DOC) in conjunction with salt or sulfhydryl reducing or blocking agents. Since none of these reagents were effective alone, the results are difficult to interpret, but one explanation is that the action of sulfhydryl reagents in high salt concentrations induces conformational changes in PSD proteins or in the actin itself that render it susceptible to solubilization in detergent.

Possible Roles of Actin in PSD. The observation described in the previous sections suggest that F-actin is the major surface element of the postsynaptic density and appears to be attached to the PSD by strong, noncovalent interactions. While actin filaments had been shown to emerge from the PSD in situ (LeBeaux & Willemot, 1975; Gulley & Reese, 1981), this is the first demonstration that filamentous actin remains tightly attached to the bulk of the PSD during its isolation. Actin is known to interact strongly with lipids (Shenouda & Pigott, 1977), with F-actin exhibiting a greater binding capacity than G-actin. This is one mechanism by means of which a shell of actin on the surface of the PSD may anchor it to the postsynaptic membrane. The 10% of PSD-associated actin that is not depolymerized by ADF may then represent G-actin linked directly to the body of the PSD, providing an attachment site for F-actin. Fifkova & Delay (1982) have recently

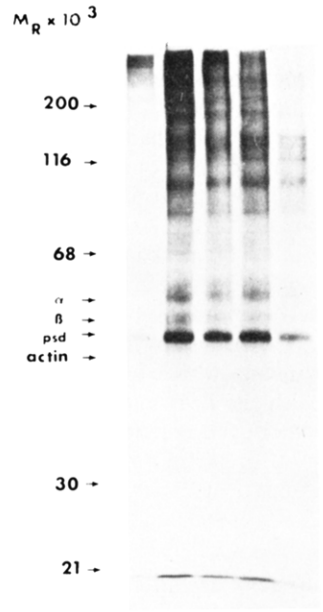


FIGURE 4: PSD proteins remaining unsolubilized following treatment with ionic detergents. Radioautograph of the void-volume fractions eluting from a Sepharose CL-6B column upon application of 0.5 mg of the PSD fraction after treatment with 1% $NaDodSO_4$–1% SLS. The significance of a high molecular weight aggregate resistant to solubilization is described in the text. α, α-tubulin; β, β-tubulin; psd, PSD protein.

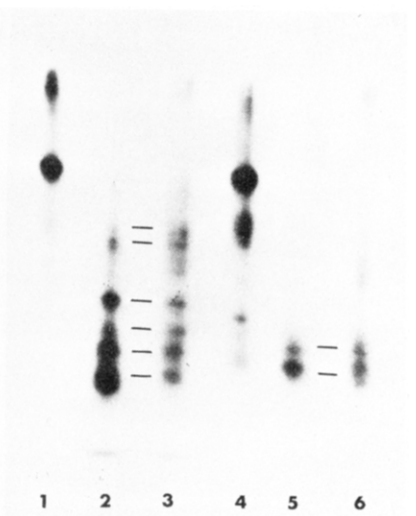


FIGURE 5: Radioautographs of peptide maps of gel bands comigrating with authentic α- and β-tubulin from iodinated aggregate samples: (1) authentic rat brain α-tubulin, undigested; (2) authentic rat brain α-tubulin, digested with 0.3 μg of *S. aureus* V-8 protease; (3) aggregate 57-kdalton band, digested with 0.3 μg of *S. aureus* V-8 protease; (4) authentic rat brain β-tubulin, undigested; (5) authentic rat brain β-tubulin, digested with 0.3 μg of *S. aureus* V-8 protease; (6) aggregate 53-kdalton band, digested with 0.3 μg of *S. aureus* V-8 protease.

shown that actin filaments identified by decoration with heavy meromyosin are associated both with the postsynaptic membrane and the PSD. Alternatively, since in situ the PSD is closely apposed to the postsynaptic membrane, it is possible that G-actin may provide a link between the two structures or that an actin–fodrin complex may be bound to membrane ankyrin (Geiger, 1982).

The attachment sites of actin to the body of the PSD are not known. As proposed above, partially or completely depolymerized actin may fulfill such a function. F-actin has also been shown to interact with tubulin in the presence of microtubule-associated proteins (Griffith & Pollard, 1978), which have been localized to the PSD immunocytochemically (Matus et al., 1981). Thus tubulin, which is present in the PSD at about twice the level of actin (Mahler et al., 1982), provides an alternative site for its attachment to the PSD. Another strong candidate is the protein doublet named fodrin, an actin binding protein recently detected in the PSD (Carlin et al., 1983), with considerable homology to erythrocyte spectrin (Levine & Willard, 1981; Burridge et al., 1982; Sobue et al., 1982). Bands in that mobility range ($M_r \simeq 250000$) become iodinated under surface-labeling conditions (Figure 1).

While actin is the major protein labeled during surface iodination, it is possible that other PSD proteins are present on the surface of the PSD, possibly in very small amounts compared to actin or lacking significant quantities of the tyrosine residues required for iodination. Figure 1 shows that many additional bands actually become iodinated under surface-labeling conditions, although most show much less incorporation of iodine per mole than does actin. The presence of specific glycoproteins in the PSD has led to speculation that these proteins protrude through the postsynaptic membrane into the synaptic cleft (Gurd, 1977, 1982; Mahler et al., 1982). If this is indeed their role, they do not become highly iodinated under surface-labeling conditions.

Protein Composition of Detergent-Insoluble PSD Aggregate. Analysis of the aggregate of proteins remaining after $NaDodSO_4$–SLS treatment and isolated in the void volume of the Sepharose CL-6B column (30% of the total PSD proteins) is provided in Figure 4. The major proteins in this aggregate comigrate on one-dimensional gels with PSD protein and authentic brain tubulin. Confirmation of the identity of tubulin in the aggregate was accomplished with partial proteolytic digests (Figure 5). Molecular masses of other major proteins in this aggregate are 290, 275, 250, 210, 190, 113, 41, and 28 kdaltons. The ratio of α-tubulin to β-tubulin in the aggregate is 1.09 ± 0.03, which is similar to this ratio in tubulin isolated from whole brain (1.14 ± 0.04) postsynaptic membranes or PSDs (Mahler et al., 1982). The M_r of the aggregate is estimated at (14–20) × 10^6 since it is excluded by Sepharose CL-4B but included by Sepharose CL-2B (data not shown). These data are in agreement with those of Kelly & Cotman (1978a,b), who found that major PSD proteins including PSD protein and a protein with M_r 55000, which they speculatively identified as tubulin, are completely excluded from a 4.5% polyacrylamide stacking gel, implying an aggregate of $M_r > 1.0 \times 10^6$. On the basis of their mole ratio, approximately 20 molecules of PSD protein and 10 tubulin heterodimers are present in each aggregate of this size. These data indicate that tubulin is an intrinsic and major structural component of the PSD in its own right, confirming its immunocytochemical localization in this organelle (Walters & Matus, 1975). This core of PSD protein and tubulin is attached to several proteins including additional PSD protein and tubulin that therefore are contained in the included volume of the Sepharose CL-6B column, and surface labeling indicates that this complex is in turn surrounded by a shell of actin.

Evidence That Aggregate Proteins Are Held Together by S–S Bonds. Since Blomberg et al. (1977) found that sulfhydryl reducing agents exposed the internal structure of the density and Kelly & Cotman (1978b) observed that proteins of postsynaptic densities required reduction in order to enter denaturing gels, we assumed that the aggregate proteins were linked by disulfide bridges. When the PSD was reduced with 80 mM DTT prior to exposure to 1% $NaDodSO_4$ plus 1% SLS as described above, it became completely solubilized. When this solution was examined by gel-filtration chromatography, it was found that over the entire elution profile all proteins

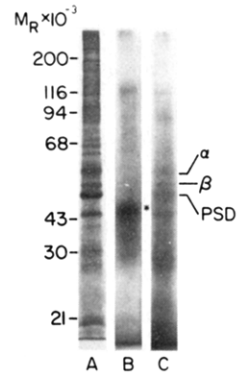


FIGURE 6: Open and closed configurations of PSD: (A) Coomassie blue staining pattern of a typical postsynaptic density preparation (PSD) from rat cerebral cortex; (B) radioautograph showing surface iodination of the same PSD preparation [band marked with an asterisk (*) comigrates with actin]; (C) radioautograph of PSD reduced with 20 mM dithiothreitol (DTT) and then alkylated with *N*-ethylmaleimide (100 mM), solubilized in 1% $NaDodSO_4$–1% SLS, and iodinated as in (B).

now eluted at positions corresponding to their intrinsic molecular weights. This observation indicates the absence of any significant residual interactions between these different proteins.

We attempted to corroborate the solubilization of PSD structure following detergent solubilization and reduction with DTT by the chloroglycouril-labeling technique. However, although reduction in 20 mM DTT showed a partial opening of the PSD, making tubulin and PSD protein relatively more accessible to iodination (Figure 6), we were not able to label PSD proteins in the proportions determined by Coomassie Blue staining. Following removal of solubilized proteins, including actin by gel filtration, proteins comigrating on $NaDodSO_4$–polyacrylamide gels with tubulin and PSD protein become iodinated by iodogen as shown in the photograph in Figure 4. Tubulin and PSD protein could have been rendered more accessible to iodination by changing their conformation within the aggregate or by removal of the solubilized shell, including actin. We cannot exclude the possibility that nonsurface proteins, originally buried within the actin shell, are removed from the aggregate by detergent treatment and permit reorientation of some of the remaining aggregate proteins so long as they remain cross-linked by disulfide bonds. We were unable to use the iodogen technique with PSDs reduced under more stringent conditions such as those employed for gel filtration, since for iodination to succeed it is necessary to remove all excess DTT. Even addition of NEM in amounts approaching its solubility limit was insufficient to alkylate the excess DTT to permit iodination. These results are comparable to those of Blomberg et al. (1977), who found that 13-nm particle arrays were still present in thin sections of pellets of PSDs that had been treated overnight at 0 °C with 50 mM DTT–0.5% DOC. Our stringent reduction conditions apparently break up these aggregates. We conclude from these data that the actin-depleted core of the PSD is composed of a group of proteins held together by disulfide cross bridges, which are dissociated only by reduction under stringent conditions.

Quantitation of Free Sulfhydryls and Disulfides in PSD. Native PSDs, PSDs solubilized in 1% $NaDodSO_4$ plus 1% SLS, and PSDs reduced with 80 mM DTT prior to solubilization were assayed to quantitate free sulfhydryl groups in the PSD (Table I). Since results were quite variable with Ellman's reagent after reduction of PSD by DTT, probably due to residual DTT still bound to the PSD even after desalting,

Table I: Protein -SH Content of Postsynaptic Densities

	μmol of -SH/mg of PSD	assay
PSD (n = 5)	0.0094 ± 0.0005[a]	Ellman's reagent
PSD + 1% $NaDodSO_4$ + 1% SLS (n = 5)	0.017 ± 0.005	Ellman's reagent
PSD + 1% $NaDodSO_4$ + 1% SLS + DTT (n = 5)	0.051 ± 0.043	Ellman's reagent
PSD, oxidized by performic acid (n = 2)	0.06	amino acid analysis

[a] Mean ± standard deviation. n, number of preparations assayed.

Table II

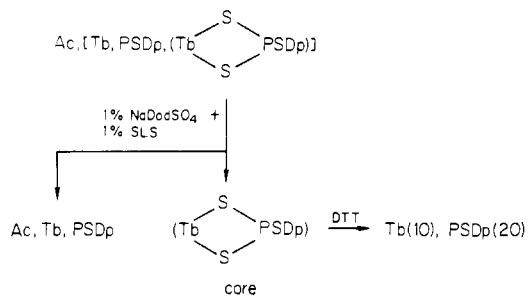
	preparation[a]				
	PSD (0.5% Triton)		PSD (0.4%	PSD (3%	
residue	expt 1	expt 2	NLS)	NLS)	SPM
Asp	90	88	90	100	96-105
Thr	53	59	56	60	54-61
Ser	57	68	86	83	71-98
Glu	131	130	125	130	110-122
Pro	63	68	63	67	44-51
Gly	79	76	78	86	70-78
Ala	87	88	78	87	75-83
Cys[b]	17	17	nd	nd	nd
Val	64	61	56	49	57-73
Met	24	18	21	13	19-30
Ile	47	47	44	37	43-53
Leu	88	87	88	79	83-93
Tyr	28	28	31	28	26-32
Phe	38	37	37	31	36-46
Lys	39	33	58	57	59-66
His	30	27	29	29	18-21
Arg	65	65	60	60	40-48
Trp	nd[c]	nd	nd	nd	nd

[a] Data are expressed as residues/1000 residues. Values for NLS PSD and SPM are taken from Banker et al. (1974). [b] Determined as cysteic acid following performic acid oxidation. [c] nd, not determined.

results were corroborated by amino acid analysis following oxidation of cyst(e)ine to cysteic acid (Table II). Of the total sulfhydryl groups in the PSD, 71% are unreactive toward Ellman's reagent unless the PSD is prereduced with DTT, indicating that most sulfhydryl groups are present in the PSD in the form of disulfide bridges.

The total amount of cyst(e)ine in the PSD is 0.06 μmol/mg of protein, or twice that of rat liver ribosomes (Steinert et al., 1974). The amino acid composition of densities isolated by extraction with Triton X-100 is quite similar to that reported by Banker et al. (1974), who analyzed PSDs obtained by SLS extraction. Both PSD fractions are enriched in histidine, arginine, glutamic acid (and/or glutamine since Gln residues are converted to Glu on acid hydrolysis), and proline. Since the amino acid sequence in the polypeptide chain controls folding and accessibility of sulfhydryls for oxidation to disulfides, differences in these sequences must govern the tendency to form high molecular weight aggregates. Comparison of amino acid content of the PSD fraction with that of the highly cross-linked wheat glutenin proteins (Heubner et al., 1976) reveals that both have a similar content in cyst(e)ine and are enriched in glutamic acid and proline. In the wheat system, it has been suggested that disulfide interchange (Goldstein, 1957), the transfer of attachment of disulfide bonds from one protein chain to another, as in the

Scheme I



Ac = actin (9F:1G)
Tb = tubulin dimer (1α:1β)
PSDp = PSD protein

scheme $P_1S\text{-}SP_2 + P_3SH \rightleftharpoons P_1S\text{-}SP_3 + P_2SH$, could allow either a relaxation of a rigid structure or a tightening of a protein network by chain elongation (Heubner et al., 1976). One method for alteration of PSD structure in response to a changing intracellular environment following depolarization of the postsynaptic cell could involve changing patterns of the disulfide bonds that cross-link PSD proteins.

Structure of PSD. Finally, using all the results just described, we propose a highly speculative model of the PSD, as shown in Scheme I. The entities in brackets are postulated to be surrounded by an actin shell and, therefore, not subject to surface iodination. All the actin plus >67% of the PSD protein and tubulin can be solubilized by treating the structure with the combination of detergents shown, leaving behind an insoluble core consisting of these two proteins held together by disulfide bridges. The numbers in parentheses indicate approximate mole ratios. The scheme makes no statement concerning the function of additional proteins including fodrin, MAPs, and glycoproteins known or surmised to form part of the PSD, except that they probably do not make a major contribution to the outer shell.

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Purification, Characterization, and Assembly Properties of Tubulin from Unfertilized Eggs of the Sea Urchin *Strongylocentrotus purpuratus*†

H. William Detrich, III,* and Leslie Wilson

ABSTRACT: Tubulin was purified from unfertilized eggs of the sea urchin *Strongylocentrotus purpuratus* by chromatography of an egg supernatant fraction on DEAE-Sephacel or DEAE-cellulose followed by cycles of temperature-dependent microtubule assembly and disassembly in vitro. After two assembly cycles, the microtubule protein consisted of the α- and β-tubulins (>98% of the protein) and trace quantities of seven proteins with molecular weights less than 55 000; no associated proteins with molecular weights greater than tubulin were observed. When analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis on urea–polyacrylamide gradient gels, the α- and β-tubulins did not precisely comigrate with their counterparts from bovine brain. Two-dimensional electrophoresis revealed that urchin egg tubulin contained two major α-tubulins and a single major β species. No oligomeric structures were observed in tubulin preparations maintained at 0 °C. Purified egg tubulin assembled efficiently into microtubules when warmed to 37 °C in a glycerol-free polymerization buffer containing guanosine 5′-triphosphate. The critical concentration for assembly of once- or twice-cycled egg tubulin was 0.12–0.15 mg/mL. Morphologically normal microtubules were observed by electron microscopy, and these microtubules were depolymerized by exposure to low temperature or to podophyllotoxin. Chromatography of a twice-cycled egg tubulin preparation on phosphocellulose did not alter its protein composition and did not affect its subsequent assembly into microtubules. At concentrations above 0.5–0.6 mg/mL, a concentration-dependent "overshoot" in turbidity was observed during the assembly reaction. These results suggest that egg tubulin assembles into microtubules in the absence of the ring-shaped oligomers and microtubule-associated proteins that characterize microtubule protein from vertebrate brain.

The assembly and disassembly of labile cytoplasmic microtubules are apparently closely coupled with the functions that they perform. Because it is available in large quantity, most studies of cytoplasmic microtubule assembly have been performed on microtubule protein isolated from vertebrate brain tissue. However, it is important to recognize that brain microtubule protein may be an atypical material for study, perhaps modified to form stable cytoskeletal elements in nonmitotic cells. Therefore, we have chosen to investigate the properties of tubulin isolated from unfertilized eggs of the purple sea urchin, *Strongylocentrotus purpuratus*.

Unfertilized sea urchin eggs contain a large pool of presynthesized tubulin (Raff & Kaumeyer, 1973; Pfeffer et al., 1976). Bibring & Baxandall (1977) have shown that most of the tubulin incorporated into the mitotic spindle during the first cleavage division in *S. purpuratus* zygotes is drawn from this pool. Doublet-specific ciliary tubulin destined for ciliogenesis during the blastula stage is also present in the unfertilized egg (Bibring & Baxandall, 1981). However, the pool of ciliary tubulin in embryos appears to be small (capable of supporting three to four rounds of ciliary regeneration; Auclair & Siegel, 1966). Furthermore, Stephens (1978) was unable to detect ciliary tubulin in the unfertilized egg by peptide mapping. These studies suggest that the majority of the tubulin in the unfertilized egg is mitotic or cytoplasmic in destiny.

Dramatic changes both in the extent of assembly of cytoplasmic microtubules and in their organization take place during the first cleavage cycle of sea urchin zygotes. Few, if any, microtubules are assembled prior to fertilization in eggs of *Arbacia punctulata* and *Lytechinus variegatus* (Bestor & Schatten, 1981). Following fertilization in *S. purpuratus*, a monaster of microtubules forms around the sperm pronucleus and then recedes, to be replaced by the interphase asters and a spiral cortical array of microtubules (Harris et al., 1980a,b). The cortical microtubules and the interphase asters in turn break down prior to the formation of the mitotic spindle and the occurrence of the first cleavage at 135 min (15 °C). Harris et al. (1980b) have proposed that the breakdown of microtubules is induced by a transient, wavelike movement of some depolymerizing factor (perhaps calcium) from the cell center to the periphery of the cell.

Our long-range objective is to understand the mechanism and, ultimately, the regulation of the assembly and disassembly of sea urchin cytoplasmic microtubules, both in vitro and in vivo. In this report, we describe a protocol for the isolation of highly purified tubulin from unfertilized eggs of *S. purpuratus*. Furthermore, we have analyzed the protein com-

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