Studying the Protein Organization of the Postsynaptic Density by a Novel Solid Phase- and Chemical Cross-linking-based Technology*

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Agarose beads carrying a cleavable, fluorescent, and photoreactive cross-linking reagent on the surface were synthesized and used to selectively pull out the proteins lining the surface of supramolecules. A quantitative comparison of the abundances of various proteins in the sample pulled out by the beads from supramolecules with their original abundances could provide information on the spatial arrangement of these proteins in the supramolecule. The usefulness of these synthetic beads was successfully verified by trials using a synthetic protein complex consisting of three layers of different proteins on glass coverslips. By using these beads, we determined the interior or superficial locations of five major and 19 minor constituent proteins in the postsynaptic density (PSD), a large protein complex and the landmark structure of asymmetric synapses in the mammalian central nervous system. The results indicate that α,β-tubulins, dynein heavy chain, microtubule-associated protein 2, spectrin, neurofilament H and M subunits, an hsp70 protein, α-internexin, dynamin, and PSD-95 protein reside in the interior of the PSD. Dynein intermediate chain, α-amino-3-hydroxy-5-methyl-4-isoxazole propionate receptors, kainate receptors, N-cadherin, β-catenin, N-ethylmaleimide-sensitive factor, an hsc70 protein, and actin reside on the surface of the PSD. The results further suggest that the N-methyl-α-aspartate receptors and the α-subunits of calcium/calmodulin-dependent protein kinase II are likely to reside on the surface of the PSD although with unique local protein organizations. Based on our results and the known interactions between various PSD proteins from data mining, a model for the molecular organization of the PSD is proposed. Molecular & Cellular Proteomics 5: 1019–1032, 2006.

Cells contain numerous supramolecules, such as the nuclear pore complex, proteosome, chlorosome, ribosome, and flagellar motor complex, and these are used to carry out a wide variety of complicated functions. A supramolecule usually consists of a population of diverse biomolecules, and these biomolecules are organized in such a way that their contributions to the function of the supramolecule are coordinated spatially and temporally. Understanding the protein organization of a supramolecule is thus essential for fully understanding the molecular mechanism by which the supramolecule functions. Several powerful techniques, such as nuclear magnetic resonance, cryoelectron microscopy, x-ray crystallography, and chemical cross-linking, have been used to investigate the protein organization of supramolecules. Here we report a novel methodology that was designed to distinguish proteins lining the surface from those embedded in the interior of supramolecules. After verification with a synthetic multilayered protein complex of known structure, this methodology was used to investigate the protein organization of a landmark structure of the asymmetric synapses in the mammalian central nervous system, the postsynaptic density (PSD).1

The PSD is a layer of densely packed protein complex lying underneath the postsynaptic membrane of the excitatory synapses in mammalian central nervous system. Under electron microscopy, the PSD appears as a planar array of granular material with filaments embedded in and extending from it (1). Accumulating evidence has suggested that the PSD plays important roles in clustering neurotransmitter receptors in the postsynaptic membrane, in processing synaptic information, and in joining pre- and postsynaptic components into an integrated synaptic unit (2–7). The morphology and protein composition of the PSD in the brain have been reported to

1 The abbreviations used are: PSD, postsynaptic density; TPC, three-layered synthetic protein complex; AFM, atomic force microscopy; DADPA, diaminodipropylamine; SAED, sulfosuccinimidyl-2-(7-azido-4-methylcoumarin-3-acetamido)-ethyl-1,3-dithiopropionate; cDHHC, the heavy chain of cytoplasmic dynein; cDIC, the intermediate chain of cytoplasmic dynein; α-CaMKII, α-subunit of calcium/calmodulin-dependent protein kinase II; MAP2, microtubule-associated protein 2; NSF, N-ethylmaleimide-sensitive factor; NMDA, N-methyl-D-aspartate; AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazole propionate; ACTH clip, adrenocorticotropic hormone corticotropin-like intermediate lobe peptide; sulfo-GMBS, N-(γ-maleimidobutyl oxy)sulfosuccinimide ester; E/T, exterior/total.
undergo activity-dependent alterations (8–14). Biochemical analyses have revealed that PSD isolated from brain tissues consists of five major proteins, including actin, α-subunit of calcium/calmodulin-dependent protein kinase II (α-CaMKII), the heavy chain of cytoplasmic dynein (cDHc), and α,β-tubulin subunits, and hundreds of other minor proteins, including neurotransmitter receptors, intracellular signaling molecules, cell junction proteins, and scaffold proteins (15–24). In contrast to the wealth of information about the protein composition of the PSD, the organization of various proteins in the PSD is not fully understood at present (25–29).

By using the methodology reported here, we determined the superficial or interior localization of five major and 19 minor constituent proteins of the PSD. Together these proteins account for more than 40% of the total protein mass of the PSD. A molecular model of the PSD is proposed on the basis of our results and those appearing in the literature.

**EXPERIMENTAL PROCEDURES**

**Materials**—The polyclonal antibodies to cDHc (dynemin R-325), actin (C-11), PSD-95 (C-20), and β-catenin (C-18) were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). The monoclonal antibodies to cytoplasmic dynein intermediate chain (cDyIC) and synaptin were obtained from Sigma. The polyclonal antibody to kainate receptor subunit GluR6/7 was purchased from Upstate Biotechnology Inc. (Lake Placid, NY). The polyclonal antibodies to AMPA receptor subunits GluR1 and GluR2/3, NMDA receptor subunits NR1 and NR2a/2b, brain spectrin (240/235E), and microtubule-associated protein 2 (MAP2); the monoclonal antibodies to β-tubulin and α-CaMKII; and the horseradish peroxidase-conjugated secondary antibodies were purchased from Chemicon Inc. (Temecula, CA). The monoclonal antibody to synaptopsin was obtained from Novocastra Laboratories Ltd. (Newcastle upon Tyne, UK). The polyclonal antibody to N-cadherin (ab12221) was purchased from Abcam Ltd. (Cambridge, UK). Diamidopropylamine (DADPA)-agarose beads, sulfoconcanimide-2-(7-azido-4-methylcoumarin-3-acetamido)-ethyl-1,3′-dithiopropionate (SAED), and disuccinimidyl tartarate were obtained from Pierce.

**Isolation of the PSD and Other Subcellular Fractions from Pig Cerebral Cortices**—Fresh pig brains were obtained from a local slaughterhouse within 1 h after the pigs were slaughtered. The white matter was removed from the forebrain, leaving mainly cerebral cortex. Brain homogenates and various subcellular fractions (including PSD, crude synaptosome, synaptic plasma membrane, synaptic junction, microsome, cytosol, mitochondria, and myelin sheath) were prepared from pig cerebral cortices by the method published previously (30). Briefly brain tissues were first suspended with 6 volumes of buffer A (0.1 mM EDTA, 0.1 mM EGTA, and 1 mM HEPES at pH 7.4) and homogenized on ice by a Polytron blender (Kinematica GmbH, Littau, Switzerland) at a setting of 6 for 30 s. The homogenate was centrifuged at 4,420 × g for 5 min, and the supernatant was saved and kept on ice. The resultant pellet was suspended in 4 volumes of the same sucrose solution and centrifuged again at 4,420 × g for 5 min. The combined supernatants were then centrifuged at 38,900 × g for 45 mm. The resultant supernatant was further centrifuged at 245,000 × g for 1 h, and the resultant supernatant and pellets were used as the cytosol and microsome fractions, respectively. The pellet obtained after the 38,900 × g centrifugation step (the crude synaptosome) was suspended in 2 volumes of 2 mM Tris acetate at pH 8.0 and applied on top of a layer of buffer A containing 1.2 mM sucrose. After centrifugation at 245,000 × g for 50 min, the pellet was saved and used in this study as the mitochondria fraction, whereas the white material in the interface was collected and applied on top of a layer of buffer A containing 0.9 mM sucrose. After centrifugation at 245,000 × g for 50 min, the white membranous material in the interface was collected and used as the myelin sheath fraction in this study, and the pellet was the synaptic plasma membrane fraction. Synaptic plasma membranes were suspended by homogenizing in HEPES/CaCl \(_2\) buffer (2 mM HEPES at pH 7.4 and 50 μM CaCl \(_2\)) and pelleted (65,000 × g for 20 min). This washing step was repeated once more. The final pellet was suspended in a solution containing 1 volume of HEPES/CaCl \(_2\) buffer and 2 volumes of 2 mM HEPES (pH 7.4) in 0.4% (v/v) Triton X-100 with a final protein concentration of 2 mg/ml and then layered on top of 1.0 mM sucrose. After centrifugation at 85,000 × g for 1 h, the resultant pellet was resuspended in Tris acetate buffer solution (50 mM Tris acetate, 0.1 mM EDTA, and 0.1 mM EGTA, pH 7.4) and subsequently centrifuged at 50,000 × g for 30 min. After repeating the above washing step once more, the resultant pellets were used as the synaptosome fractions here. Synaptic junctions were diluted to 4 mg of protein/ml by solution B (50 μM CaCl \(_2\) and 6 mM Tris-HCl at pH 8.1), mixed with an equal volume of solution C (0.32 mM sucrose, 1% (v/v) Triton X-100, and 12 mM Tris-HCl at pH 8.1), incubated on ice for 15 min, and finally centrifuged at 32,800 × g for 20 min. The resulting pellet was suspended in solution B and applied on top of step gradients consisting of 1.0, 1.5, and 2.0 mM sucrose containing 1 mM NaHCO \(_3\). After centrifugation at 200,000 × g for 2.5 h, the band in the interface between the 1.5 and 2.0 mM sucrose layers was collected. The collected sample was then mixed with an equal volume of a solution containing 150 mM KCl and 1% (v/v) Triton X-100 and centrifuged at 200,000 × g for 20 min. The resulting pellet was suspended in solution B containing 40% (v/v) glycerol and used as the PSD fraction. Lodoacetamide (2 mM) was added to all buffer solutions used for isolating the PSD to prevent the random formation of interprotein disulfide bonds. A protease inhibitor mixture containing (final concentration) 1 μg/ml leupeptin, 1 μg/ml pepstatin A, 1 mM benzamidine, and 0.25 mM PMSF was also added to all buffer solutions. Protein concentrations were determined by the method of Lowry (31).

**SDS-PAGE and Western Blotting**—SDS-PAGE was carried out using a minigel apparatus (Mini-Protein 3; Bio-Rad) and either 9% polyacrylamide gels according to the method of Laemmli (32) or 3.5% polyacrylamide gels according to the method of Weber and Osborn (33) to analyze the cytoplasmic dynein heavy chain. The SDS-PAGE was performed under reducing conditions. Western blotting was performed as described previously (34). The relative intensities of Coomassie Blue-stained or immunostained bands were quantified by the TotalLab image analysis software system (Nonlinear Dynamics, Newcastle, UK).

**Electron Microscopy**—Thin sections of the PSD prepared and fixed according to the procedures described by Lai et al. (30) were examined with a transmission electron microscope (Hitachi model H-600-3). For scanning electron microscopic analyses, the PSD (0.06 mg of protein/ml in 5 mM Tris-HCl at pH 7.2) was applied to the surface of 6 × 6-mm \(^2\) glass coverslips. After removing the excessive liquid, the coverslips were air-dried, coated with platinum by an ion coater (E-1030; Hitachi, Tokyo, Japan), and examined under a scanning electron microscope (Hitachi model S-4700). All data were collected at a magnification of 30,000. The areas and contour lengths of the PSDs in the scanning electron micrographs were determined by the image quantification program MetaMorph Version 6.2r6 (Universal Image, West Chester, PA).

**Isolation of Surface Proteins from the PSD**—The procedure for isolating surface proteins from the PSD is illustrated in Fig. 1C. One milliliter of DADPA-agarose beads was mixed with 1 ml of 20 mM borate buffer at pH 8.2 and then with 20 μl of 50 mM SAED in
dimethylformamide at 4 °C in the dark. Aliquots of 20 µl of SAED solution were added to the reaction mixture at 5, 10, and 15 min later. The reaction was stopped by adding Tris-HCl at pH 8.4 (final concentration, 50 mM) 1 h after the final addition of SAED. Unreacted SAED was removed from the beads by filtering the reaction mixture through a sintered glass filter. SAED-conjugated DADPA beads were incubated with either the PSD (in 6 mM Tris-HCl at pH 8.1, 50 µM CaCl2, 40% (v/v) glycerol, and 2 mM iodoacetamide) or SDS-treated PSD (PSD in 3% (w/v) SDS and heated in a boiling water bath for 5 min) at 4 °C in the dark with gentle mixing. Two minutes later, the samples were exposed to UV light (365-nm wavelength light emitted from a transilluminator (TLW-20; UVP Inc., Upland, CA)) at a distance of 1 cm for different lengths of time while the sample was kept on ice.

After the photolytic reaction, SDS (final concentration, 3% (w/v)) and Tris-HCl at pH 6.8 (final concentration, 125 mM) were added to the sample, and the sample was heated in a boiling water bath for 5 min. The beads were collected by filtration and washed repeatedly with a solution containing 3% (w/v) SDS, 10% (v/v) glycerol, and 125 mM Tris-HCl at pH 6.8. Finally the beads were resuspended in a solution containing 3% (w/v) SDS, 10% (v/v) glycerol, 125 mM Tris-HCl, and 5% (v/v) β-mercaptoethanol at pH 6.8 and heated in a boiling water bath for 5 min. After removing the beads by filtration, the resultant sample was subjected to electrophoresis and Western blotting analyses. The fluorescence photographs of the SDS gels containing the pulled out proteins were taken on top of a transilluminator (365 nm) through a 435 nm cutoff filter (CVI Laser Co, Albuquerque, NM) after the gels were washed four times with 10% (v/v) acetic acid for 15 min.

Two-dimensional Gel Electrophoresis—Twenty micrograms of PSD proteins and pulled out proteins were first precipitated by incubating with acetone at −20 °C overnight. Proteins were then pelleted by centrifugation at 18,000 × g for 30 min. The protein pellet was dissolved in the rehydration solution consisting of 5 µl Tris-HCl (pH range 3–10, Amersham Biosciences) or SDS-treated PSD (PSD in 3% (w/v) SDS and heated in a boiling water bath for 5 min) at 4 °C in the dark with gentle mixing. Two minutes later, the samples were exposed to UV light (365-nm wavelength light emitted from a transilluminator (TLW-20; UVP Inc., Upland, CA)) at a distance of 1 cm for different lengths of time while the sample was kept on ice. After the photolytic reaction, SDS (final concentration, 3% (w/v)) and Tris-HCl at pH 6.8 (final concentration, 125 mM) were added to the sample, and the sample was heated in a boiling water bath for 5 min. The beads were collected by filtration and washed repeatedly with a solution containing 3% (w/v) SDS, 10% (v/v) glycerol, and 125 mM Tris-HCl at pH 6.8. Finally the beads were resuspended in a solution containing 3% (w/v) SDS, 10% (v/v) glycerol, 125 mM Tris-HCl, and 5% (v/v) β-mercaptoethanol at pH 6.8 and heated in a boiling water bath for 5 min. After removing the beads by filtration, the resultant sample was subjected to electrophoresis and Western blotting analyses. The fluorescence photographs of the SDS gels containing the pulled out proteins were taken on top of a transilluminator (365 nm) through a 435 nm cutoff filter (CVI Laser Co, Albuquerque, NM) after the gels were washed four times with 10% (v/v) acetic acid for 15 min.

**Fig. 1. The synthesis and application of SAED-conjugated DADPA-agarose beads.** A, chemical structure of SAED and the reaction for conjugating SAED to DADPA-agarose beads. B, scheme for pulling out the surface proteins from a synthetic TPC with SAED-conjugated DADPA beads. The TPC consists of a bottom layer of actin, a middle layer of the primary antibody to actin (1Ab), and a top layer of the horseradish peroxidase-conjugated secondary antibody to the primary antibody (2Ab) on the surface of the glass coverslip, which has been modified with sulfo-GMBS. SAED-conjugated DADPA beads are incubated with TPC. After photolysis, the topmost layer proteins form covalent links with the beads. The proteins non-covalently bound to the beads are washed by a solution containing SDS. Afterward the proteins covalently bound to the beads are released by washes with a solution containing SDS and β-mercaptoethanol. C, scheme for pulling out the surface proteins from the PSD. The procedure is the same as that described in B, >, coumarin fluorophore; ⚫, surface PSD proteins; ⚫, interior PSD proteins. NHS ester, sulfo-N-hydroxysuccinimidyl ester.

Synthesis of Three-layered Protein Complex (TPC) on Glass Coverslips—Actin isolated from porcine skeletal muscle (36) was coated on the surface of sulfo-GMBS-coupled glass coverslips (37). Three-layered protein complex was made by incubating the resultant actin-coated glass coverslips with polyclonal goat anti-actin antibody (1:200 dilution in TTBS (0.05% Tween 20, 20 mM Tris-HCl, 50 mM NaCl, pH 7.4) for 1 h and then with horseradish peroxidase-conjugated donkey anti-goat IgG antibody (1:200 dilution in TTBS) for another hour at room temperature. To analyze the composition of proteins coated on the coverslips, each coverslip was covered with 100 µl of a solution containing 3% (w/v) SDS, 10% (v/v) glycerol, 125 mM...
Tris-HCl at pH 6.8, and 5% (v/v) β-mercaptoethanol. Ten minutes later, the solution was collected and then subjected to SDS-PAGE analysis. The procedure for isolating the proteins forming the top layer of TPC by DADPA beads is illustrated in Fig. 1B. Each coverslip coated with TPC was also mixed with 40 μl of the SAED-conjugated DADPA beads (wet volume) and 360 μl of TTBS. After being irradiated by UV light (365 nm) for 10 min, the beads were collected from the coverslip by extensive washing with a solution containing 3% (w/v) SDS, 10% (v/v) glycerol, and 125 mM Tris-HCl at pH 6.8 and then heated in a boiling water bath for 5 min. The proteins covalently bound to the collected beads were analyzed by the same procedures as described above.

**AFM Imaging of TPC—** Before AFM analysis, protein structures on coverslips were strengthened by treatment with 0.5 mM disuccinimidyl tartarate in a solution containing 10 mM phosphate at pH 7.4 for 30 min at room temperature. Coverslips were then rinsed with distilled water and air-dried. Scratches were made across the surface of coverslips by a steel surgical blade (Number 10; Feather Safety Razor LTD., Osaka, Japan). Images of the scratches were taken by an atomic force microscope (NS3a controller with D3100 stage; Digital Instruments, Santa Barbara, CA) operated in the tapping mode with silicon nitride cantilevers of 100 μm long with narrow arms (Digital Instruments). The depths of the scratches on coverslips were determined by NanoScope Version 5.12r5 software (Digital Instruments).

**RESULTS**

**Synthesis of SAED-conjugated DADPA Beads and Verification of Their Usefulness**— A novel methodology (as illustrated in Fig. 1) was developed in this study for isolating proteins residing on the surface of supramolecules. SAED is a cleavable, heterobifunctional cross-linking reagent (Fig. 1A). SAED contains a sulfo-N-hydroxysuccinimidy l ester that can form covalent links with free amines. SAED also contains an azido group of SAED can be activated by UV irradiation and then forms aryl nitrene inter-
mediates that can form covalent links with neighboring amino acid residues in a non-selective fashion (38). In addition, SAED contains a hydrolyzable disulfide bond in the middle. This compound has been used to study a variety of molecular interactions (39). In this study, SAED was conjugated to DADPA-agarose beads (average diameter, ~80 μm) via covalent links formed between its sulfo-N-hydroxysuccinimidyl ester and free amino groups on the beads (Fig. 1A). The distance between the bead surface and terminal azido group was estimated to be 3.5 nm by the RasMol Molecular Visualization Software (Version 2.5, enhanced by the MultiCHEM Facility, University of California, Berkeley, CA). When mixed with supramolecules, such as the TPC or the PSD isolated from porcine brain (as illustrated in Fig. 1, B and C), the azido groups on the SAED-conjugated DADPA beads were expected to be able to penetrate into supramolecules only in regions within 3.5 nm from the surface. Therefore, after photolysis, only the proteins residing within the reach of the azido groups, i.e. the superficial region of the supramolecules, could form covalent linkages to the beads. After washing away the non-covalent bound proteins from the beads by a solution containing SDS, the covalently bound proteins were released from the beads by washing with a solution containing SDS and β-mercaptoethanol. Upon the breakage of the disulfide bond, the coumarin fluorophore was transferred to those proteins that had been linked to the SAED-conjugated DADPA beads.

To test whether the SAED-conjugated DADAP-agarose beads would indeed work as expected, we synthesized a TPC consisting of a bottom layer of actin, a middle layer of the peroxidase-conjugated secondary antibody to the primary antibody on the surface of glass coverslips (Fig. 1B). AFM analyses indicated that the thickness of the TPC was 17.22 ± 1.21 nm (n = 4) (Fig. 2, A and B). The thickness of a complex containing only the middle and bottom layers was 10.58 ± 1.22 nm (n = 5), and the thickness of the bottom layer attached to the glass surface was 5.71 ± 1.70 nm (n = 3). The thickness of the GMBS coating was 1.71 ± 0.19 nm (n = 6). Thus, the thickness of the bottom, middle, and top layers was calculated to be 4 ± 1.71, 4.87 ± 2.09, and 6.64 ± 1.72 nm, respectively. These results suggest that the TPC consisted of three single layers of actin, primary antibody, and peroxidase-conjugated secondary antibody, respectively, from bottom to top. SDS-PAGE analyses also indicated that the major protein washed out of a two-layered complex was the primary antibody and that the major proteins washed out of a three-layered complex were the primary antibody and peroxidase-conjugated secondary antibody (Fig. 2C, left panel, left and right lanes). Weak bands of ~45 kDa in these gels (Fig. 2C, left panel) indicated that trace amounts of actin were also washed out from these complexes.

SAED-conjugated DADPA beads were applied onto the coverslips containing the TPC, and the mixture was then subjected to UV irradiation. Afterward the proteins covalently bound to the beads were collected by the procedure as illustrated in Fig. 1B. SDS-PAGE analysis indicated that the major protein pulled out by the beads was the one that constituted the top layer of the TPC, the peroxidase-conjugated secondary antibody (Fig. 2C). The middle layer protein of the TPC was not detected in the pulled out sample. Under UV light, the major protein band in the pulled out sample was strongly fluorescent (Fig. 2C). These observations indicated that the SAED-conjugated DADPA-agarose beads indeed could be used to selectively pull out the proteins lining the
FIG. 4. Interactions of PSD proteins with free SAED and SAED conjugated to DADPA beads. A, free SAED non-discriminatively labels various proteins in the PSD. Upper panel, PSD (1 mg of protein/ml) was incubated with 2 mM SAED (whose sulfo-N-hydroxysuccinimidyl ester group was inactivated by treatment with 1 M Tris-HCl at pH 8.5 at 4 °C for 1 h). After exposure to UV light for 10 min, 20 μg of the resultant sample was treated with 5% β-mercaptoethanol and then subjected to 9% SDS-PAGE analysis. After being rinsed four times with 10% (v/v) acetic acid, photographs of the resultant gel were taken on a UV transilluminator (right). The same gel was rinsed extensively with water and then stained with Coomassie Blue (left). Lower panel, the intensities of various bands in Coomassie Blue-stained gel (S) exhibit a linear relationship (correlation coefficient of 0.918) with their fluorescence intensities in the fluorograph (F) (both intensities are in arbitrary units). B, SDS-PAGE analyses of the PSD proteins and the proteins pulled out from the PSD by SAED-conjugated beads. The PSD (1 mg of protein) was incubated with SAED-conjugated beads (50-μl wet volume). After exposure to UV irradiation for 10 min, the beads were first washed with a solution containing SDS (3%) and then with a solution containing SDS (3%) and 5% β-mercaptoethanol. Twenty micrograms of the proteins in the last wash (lane 1) and 20 μg of the original PSD (PSD) were subjected to SDS-PAGE analysis and then stained with Coomassie Blue. Lane 2 is the photograph of lane 1 taken before the Coomassie Blue staining step on top of a UV transilluminator. C, SDS-PAGE analysis of the proteins pulled out by SAED-conjugated beads from PSD pretreated with SDS. The PSD (1 mg of protein) was first incubated with SDS (3% (w/v)) in a boiling water bath for 5 min and then incubated with SAED-conjugated beads (50-μl wet volume). After exposure to UV irradiation for 10 min, the beads were first washed with a solution containing SDS (3%) and then with a solution containing SDS (3%) and 5% β-mercaptoethanol. Twenty micrograms of the proteins in the last wash (lane 1) and 20 μg of the original PSD (PSD) were subjected to SDS-PAGE analysis and then stained with Coomassie Blue. Lane 2 is the photograph of lane 1 taken before the Coomassie Blue staining step on top of a UV transilluminator. D, SDS-PAGE analyses of the proteins pulled out from the PSD by SAED-conjugated beads after being exposed to UV light for varied durations. The PSD (20 μg of protein) and the proteins (20 μg) pulled out by 50 μl of SAED-conjugated beads from 1 mg of PSD proteins after being exposed to UV light for 3 (lane 1) and 25 (lane 2) min were subjected to SDS-PAGE analysis and then stained with Coomassie Blue. Lanes 3 and 4 are the photographs of lanes 1 and 2, respectively, taken before the Coomassie Blue staining step on top of a UV transilluminator. E, percentages of the proteins being pulled out by varied amounts of SAED-conjugated beads from original PSD (●) and SDS-treated PSD (●) under conditions described in B and C, respectively. Data are the means ± S.D. of three experiments. F, percentages of the amount of the proteins being pulled out by SAED-conjugated beads after exposure to UV light for varied lengths of time under conditions as described in D. Data are the means ± S.D. of three experiments.
surface of supramolecules with concomitant transfer of the coumarin fluorophore of SAED to these proteins.

Characterization of the PSDs Isolated from Porcine Cerebral Cortices—Electron microscopy was used to characterize the morphology of the PSD isolated from porcine brain. Under scanning electron microscopy, the PSDs appeared as porous, disklike objects with irregular contours (Fig. 3A). If the size of the PSDs is represented by the diameter of a circular disk with a surface area equivalent to the PSD, the average diameter of the isolated PSDs can be calculated to be 265.3 ± 100.6 nm (mean ± S.D., n = 343, between 100 and 650 nm) (Fig. 3B), similar to those reported in other studies (27, 40). Transmission electron microscopic analyses of the ultrathin sections of PSD samples indicated the average thickness of the PSD to be 41.1 ± 6.0 nm (mean ± S.D., n = 50) (Fig. 3C and D), also conforming to the reported thickness of the PSD (41–43).

The isolated PSD samples were then characterized biochemically by Western blotting. It was found that PSD-95, a synaptic scaffold protein and a core component of the PSD (44), was greatly enriched in the PSD fraction, and the content of PSD-95 in the PSD fraction was much higher than those in the other subcellular fractions, including the crude synaptosome, synaptic plasma membrane, synaptic junction, microsome, cytosol, mitochondria, and myelin sheath, that were also isolated from porcine cerebral cortices (Fig. 3E). NR1, an NMDA receptor subunit also enriched at excitatory postsynaptic terminals (45), was greatly enriched in the synaptic junction and PSD fractions (Fig. 3E). N-cadherin, a cell adhesion protein found at synapses (46), and its cytoplasmic binding partner, β-catenin, were also both enriched in the PSD fraction (Fig. 3E). On the other hand, synaptophysin, a marker for presynaptic terminals, in the PSD fraction was below the...
detection limit for our Western blotting analysis (Fig. 3E). Syntaxin, another protein enriched in presynaptic terminals, in the PSD fraction was only barely detectable (Fig. 3E). These results indicated that the PSD fraction used in this study was primarily of postsynaptic origin and largely free of the contamination from presynaptic terminals.

**Labeling PSD Proteins with Free SAED or with SEAD Bound to DADPA-Agarose Beads**—We then examined whether SAED by itself could penetrate into the PSD structure. We also tested whether or not free and bead-bound SAED could interact and label various PSD constituent proteins non-discriminatively. When the PSD was incubated with free SAED, exposed to UV light, and then treated with β-mercaptoethanol, the coumarin fluorophore of SAED could be transferred to various PSD proteins almost equally well (Fig. 4A), indicating that free SAED could interact with various PSD constituent proteins irrespective of their localization in the PSD or their biochemical nature. When SAED was conjugated to DADPA beads, it could still pull down and label all the various PSD proteins non-discriminatively if the PSD structure had been disrupted by prior treatment with SDS (Fig. 4C), and the amount of proteins being pulled down from the same amount of PSD increased almost linearly as the amount of SAED-conjugated beads increased (Fig. 4E). In contrast, when PSD with an intact structure was used in the same experiments, the proteins being pulled down from the PSD exhibited a pattern distinctly different from that of the original PSD (Fig. 4B), and there appeared to be a maximum amount of proteins that could be pulled out from the same amount of PSD when the amount of SAED beads used in the experiment was increased (Fig. 4E). It was further found that lengthening of the UV exposure time from 3 to 25 min did not noticeably affect the amount or species of proteins being pulled out of the PSD (Fig. 4, D and F).

**Characterization of the Proteins Pulled Out from the PSD by SAED-conjugated DADPA-Agarose Beads**—The proteins being pulled out from the PSD by SAED-conjugated DADPA beads and the proteins of the original PSD were then subjected to SDS-PAGE analysis. Densitometric scans of the resultant SDS gels after Coomassie Blue staining (Fig. 5A) indicated that the majority of the more abundant and discernible protein bands (11 out 16 protein bands) of the PSD exhibited decreases or increases in their abundances after being pulled out by SAED-conjugated beads. The abundances of proteins with sizes of 75, 66, 64, 45, and 38 kDa in the pulled out sample were higher than those of their counterparts in the PSD (Fig. 5A), and the abundances of proteins with sizes of 280, 230, 200, 58, 56, and 31 kDa in the pulled out sample were lower than those of their counterparts in the PSD. There was no significant difference between the abundances of the proteins with sizes of 114, 107, 80, 70, and 50 kDa in these two samples.

Western blotting analyses of the proteins pulled out from the PSD by SAED-conjugated beads and the original PSD proteins with 16 antibodies to PSD proteins also indicated diverse changes in the abundances of these proteins in these two samples (Fig. 5B). The ratios of the intensities of immunostained bands in the pulled out sample to those of their counterparts in the original PSD, called the exterior (E)/total (T) ratios here, were calculated and are listed to the right in Fig. 5B. The antibody to NR1 used here recognized two bands with the sizes of 120 and 116 kDa, and their E/T ratios were 1.17 ± 0.27 and 1.84 ± 0.46, respectively. The E/T ratio calculated from the sums of the intensities of these two immunostained bands in the pulled out sample and PSD are shown in Fig. 5B.

The proteins pulled out from the PSD by SAED-conjugated beads and the original PSD proteins were also subjected to two-dimensional gel electrophoresis, and the resultant gel was silver-stained (Fig. 6, A and B). To illustrate the differences between the protein patterns of these gels, we overlaid these two gels and colored the proteins of the pulled out...
Protein Organization of the Postsynaptic Density

TABLE I
PSD proteins identified by MALDI-TOF MS

<table>
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<tr>
<th>Spot no.</th>
<th>Protein identitya</th>
<th>NCBI accession no.</th>
<th>Molecular mass Nominalb</th>
<th>Measuredc</th>
<th>Reported</th>
<th>Number of matched/searched mass values</th>
<th>Sequence coverage</th>
<th>Error</th>
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<td>1</td>
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<td>285.150</td>
<td>232.1</td>
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<td>29</td>
<td>128</td>
</tr>
<tr>
<td>2</td>
<td>Neurofilament heavy subunit (NF-H)</td>
<td>gi</td>
<td>453250</td>
<td>113.367</td>
<td>210.1</td>
<td>200 (15); 210 (72)</td>
<td>15/21</td>
<td>12</td>
<td>17</td>
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<tr>
<td>3</td>
<td>Neurofilament medium subunit (NF-M)</td>
<td>gi</td>
<td>7428714</td>
<td>60.003</td>
<td>181.1</td>
<td>150 (15); 160 (72)</td>
<td>22/30</td>
<td>43</td>
<td>32</td>
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<td>4</td>
<td>Dynamin-1</td>
<td>gi</td>
<td>76630621</td>
<td>95.536</td>
<td>99.8</td>
<td>100 (15, 73)</td>
<td>21/27</td>
<td>27</td>
<td>107</td>
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<tr>
<td>5</td>
<td>Dynamin-1</td>
<td>gi</td>
<td>76630621</td>
<td>95.536</td>
<td>99.8</td>
<td>100 (15, 73)</td>
<td>29/34</td>
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<td>6</td>
<td>NSF gi</td>
<td>gi</td>
<td>73965161</td>
<td>83.773</td>
<td>77.5</td>
<td>78 (15); 83 (74)</td>
<td>20/29</td>
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<tr>
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<td>NSF gi</td>
<td>gi</td>
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<td>83.773</td>
<td>77.5</td>
<td>78 (15); 83 (74)</td>
<td>22/31</td>
<td>28</td>
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<td>Heat shock 70-kDa protein 8 isoform 2</td>
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<td>62896815</td>
<td>53.580</td>
<td>67.3</td>
<td>72 (68)</td>
<td>16/31</td>
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<td>9</td>
<td>Internexin, α isoform 1</td>
<td>gi</td>
<td>76654993</td>
<td>55.533</td>
<td>60.2</td>
<td>66 (58, 75)</td>
<td>32/42</td>
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<td>gi</td>
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<td>51.941</td>
<td>58.7</td>
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<td>12/29</td>
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<td>92930</td>
<td>50.361</td>
<td>53.5</td>
<td>53 (15); 56 (76)</td>
<td>22/43</td>
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<td>155</td>
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<td>gi</td>
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<td>45 (15); 46 (76)</td>
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<td>13</td>
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<td>gi</td>
<td>73998888</td>
<td>82.481</td>
<td>76.0</td>
<td>68 (15); 72 (68)</td>
<td>26/34</td>
<td>32</td>
<td>132</td>
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</table>

*a The spot numbers are those shown in Fig. 6C.
*b The protein identities were consistently obtained from protein spots with the same pl/MW mobility of two or three two-dimensional gels.
*c Nominal molecular masses were obtained from the NCBI database.
*d Measured molecular masses were calculated from protein mobility in Fig. 6C.

Sample in green and those of the original PSD in red (Fig. 6C). Thirteen well separated and more abundant protein spots (as indicated by numbers in Fig. 6C) were selected for more detailed analysis. Quantification of the abundances of these 13 protein spots indicated that they together accounted for 42.3% of the total protein in the same gel. The E/T ratios of these 13 proteins were listed in Table I. These protein spots were then excised from the gel, subjected to in-gel digestion, and analyzed by MALDI-TOF mass spectrometry. Using the results of peptide mass fingerprinting, these proteins were able to be identified (Table I). All of the proteins identified here had also been found in the PSD fraction in earlier studies (15–24).

Estimation of the E/T Ratios of PSD Proteins—The E/T values of proteins residing on the surface or in the interiors of the PSD or evenly distributed throughout the PSD were estimated by the following calculations. The PSD was assumed to be a disk-shaped object with a uniform thickness of 41 nm and to consist of spherical proteins of 5 nm in diameter, the average size of PSD constituent proteins (47). For a sample containing n PSDs, the E/T ratio of a particular protein a was equal to

$$E/T_a = \frac{n_a \sum_{j=1}^{n} V_j}{N_a \sum_{j=1}^{n} V_j}$$

(Eq. 1)

where \(n_a\) and \(N_a\) were the numbers of protein a in the pulled out sample and the original PSD sample, respectively, and \(V_j\) were the volumes occupied by the surface proteins of a PSD and the total volumes of a PSD, respectively. Because

the PSDs are sheetlike objects with similar thickness (~41 nm) and irregular contours (with the surface area A and contour length C), the total volume of PSDs and the total volume occupied by surface proteins could be calculated, respectively, as

$$V_{total} = \sum_{j=1}^{n} V_j$$

and

$$V_{surface} = \frac{\sum_{j=1}^{n} (2A_j \times 5 + C(41 - 5 \times 2) \times 5)}{\sum_{j=1}^{n} 10A_j + 155C_j}$$

(Eq. 3)

Therefore, the E/T ratio of the protein a is

$$E/T_a = \frac{n_a \sum_{j=1}^{n} 10A_j + 155C_j}{N_a \sum_{j=1}^{n} 41A_j}$$

(Eq. 4)

The A and C values of a total of 343 individual PSDs were estimated by the procedures described under “Experimental Procedures.” For a protein residing exclusively on the cytoplasm-facing and/or the postsynaptic membrane of the PSD, \(n_a = N_a\), and its E/T ratio was estimated as 3.2. For a protein evenly distributed in the PSD, its E/T ratio was estimated as 1. For an interior protein not residing on the cytoplasm- or membrane-facing surface, when the SAED bound to agarose beads could still approach them from the exposed lateral
Protein Organization of the Postsynaptic Density

Here we describe a methodology that was designed to help us determine the interior/exterior localization of various proteins in supramolecules. This methodology involves using agarose beads carrying a photoreactive, cleavable, bifunctional chemical cross-linker on the surface to selectively pull out the proteins making up the topmost layers of supramolecules. Whether a protein resides on the surface or in the interiors of the supramolecule can then be determined on the basis of the ratio of the abundance of this protein in the sample collected from the beads to its original abundance in the supramolecule. Using a synthetic protein complex consisting of three layers of proteins, this methodology was demonstrated to work as designed. Finally, this methodology was

\[
\text{E/T ratio} = \left( \sum_{i=1}^{n} \frac{C_i}{A_i} \right) \times \left( \frac{N_o}{\sum_{i=1}^{n} 10A_i + 155C_i} \right)
\]

By substituting the values of each \( A_i \) and \( C_i \) of all PSD, obtained by electron micrographs, The E/T ratio was estimated to be 0.29. For a protein residing exclusively in the interior of the PSD, i.e. does not reside on the cytoplasmic or membrane surface or on the lateral surface of the PSD, its E/T ratio should be 0.

**Prediction of the Interior/Exterior Locations of Various PSD Proteins by Their E/T Ratios**—By Western blotting, the E/T ratios of 16 proteins in the PSD were determined (Fig. 5B), and by two-dimensional gel electrophoresis analysis, the E/T ratios of 11 proteins were determined (Table I). The E/T ratios of three PSD proteins, spectrin, actin, and \( \beta \)-tubulin, were determined by both methods. The E/T ratios of spectrin and actin determined by Western blotting (0.47 ± 0.18 and 2.63 ± 0.17, respectively) matched rather well with those determined by two-dimensional gel electrophoresis (0.50 ± 0.13 and 2.40 ± 0.84, respectively). The different E/T ratios of \( \beta \)-tubulin determined by Western blotting and two-dimensional gel electrophoresis (0.2 ± 0.04 and 0.6 ± 0.06, respectively), however, indicated that the E/T ratio of a protein might vary when different methods were used. This difference in the E/T ratio of \( \beta \)-tubulin may be due in part to the finding that the protein spots identified as \( \beta \)-tubulin in two-dimensional gels were rather large (Fig. 6) and likely to contain other minor proteins. Because sums of the intensities of these contaminating proteins and \( \beta \)-tubulin were used to calculate the E/T ratio for \( \beta \)-tubulin, the E/T ratio thus determined may not be as accurate as that determined by Western blotting. Nevertheless the E/T ratios of various PSD proteins, disregarding how they were determined, all fell within the range between that predicted for exclusively superficial ones, 3.2, and that predicted for exclusively interior ones, 0.

The E/T ratios of AMPA receptor subunits GluR1 and GluR2/3, kainate receptor subunit GluR6/7, N-cadherin, \( \beta \)-catenin, cDIC, heat shock 70-kDa protein 8 isoform 2 (a member of hsc70 (48)), N-ethylmaleimide-sensitive factor (NSF) (a binding partner of GluR2 (49)), NMDA receptor subunit NR1, and actin fell between the predicted ratio for exclusively superficial ones, 3.2, and that predicted for evenly distributed ones, 1, hence suggesting their more superficial localizations in the PSD. It was further found that the E/T ratios of N-cadherin and its intracellular binding partner \( \beta \)-catenin (Fig. 5B) were not significantly different from the E/T ratios of these proteins determined in the presence of 3.5 mM EDTA, a condition under which the homophilic interactions between cadherin molecules would be interrupted (50). The E/T ratios of cDHc, MAP2A/2B, spectrin, PSD-95, MAP2C/2D, neurofilament heavy subunit, neurofilament medium subunit, heat shock 70-kDa protein 12A (a member of hsp70 (51)), \( \alpha \)-internexin, dynamin-1, \( \alpha \)-tubulin, and \( \beta \)-tubulin were between the E/T ratio predicted for evenly distributed proteins, 1, and that predicted for exclusively interior proteins, 0, hence suggesting their more interior localization in the PSD. The E/T ratios of \( \alpha \)-CaMKII and NMDA receptor subunits NR2a/2b were found to be around 1, suggesting their even distribution in the PSD or that they have a unique local protein organization.

**DISCUSSION**

Here we describe a methodology that was designed to help us determine the interior/exterior localization of various proteins in supramolecules. This methodology involves using agarose beads carrying a photoreactive, cleavable, bifunctional chemical cross-linker on the surface to selectively pull out the proteins making up the topmost layers of supramolecules. Whether a protein resides on the surface or in the interiors of the supramolecule can then be determined on the basis of the ratio of the abundance of this protein in the sample collected from the beads to its original abundance in the supramolecule. Using a synthetic protein complex consisting of three layers of proteins, this methodology was demonstrated to work as designed. Finally this methodology was
The PSD is assumed to be a disk-shaped protein complex of ~41 nm in thickness spanning from beneath the postsynaptic membrane to the cytoplasm of dendritic spines. A layer of phospholipid membrane is added to the PSD model to indicate the side of the PSD that faces the postsynaptic membrane. The cDIC, AMPA receptors, kainate receptors, N-cadherin, β-catenin, NSF, heat shock 70-kDa protein 8 (a member of hsc70), and actin reside primarily in the space within 3.5 nm from the surface, whereas the α,β-tubulin subunits, cDHHC, MAP2A/2B, MAP2C/2D, spectrin, neurofilament heavy subunit (NF-H), neurofilament medium subunit (NF-M), heat shock 70-kDa protein 12A (a member of hsp70), α-internexin, dynamin-1, and PSD-95 reside primarily in a region deeper than 3.5 nm from the surface. The results indicate that dynein intermediate chain, AMPA receptors, kainate receptors, N-cadherin, β-catenin, N-ethylmaleimide-sensitive factor, heat shock 70-kDa protein 8 isoform 2 (a member of hsc70), and actin reside primarily on the surface and that α,β-tubulin subunits, dynein heavy chain, MAP2A/2B, MAP2C/2D, spectrin, neurofilament heavy and medium subunits, heat shock 70-kDa protein 12A (a member of hsp70), α-internexin, dynamin-1, and PSD-95 occupy a space deeper than ~3.5 nm from the surface of the PSD. Our prediction that PSD-95 and actin, respectively, reside in the interior and on the surface of the PSD agree with previous findings obtained by using immunogold labeling and surface iodination methods (25–27). The E/T ratio of α-CaMKII was 1.06 ±0.02, indicating that α-CaMKII subunits are distributed evenly in the PSD. However, the α-CaMKII pulled out from the PSD exhibited very weak fluorescence (Fig. 4, B and D), indicating that the majority of this pulled out protein did not form covalent linkages with the SAED-conjugated beads directly. To account for these observations, it is hypothesized that the α-CaMKII subunits assemble into large complexes that reside on the edge of the PSD as they do in the model proposed by Petersen et al. (27). With this spatial arrangement, only a small portion of the subunits of each of these complexes could be reached by and thus link to SAED-conjugated beads. Because it is well known that binding interactions among α-CaMKII subunits is exceptionally strong (40), it is further hypothesized that the small portion of subunits that are themselves linked to the beads are able to bring down some of the remaining subunits in the same complexes and subsequently washes. The balance of these two effects will lead to an E/T ratio of ~1. Nevertheless we could not completely rule out the possibility that the presence of small α-CaMKII-enriched aggregates of ~100 nm in diameter in the PSD sample (40) may also influence the E/T ratio of α-CaMKII subunits obtained here. The E/T ratio of NMDA receptor subunit NR2a/2b was ~1, also indicating an even distribution in the PSD. On the other hand, the E/T ratio of NR1, another NMDA receptor subunit, was 1.41, suggesting a more superficial localization. Because NMDA receptor subunits are integral membrane proteins (45), they are unlikely to reside in the interior of the PSD. An alternative possibility is that the NMDA receptors in the PSD may be surrounded by some large proteins, such as N-cadherin (52), ErbB4 (53–55), or neural ligands (55, 56), and these nearby large proteins may reduce the chance of the NR1 subunits (but less effectively than for the NR2 subunits in a NMDA receptor), to be reached by and to react with the SAED attached to agarose beads.

Based on the results obtained here together with those in earlier reports, we propose a model for the protein organization in the PSD (Fig. 8). Models for describing how scaffold proteins organize the various constituent proteins, how the signaling molecules associated with NMDA receptors are organized, and how the complexes of α-CaMKII subunits are localized in the PSD have been proposed in a number of earlier studies (26, 27, 55). The model shown in Fig. 8 is an extension of the above models by including the results obtained in this study, i.e. the information concerning the spatial

![Image](image-url)
localization of the five major and 19 minor constituent proteins in the PSD. These 24 PSD proteins together account for at least 40% of the total protein mass of the PSD. Therefore, this model may be regarded as a description of the molecular basis of the PSD architecture.

In our proposed model (Fig. 8), the PSD is a disk-shaped object that is ~41 nm thick and has a broad surface facing the postsynaptic membrane and another broad surface facing the cytoplasm of dendritic spines. Ratner and Mahler (25) have suggested that the actin in the PSD polymerizes into filaments lying on the surface of the PSD. This highly exposed arrangement is consistent with our observation of strong fluorescent labeling of pulled out actin protein. Actin filaments may be cross-linked by underlying spectrin into a cortex-like structure beneath the postsynaptic membrane, similar to the cortex structure underneath the plasma membrane of red blood cells (57). α-Internexin, a 66-kDa neurofilament, may bind to spectrin via protein 4.1 (58, 59). Actin filaments may also partially cover the PSD surface facing the cytoplasm, and these actin filaments could bind to α-CaMKII complexes (60) and the light chain of dynein via myosin V (61) that also lies on the same PSD surface. The CaMKII subunits could form large complexes that reside on the edge of the PSD facing the cytoplasm as suggested by Petersen et al. (27), and only a small portion of the subunits of each complex resides on the surface of the PSD. The cDHc in the core of the PSD may dimerize, and cDHc dimers may form complexes with cDIC and the light chain of dynein as they do in a cytoplasmic dynein complex (62). α,β-Tubulin dimers, cysteine-rich interactor of PDZ three (CRIPt), and MAP2 also seem to lie in the core of the PSD, and these proteins may form complexes with the cDHc dimers. NMDA receptors embedded in the postsynaptic membrane may be associated with the actin filaments via actinin (63), with PSD-95 protein (64), and with α,β-tubulin dimers (65). N-cadherin in the postsynaptic membrane may be associated with the actin filaments of the PSD via α-and β-catenins, which may in turn bind to the dynein complex (66). In the core, PSD-95 protein may also bind to intracellular signaling molecules including neuronal nitric-oxide synthase and guanylate kinase-associated protein, microtubule-associated protein CRIPt (26), and trans-synaptic signaling molecules such as neuroligin (55, 56) and ErbB4 (53–55). A possibility exists that the NMDA receptors are in close association with large surface proteins such as N-cadherin, whose extracellular domains could extend ~10–15 nm out of the postsynaptic membrane (67); ErbB4; or neuroligin. hsp70 and hsc70 have been identified in the PSD (68). Here our results further indicate that hsp70 and hsc70, respectively, reside in the interior and on the surface of the PSD. In the core of the PSD, hsp70 may interact with the α,β-tubulin dimers that are also enriched there (69).

Proteomic analyses of the PSD have revealed that this subcellular specialization consists of more than 400 different proteins (19–22). The present model (Fig. 8) describes the probable spatial arrangement of 35 proteins in the PSD. In the future, more efforts are needed not only to verify the arrangement of these 35 proteins in the PSD as proposed here but also to learn the organization and localization of the remaining constituent proteins of the PSD. The PSD model at the present stage nevertheless raises some interesting questions. These include the physiological role(s) of the α,β-tubulin subunits and various microtubule-binding proteins such as MAPs, dynein, dynamin, and CRIPt in the interior of the PSD when the PSD in neurons lies in the cytoplasm of dendritic spines that is largely devoid of microtubules. It will also be interesting to know something about the physical arrangement of the proteins surrounding NMDA receptors at postsynaptic membrane. Finally the roles of hsp70 and hsc70, which reside in the interior and on the surface of the PSD, respectively, at synapse remain to be investigated (70).

The methodology using SEAD-conjugated DADAP-agarose beads as reported here is a useful tool to pull out the superficial proteins from supramolecules in quantities large enough for carrying out more detailed biochemical analyses, such as Western blotting and proteomic analyses by two-dimensional gel electrophoresis followed by mass spectrometric analyses as demonstrated here. By replacing the SAED used in this method with other similar bifunctional cross-linkers with longer linker lengths, the usefulness of this method could be further extended to investigate the protein compositions at different depths from the surface of supramolecules. In the future, this method could also be used to help study the composition of the proteins lining the surface of organelles and cells under various manipulations.

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REFERENCES

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