INTERMOLECULAR DISULFIDE BONDS AT CENTRAL NERVOUS SYSTEM SYNAPTIC JUNCTIONS

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SUMMARY

Most proteins in isolated synaptic junctions and nearly all those in postsynaptic densities (the fibrous protein matrix underlying the postsynaptic membrane at the synapse) are extensively cross-linked by disulfide bonds into polymers with a molecular weight of 350,000 or greater. Since the postsynaptic density appears to consist primarily of a matrix of cytoplasmic proteins, such as tubulin and neurofilament protein, our results indicate that at the membrane such proteins may use disulfide bonds to differentiate into the postsynaptic density and tie into the postsynaptic membrane.

INTRODUCTION

It has been shown in a number of cell types that certain fibrous proteins, such as microtubules and microfilaments, are present in the cytoplasm in close proximity to the plasma membrane. Moreover, microtubules and microfilaments appear to play a direct role in regulating the lateral mobility of integral membrane proteins through non-covalent or covalent interactions to one another or to the membrane (1). A particularly interesting and unusual example of this is found at synaptic junctions in the central nervous system where a matrix of fibrous proteins lies immediately beneath the plasma membrane (2,3,4). Recent evidence indicates that this matrix, called the postsynaptic density, contains appreciable quantities of two proteins which appear to be similar to tubulin (5,6) and the major neurofilament protein (7). At present, however, little is known about the nature of the interactions between cytoplasmic fibrous proteins at the membrane or between such fibrous proteins and membrane proteins.

In this communication we present evidence that specific polypeptides in isolated postsynaptic densities and synaptic junctions are cross-linked by intermolecular disulfide bonds. Our data suggest that interprotein disulfide

bonds may be essential in determining synaptic structure and thereby regulating function. Disulfide bond reduction and oxidation has been implicated in regulating transmitter release (8) and appears to modify receptor mediated permeability changes at the postsynaptic membrane (9).

MATERIALS AND METHODS

Materials: 5,5'-dithio *bis* [2-nitrobenzoic acid] (DTNB) was obtained from Sigma Chemical Co. Reagents used in electrophoresis were purchased from Eastman Kodak Co. Sodium dodecyl sulfate (SDS) was obtained from BDH Chemicals Ltd. N-ethylmaleimide was purchased from Cal Biochem. All other chemicals were of reagent grade.

Subcellular Fractionation: Sprague-Dawley male rats (50-100 days of age) purchased from Simonsen Laboratories (Gilroy, CA.) were used in these experiments. A purified synaptic junction fraction was prepared according to the method of Cotman and Taylor (3), except that the junction fraction was obtained by centrifugation of the Triton-treated synaptic plasma membrane fraction through 1.0 M sucrose. The isolation of postsynaptic densities was carried out as previously described (10) by treatment of synaptic plasma membranes with 3.9% (w/v) sodium-lauryl sarcosinate.

Protein Solubilization and Polyacrylamide Gel Electrophoresis: Postsynaptic and synaptic junction fractions frozen at -70°C or prepared fresh produced indistinguishable results. Blockage of free sulfhydryl groups with N-ethylmaleimide was conducted as described by Peretz et al. (11) with minor modifications. Isolated fractions at 1-2 mg protein/ml were made 10 mM Bicine (pH 7.5) and 20 mM N-ethylmaleimide. Samples were incubated for 30 min at 37°C and centrifuged for 5 min at 10,000 g. The resulting pellet was then solubilized in one-tenth the volume of 10% (w/v) SDS. At this point protein determinations were carried out by the method of Lowry et al. (12) and then 2-mercaptoethanol was added to appropriate samples to a final concentration of 5% (v/v).

The discontinuous SDS-buffer system of Laemmli (13) was used. Exponential gradient slab-gels were cast as previously described (14). Following electrophoresis, gels were fixed and stained with Coomassie blue as described elsewhere (14).

Sulfhydryl Titrations: The reaction of DTNB with -SH groups was carried out according to the method of Ellman (15). The assay mixture contained 50 µg of synaptic junction or postsynaptic density protein, 1.4 mM DTNB and 0.1 M Tris HCl, pH 7.5, in a final volume of 0.2 ml. The reaction was carried out at 22°C and its change in absorbance at 412 nm was monitored spectrophotometrically. All values were corrected for background by subtraction of control values from reaction mixtures not containing density or junction proteins.

RESULTS

Postsynaptic density fractions solubilized in SDS and not reduced with mercaptoethanol produced strikingly altered electrophoretic patterns when compared to completely reduced samples (compare Fig. 1a and 1b). The major

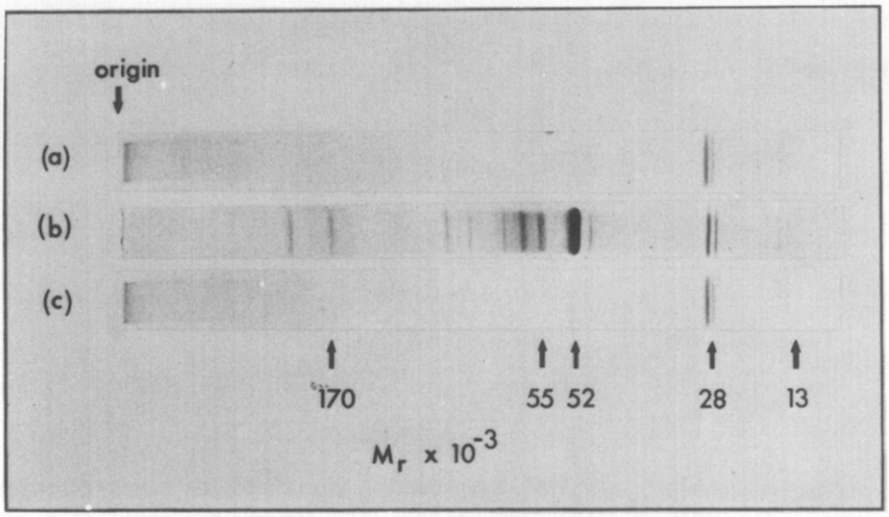

Figure 1. Polyacrylamide slab gel electrophoresis of postsynaptic density fractions (50 µg each) treated with (a) 2.3% (w/v) SDS only (b) 2.3% (w/v) SDS plus 5% (v/v) 2-mercaptoethanol and (c) postsynaptic density fractions treated with 20 mM N-ethylmaleimide prior to solubilization in 2.3% SDS. Gels were 8%–20% exponential-linear gradients.

density polypeptide (molecular weight [M_r] ~ 52,000) was completely absent from electrophoretic patterns of non-reduced samples. The second most prominent polypeptide (M_r ~ 55,000) was also absent from samples electrophoresed without reduction. The dependence on reduction of these major polypeptides appears quite specific, since without reduction the 26,000 and 28,000 M_r components entered the gel in the same quantities and with the same apparent M_r as in completely reduced and SDS-solubilized samples.

It seemed possible that random intermolecular disulfide bond formation following solubilization in SDS could be responsible for the major postsynaptic density polypeptides not entering the gels in the absence of reduction. To test this possibility the postsynaptic density fraction was treated with N-ethylmaleimide, which forms a stable thio-ether with free sulfhydryl groups at molar concentrations exceeding the half-cystine content of these fractions (16). As shown in Fig. 1c, the electrophoretic patterns were indistinguishable with or without N-ethylmaleimide treatment. Therefore, random disulfide bond

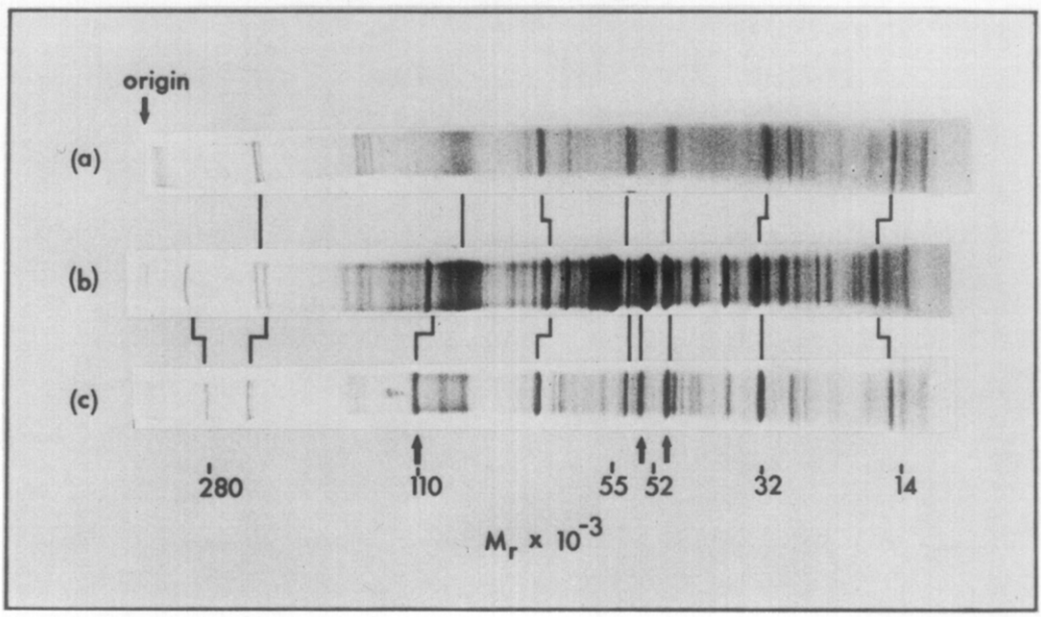

Figure 2. Electrophoresis of synaptic junction proteins (60 μg each) treated with (a) SDS only (b) SDS plus 2-mercaptoethanol and (c) N-ethylmaleimide prior to SDS solubilization, as in Figure 1. The bold lines between (a), (b) and (c) connect bands of equal apparent M_r.

formation following SDS solubilization does not appear to contribute to the creation of non-native interprotein aggregates.

Since the resolving gels used in these experiments were 8% (w/v) acrylamide at the top, proteins or protein aggregates excluded from the gel under SDS denaturing conditions must have an apparent M_r greater than 300,000-350,000. Furthermore, most of these protein aggregates did not enter the 4.5% (w/v) acrylamide stacking gel, which implies an apparent $M_r \geq 1{,}000{,}000$ (not shown). The dependence on reduction for individual protein components to enter the gels appeared to be an all or none phenomenon, since a polypeptide was either present in quantities indistinguishable from completely reduced samples or was absent altogether.

In addition the vast majority of proteins in synaptic junctions was coupled via disulfide bridges into large aggregates or was readily induced to form such cross-linked aggregates. The majority of polypeptides was cross-linked in samples solubilized in SDS in the absence of reduction (Fig. 2a vs. 2b). This

observation is consistent with the finding that junction polypeptides contain substantial quantities of -SH groups (approximately 4 x 10^6/mg protein as determined by titration with DTNB), which may associate via oxidation to form covalent linkages between synaptic proteins. In particular, the 52,000 and 55,000 bands did not enter the gel as expected from the fact these components presumably belonged to the postsynaptic density. Other bands were also excluded, suggesting that membrane proteins may be covalently coupled as well. The exclusion of junctional proteins reflected, in part, a cross-linking of particular proteins after isolation, since samples treated with N-ethylmaleimide prior to solubilization in SDS exhibited 3 major bands not present in non-reduced samples (Fig. 2c, arrows). Neither the 52,000 or the 55,000 band was affected by N-ethylmaleimide. This finding indicates that the cross-linking was selective. Taken together, these results suggest that junction proteins have an intrinsic ability to form disulfide bridges and that this is selective to particular proteins.

DISCUSSION

These data illustrate that all polypeptides in isolated postsynaptic densities, except 2 bands with M_r of 26,000 and 28,000, are cross-linked by disulfide bonds into supramolecular aggregates. The aggregates have an apparent M_r of 350,000 or greater. In addition most, but not all, polypeptides in isolated junctions can be cross-linked by disulfide bonds. These data indicate that most polypeptides of these complexes possess a capacity to develop extensive supramolecular lattices via disulfide bond formation. This property may help in providing the synapse with a structural rigidity required to maintain a strong connection between nerve cells.

The extensive intermolecular disulfide bond formation is not general to all membranes but appears, in part, unique to the synaptic membrane complex. We found that myelin membranes from brain, for example, displayed no intermolecular disulfide bond formation. Also, it is known that proteins in erythrocyte membranes are not cross-linked by disulfide bridges, since the

electrophoretic patterns are not affected by reducing agents (17). Moreover, intermolecular disulfide bonds are not formed when these membranes are solubilized in SDS (17).

We would suggest that disulfide bond formation may play a critical role in interconnecting fibrous cytoplasmic proteins at the plasma membrane. In the electron microscope microtubules and neurofilaments appear, in some cases, juxtaposed and actually interwoven into the postsynaptic density (18). It may be that tubulin, neurofilament protein and perhaps other fibrous proteins use intermolecular disulfide bonds to differentiate into the postsynaptic density matrix and tie into the membrane. In this way such proteins may be directly linked to the membrane. To our knowledge our results are the first to indicate that certain fibrous proteins juxtaposed to the membrane are extensively cross-linked via sulfhydryl bridges.

Recent studies suggest that the state of protein polymers in certain cells may be regulated, in part, by the *in vivo* thio-disulfide status of the cells (19,20). Transhydrogenases exist which catalyze the exchange of hydrogens between sulfhydryls and disulfides in proteins (21), and it has been suggested that tubulin can participate in such reactions (22). Moreover, Mellon and Rebhun (20) have shown that *in vitro* tubulin polymerization depends on its free sulfhydryl content. Although interprotein disulfide bonds do not appear to be created during microtubule polymerization, they may be the necessary prerequisite in the formation of a fibrous matrix such as the post-synaptic density. Tubulin has 8-10 sulfhydryl groups (20) and a portion of these may be used to form a stable covalent matrix. However, at present we can only speculate on the status of synaptic sulfhydryl groups *in vivo:* this would depend on the *in vivo* organization of reactive groups, the location of appropriate enzymes and cellular metabolism. Recent studies have shown that the reduction/oxidation state of protein thiol and disulfide groups may be directly coupled to cellular metabolism through the enzymatic processes that interconvert reduced and oxidized glutathione (19). This raises the intriguing

possibility that certain proteins of central nervous synapses may, through their sulfhydryl groups, respond to specific changes in neuronal metabolism. Kosower (23) has argued that changes in disulfide bond formation at synapses may alter the efficiency of transmitter release during learning and memory. This notion takes on a new perspective in view of the possibility that post-synaptic structures may encode through altered states of polymerization the previous metabolic history of the neuron. This could, for example, result in a larger and therefore more efficient synapse.

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