Detection by chemical cross-linking of bovine brain synapsin I self-association

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Synapsin I is believed to play an important role in the regulation of neurotransmitter release, since it is able to bind to synaptic vesicles, to the cytoskeleton and to membrane proteins; in addition, it bundles F-actin and microtubules. These properties, which are controlled by phosphorylation, could be explained if synapsin has different and multiple binding sites or if synapsin I is able to form polymers by self-association. In this study we present experimental evidence that synapsin I at low concentration forms self-associated dimers, as revealed after mild treatments with cross-linking agents. We have especially studied here the effects of copper/*o*-phenanthroline, a zero-length cross-linking agent which forms covalent links by oxidative formation of S–S bridges between adjacent cysteines. The time course and concentration-dependence of synapsin-dimer formation are studied; interestingly, these experiments could suggest a different behaviour of the two polypeptides. Limited proteolysis of phosphorylated synapsin I by V8 protease, α -chymotrypsin or collagenase, performed on the isolated dimer and monomer, allows us to localize tentatively in the central hydrophobic core of the molecule the cysteine residues the oxidation of which by copper/*o*-phenanthroline gives rise to synapsin dimers.

INTRODUCTION

Since its discovery by Johnson *et al.* (1972) as an endogenous substrate for protein kinases, synapsin I has been extensively studied (for a review see Nestler & Greengard, 1986). As reported then, synapsin I consists of two closely related basic proteins, synapsin Ia and synapsin Ib, which are mainly associated with synaptic vesicles. Synapsin I consists of two domains: a *N*-terminal collagenase-resistant domain and a proline-rich *C*-terminal domain susceptible to degradation by collagenase. Its primary structure has been deduced from rat brain cDNA (McCaffery & De Gennaro, 1986) and recently partially modified (Czernik *et al.*, 1987). In addition, synapsin I can be phosphorylated either by cyclic-AMP-dependent protein kinase and Ca^{2+} /calmodulin-dependent protein kinase I at site 1 in the *N*-terminal region of the molecule or by Ca^{2+} /calmodulin-dependent protein kinase II at sites 2 and 3 in the collagenase-sensitive region of the molecule.

Synapsin I is believed to play a major role in the regulation of neurotransmitter release. Previous studies have shown that synapsin I is able to bind to small synaptic vesicles (Schiebler *et al.*, 1986), and to brain spectrin (Baines & Bennett, 1985; Krebs *et al.*, 1987a) with high affinity, to associate with membrane proteins and neurofilaments (Steiner *et al.*, 1987a), and to bundle F-actin (Bähler & Greengard, 1987; Petrucci & Morrow, 1987) and microtubules (Baines & Bennett, 1986). All these interactions could be of physiological significance, since they are modulated by the phosphorylation state of the protein. As suggested by Baines & Bennett (1986), microtubule bundling (as well as actin bundling) implies either that synapsin has two or more binding sites for microtubules, or that synapsin I has one binding site and is able to induce bundling by self-association.

Cross-linking agents are interesting tools with which to study protein self-association, since they are able to induce covalent cross-linking between interacting molecules [for reviews, see Ji (1979) and Gaffney (1985)]. We have previously utilized these agents to study self-association of mitochondrial creatine kinase (Font *et al.*, 1987). In the present study we report evidence that purified synapsin I forms self-associated dimers which are covalently linked by cross-linking agents, especially Cu^{2+} /phenanthroline, in carefully controlled experimental conditions.

In addition, limited enzymic proteolysis of synapsin phosphorylated at site 1 allows us tentatively to localize the cysteines involved in synapsin cross-linking by Cu^{2+} /phenanthroline in the central hydrophobic region of the molecule.

MATERIALS AND METHODS

Synapsin I purification

Synapsin I is purified from frozen bovine brain by the method of Bennett *et al.* (1986). In our hands, this procedure, which consists of extraction of synapsin I from demyelinated membranes, followed by batch adsorption on CM-cellulose, gives better results than the CHAPS extraction method of Schiebler *et al.* (1986) or than the original acid-extraction procedure of Ueda & Greengard (1977). After elution of synapsin I from CM-cellulose and HA Ultrogel columns in line, fractions containing purified synapsin I are freeze-dried, dissolved in a small volume of 20 mM-Hepes, pH 7.4, dialysed against this buffer and stored at -80°C . All the purification steps except dialysis are performed in the presence of 2 mM- (extraction buffer) or 0.5 mM- (column buffer) dithiothreitol. About 7 mg of synapsin I is obtained from

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300–400 g (wet wt.) of bovine brain. The protein is 98 % pure, as estimated by SDS/polyacrylamide-gel electrophoresis (see Fig. 1, lane 4).

Phosphorylation of synapsin I

Synapsin I is phosphorylated on the cyclic-AMP-dependent site by the method of Schiebler *et al.* (1986) with slight modifications. Synapsin I at 0.1 mg/ml is incubated in 25 mM-NaPipes/0.1 M-NaCl/5 mM-MgCl₂/1 mM-EGTA, pH 7.2, with 24 units of cyclic-AMP-dependent protein kinase (catalytic subunit from bovine heart; Sigma), final volume 0.1 ml. Phosphorylation starts with the addition of [γ -³²P]ATP (27 μ M; about 1.5×10^6 c.p.m./nmol) and is performed at 30 °C for 10 min. At the end of the incubation period, the protein and the remaining labelled nucleotide are quickly separated by the centrifuge-column procedure of Penefsky (1977), with 1 ml columns filled with Sephadex G-50 previously equilibrated with 20 mM-Hepes, pH 7.4. In these conditions, the amount of phosphate bound to synapsin I is about 0.5 mol of ³²P/mol of synapsin I. Furthermore, phosphorylation takes place only in the 10–15 kDa N-terminal fragment of synapsin I obtained after proteolytic digestion of the phosphorylated protein by V8 protease [Huttner & Greengard (1979), and Fig. 4 below, autoradiography lanes 1–2].

Iodination of synapsin I

Synapsin is radioiodinated with Iodogen (from Pierce) as oxidizing agent; with this reagent tyrosine is considered to be the major site of substitution. Iodogen (60 nmol) dissolved in dichloromethane is added to polypropylene Microfuge tubes, and the solvent is evaporated to dryness; then synapsin (6.5 nmol in 20 mM-Hepes, pH 7.4) and Na¹²⁵I (20 nmol, about 10⁷ d.p.m.) are added and incubated for 10 min at room temperature. At the end of the incubation period, synapsin and the free NaI in excess are separated by the centrifuge-column procedure as described above.

Synapsin I cross-linking by Cu²⁺/phenanthroline

Unless mentioned elsewhere, purified synapsin I in 20 mM-Hepes is incubated at 0.5–1 mg/ml final concn. with 1 mM-CuSO₄ and 2 mM-*o*-phenanthroline for 30 min at 30 °C. In all the experiments described the molar ratio Cu²⁺/phenanthroline is 1:2. At the end of the incubation period, 5 mM-EDTA and 1.8 mM-N-ethylmaleimide are added and the samples are stored at 0 °C for 30 min. EDTA is added to impede further oxidation of thiol groups, since, as shown by Kobashi (1968), EDTA immediately stops the oxidation of thiol groups but does not reverse the effect of Cu²⁺/phenanthroline. N-Ethylmaleimide is added to block available free thiol groups and prevent disulphide exchange during storage of the sample. Before electrophoresis, a 5-fold volume of cold acetone is added to samples, which are then stored for 2 h at –20 °C and then centrifuged for 10 min at 15000 g. The supernatants are discarded and the pellets resuspended in sample buffer without β -mercaptoethanol.

Electrophoresis

SDS/polyacrylamide-gel electrophoresis is usually performed with the discontinuous system of Laemmli (1970), with a 3.5%-polyacrylamide stacking gel and either a 7.5 % or 12.5 % separating gel or a 4–12 % gradient of polyacrylamide. In some experiments, electrophoresis

is performed with the continuous system of Weber & Osborn (1969). After electrophoresis, gels are stained with Coomassie Blue and dried between two layers of cellophan, and autoradiography is performed for various times (3–8 days) at –70 °C with an intensifying screen. Densitometry of the stained gels is carried out with a Vernon densitometer.

Limited proteolysis of synapsin I

Enzymic proteolysis is performed by the original method of Cleveland *et al.* (1977), with slight modifications. Synapsin I dimers and monomers are separated by SDS/polyacrylamide-gel electrophoresis, and gels are stained and dried. The protein bands (about 25 μ g) corresponding to monomer or dimer are cut and soaked in 125 mM-Tris/HCl (pH 6.8)/0.1 % SDS, introduced in the bottom of a second slab gel and overlaid with 20 μ l of the same buffer containing 0.06 % Bromophenol Blue and either 10 μ g of V8 protease (Endoproteinase glu-C; Boehringer) or 10 μ g of α -chymotrypsin (type VII; tosyl-llysylchloromethane-treated; Sigma). When Bromophenol Blue reaches the bottom of the stacking gel, power is turned off for 30 min, and then the electrophoresis is carried out normally.

Collagenase treatment of synapsin fragments

Radioiodinated synapsin monomer (about 500 μ g) is hydrolysed by V8 protease (60 μ g) as described above. The 35/32 kDa doublet and the 28 kDa band are excised from the stained gel, and peptides are recovered by electroelution as described by Jacobs & Clad (1986). The electroeluted peptides are recovered by centrifugation (15000 g for 10 min) after precipitation with cold acetone, redissolved in 50 μ l of 40 mM-Tris/HCl (pH 7.4)/20 mM-CaCl₂ and incubated with or without 7 units of collagenase from *Clostridium histolyticum* (type VII; Sigma). After 1 h at 30 °C, samples are denatured and peptides are separated by electrophoresis.

Protein assay

This is routinely done by the method of Peterson (1977), with bovine serum albumin as standard.

RESULTS

Utilization of different cross-linkers to reveal synapsin I self-association

It has been reported (Baines & Bennett, 1986; Bähler & Greengard, 1987; Petrucci & Morrow, 1987) that some of the effects of synapsin I could be explained by synapsin I self-association. In the present experiments, we have tested the ability of various cross-linking agents to disclose the association of synapsin with itself. Results in Fig. 1 show that dimers of synapsin I appear in less than 15 min in the presence of 1 mM-Cu²⁺/phenanthroline (lane 5). This zero-length cross-linker is known to catalyse the formation of disulphide bonds from neighbouring thiol groups. In contrast, in the presence of other zero-length reagents such as 1-ethyl-3-(3-dimethylamino-propyl)carbodi-imide or N-cyclohexyl-N'-[β -(N-methylmorpholino)ethyl]carbodi-imide toluene-*p*-sulphonate at concentrations as high as 10 mM, synapsin polymers do not appear (results not shown). Bis-succinimidyl reagents such as ethylene glycol bis(succinimidyl succinate) (lane 2) or dithiobis(succinimidyl propionate) (lane 6) are also active in promoting formation of covalently linked

Fig. 1. Electrophoretic pattern of cross-linked synapsin I

Synapsin I (1 mg/ml) is incubated for 15 min at 30 °C in 20 mM-sodium phosphate, pH 7.2, with: (1) control; (2) 5 mM-ethylene glycol bis(succinimidyl succinate); (3) 0.03 % glutaraldehyde; (4) purified synapsin without incubation; (5) 1 mM- Cu^{2+} /phenanthroline; (6) 5 mM-dithiobis(succinimidyl propionate); (7) 5 mM-*N*-succinimidyl-6-(4'-azido-2'-nitrophenylamino)hexanoate incubated for 10 min in the dark and then photoactivated for 5 min under visible light (M, molecular-mass markers). Electrophoresis was in a 3–7.5%-polyacrylamide linear gradient gel as described by Weber & Osborn (1969).

synapsin I polymers (essentially dimers). The same results are obtained with the photoactivatable heterobifunctional reagent *N*-succinimidyl-6-(4'-azido-2'-nitrophenylamino)hexanoate (lane 7). In contrast, cross-linking with glutaraldehyde (lane 3) gives rise to several protein bands present at low concentration. In these experiments the continuous buffer system of Weber & Osborn (1969) is used, and the synapsin I doublet in control assays (lanes 1 and 4) migrates as a single polypeptide. Such behaviour has been reported by Krebs *et al.* (1987b) for human erythrocyte protein 4.1, which is immunologically related to synapsin I.

Covalent cross-linking of synapsin I self-associated dimers with Cu^{2+} /phenanthroline: effect of Cu^{2+} /phenanthroline concentration and influence of the incubation time

Results in Fig. 2 show that with a concentration of synapsin I of 10 μM (based on a molecular mass of 75 kDa) covalently linked dimers already appear in the presence of 0.025 mM- Cu^{2+} /phenanthroline. When the concentration of catalyst is raised, the amount of synapsin I dimers increases and reaches a plateau for Cu^{2+} concentration greater than 0.25 mM. In addition to synapsin Ia and Ib monomers and to synapsin dimers, another band with a slower migration than synapsin Ia appears at concentrations of Cu^{2+} greater than 0.025 mM. This band could correspond to synapsin I monomer with intramolecular disulphide bridge. As shown in Fig. 3, with a fixed Cu^{2+} /phenanthroline concentration of 1 mM, this additional band appears very rapidly (in less

[Cu^{2+} /Phen] (mM) ... 0.025 0.1 0.25 1

Fig. 2. Influence of Cu^{2+} /phenanthroline (Phen) concentration on the amount of synapsin I dimer

Lower part, electrophoretic pattern; upper part, percentage of protein in dimer, determined by densitometry. Synapsin I (1 mg/ml) is incubated for 15 min at 30 °C in 20 mM-Hepes, pH 7.4, with different concentrations of Cu^{2+} /phenanthroline. Electrophoresis is performed with a 4–12 % polyacrylamide gradient as described by Laemmli (1970).

than 30 s). The appearance of synapsin I dimers formed by intermolecular cross-linking is detected only after 1 min in our experimental conditions and reaches a maximum value after 5 min of incubation.

Another interesting point emerges from the results shown in Fig. 3: synapsin Ib, which is the most abundant form in our untreated preparation, as in preparations from other laboratories (see, e.g., Ueda & Greengard, 1977), becomes apparently the less abundant one after dimer formation in the presence of Cu^{2+} /phenanthroline (compare, e.g., control and time 5 min in Fig. 3).

Localization of thiol groups involved in synapsin I interaction as revealed by chemical cross-linking with Cu^{2+} /phenanthroline

In a further set of experiments, we have tried to localize the thiol groups whose oxidation by Cu^{2+} /phenanthroline stabilizes by disulphide bonds the self-associated synapsin dimers. Synapsin I is phosphorylated in the presence of [γ - ^{32}P]ATP and cross-linked by Cu^{2+} /phenanthroline; monomers and dimer are separated by SDS/polyacrylamide-gel electrophoresis, and then proteolytic digestion by V8 protease or α -chymotrypsin is conducted as described in the Materials and methods section. After electrophoresis, gels are stained by Coomassie Blue and autoradiographed.

Reproducible results are obtained from six experiments with three different synapsin preparations. After incubation with V8 protease, the main difference between synapsin I dimer (Fig. 4, lane 1) and synapsin I monomer

Fig. 3. Time course of the formation of synapsin I dimer

Fixed Cu^{2+} concentration is 1 mM. C, control (synapsin I incubated for 15 min without cross-linking agent); other experimental conditions were as in Fig. 2. Inter, intermolecular cross-linking; Intra, this band could be due to intramolecular cross-linking.

digestion products (lane 2) is the disappearance in lane 1 of a 28 kDa band present in synapsin I monomer, with a concomitant appearance of a 57 kDa band. This difference between synapsin I monomer and dimer degradation fragments disappears when pieces of gel are equilibrated in sample buffer containing β -mercaptoethanol before electrophoresis [compare lanes 6 (dimer) and 7 (monomer) in Fig. 4]. In addition, two major bands, of apparent molecular masses 35 and 32 kDa, are present in lanes 1, 2, 6 and 7.

The corresponding autoradiogram shows that only a 12–14 kDa fragment is labelled after synapsin digestion by V8 protease. This result confirms that, under our experimental conditions, the only site phosphorylated by cyclic-AMP-dependent protein kinase is site 1, located in the *N*-terminal part of the molecule. Furthermore, the same result is obtained with synapsin dimer. Thus disulphide bonds formed within synapsin I dimers by the action of Cu^{2+} /phenanthroline are not located in the 12–14 kDa *N*-terminal fragment.

When partial hydrolysis is induced by α -chymotrypsin, an autoradiogram of the gel shows three bands, of apparent molecular masses about 26, 19 and 13 kDa, for synapsin monomer (Fig. 5, lane 1). The same pattern is obtained after partial hydrolysis of synapsin I dimer (lane 2). Clearly, polymerization induced by Cu^{2+} /phenanthroline does not give rise to phosphorylated peptides of apparent molecular mass higher than those observed with synapsin I monomer. Thus, thiol groups whose oxidation stabilizes synapsin I dimers are not located in the 26 kDa phosphorylated *N*-terminal peptide obtained after α -chymotrypsin hydrolysis. To locate more rigorously the cysteine residues involved in the cross-linking, peptides obtained after partial hydrolysis by V8 protease of synapsin I labelled with ^{125}I are treated with collagenase. As clearly shown in Fig. 6, bands of apparent molecular masses 35 and 32 kDa disappear from the collagenase-treated sample as compared with the control (Fig. 6a, respectively lanes d and e). In contrast, the band corresponding to the 28 kDa peptide obtained after partial hydrolysis by V8 protease, and which is only clearly visible on the autoradiogram, does not disappear when incubated with collagenase (Fig. 6b, lane a). Thus the 28 kDa peptide is entirely different from the 35/32 kDa collagenase-sensitive doublet, and the cysteine residues implicated in synapsin I

Fig. 4. Limited proteolysis of phosphorylated synapsin I dimer or monomer by V8 protease

Synapsin I dimers and monomers are isolated and hydrolysed as described in the Materials and methods section. Lane 1: synapsin I dimer. Lane 2: synapsin I monomer. Lanes 3 (dimer) and 4 (monomer): controls without protease. Lane 5: V8 protease alone. Lanes 6 and 7: synapsin I dimers and monomers preincubated with 2% β -mercaptoethanol before electrophoresis (12.5%-acrylamide gels).

Fig. 5. Autoradiogram of the phosphorylated fragments obtained after partial proteolysis by α -chymotrypsin

Hydrolysis of synapsin I monomer (lane 1) and dimer (lane 2) by α -chymotrypsin is performed as described in the Materials and methods section.

cross-linking should be located on this 28 kDa peptide (cf. Fig. 7, below).

DISCUSSION

Several examples of protein cross-linking via disulphide formation have been described for neuronal proteins or collagens (Rehm *et al.*, 1986; Carden & Eagles, 1986; Correia *et al.*, 1987; Weber *et al.*, 1988). Preliminary evidence of synapsin I polymer formation has been reported by several groups (Baines & Bennett, 1986; Bähler & Greengard, 1987; Steiner *et al.*, 1987b), but without any experimental details. Furthermore, it

has been suggested that formation of synapsin I polymers could explain its actin- or microtubule-bundling activity (Petrucchi *et al.*, 1988). Such an effect has been described by Lynch *et al.* (1987), who have shown that the F-actin bundling activity of caldesmon results from protein cross-linking by thiol-group oxidation.

Results of the present experiments show that synapsin I in solution could exist as non-covalently self-associated dimers which are able to form covalently cross-linked dimer species in the presence of some cross-linking reagents. Under our experimental conditions, i.e. diluted protein solution (about 10 μ M) and short times of incubation (usually 15 min), dimer formation is not due to collisional cross-linking, since, as reported by Middaugh *et al.* (1983), it occurs only at high protein concentration and with long periods of incubation.

Results reported in Fig. 1 show that Cu^{2+} /phenanthroline is one of the most efficient agents able to promote covalent links between self-associated synapsin I molecules. The results obtained with Cu^{2+} /phenanthroline are not observed with other zero-length reagents such as 1-ethyl-3-(3-dimethylaminopropyl)-carbodi-imide or *N*-cyclohexyl-*N'*-[β -(*N*-methyl-morpholino)ethyl]carbodi-imide toluene-*L*-sulphonate (results not shown). Furthermore, results in Fig. 3 show that, in addition to synapsin Ia and Ib monomers and synapsin dimer, another band with a lower electrophoretic mobility than synapsin Ia appears in less than 30 s; we suggest that this band results from intramolecular disulphide-bridge formation and that a small fraction of the synapsin I molecules is folded in such a way that two cysteine residues are in close contact and readily form an intramolecular disulphide after addition of Cu^{2+} /phenanthroline.

Synapsin I is always present in two forms, synapsin Ia and synapsin Ib, which are probably encoded by a single gene. It shares the former property with other important neuronal cytoskeletal proteins (for example α and β tubulin, α and β fodrin), which, however, unlike synapsin, are encoded by separated genes. At present, the biochemical difference between synapsin Ia and Ib remains an open question. This difference is located in the collagenase-sensitive region of the molecule (Huttner *et al.*, 1981), and McCaffery & De Gennaro (1986) suggest

Fig. 6. Collagenase treatment of synapsin I peptides obtained by partial hydrolysis with V8 protease

(a) Coomassie-Blue-stained gel; (b) autoradiogram. The 28 kDa peptide (lanes a and b) and the 35/32 kDa doublet (lanes d and e) obtained after limited proteolysis by V8 protease are incubated with (lanes a and d) or without (lanes b and e) collagenase as described in the Materials and methods section. Lane c, collagenase alone; m, molecular-mass markers.

that synapsin Ia differs from synapsin Ib only by about 40 additional amino acids at the C-terminal end of the molecule. Until now, studies on synapsin I interactions with various components of the neuronal cell (microtubules, actin, synaptic vesicles etc.) have never shown different properties of synapsin Ia and synapsin Ib. For the first time to our knowledge, the present study could suggest a different behaviour of these two polypeptides (see Figs. 2 and 3): in the presence of Cu^{2+} /phenanthroline, synapsin I dimer seems to be formed primarily at the expense of protein Ib, which is the predominant molecular species in the absence of Cu^{2+} /phenanthroline. However, we cannot exclude the possibility that, owing to intramolecular disulphide formation, part of synapsin Ib is converted into material running with synapsin Ia.

In the present discussion, we assume that bovine brain synapsin I has a high degree of similarity to rat brain synapsin I, the sequence of which has been determined (McCaffery & De Gennaro, 1986). Such a similarity has been pointed out by Czernik *et al.* (1987) for the peptides corresponding to the different phosphorylation sites. In addition, Petrucci *et al.* (1988) have reported that human and bovine brain synapsin I appear to share at least 88 % sequence identity.

From this sequence we can speculate on the position of the cysteine residues whose oxidation by Cu^{2+} /phenanthroline stabilizes synapsin I dimer. Results of our different experiments are put together in the schematic illustration presented in Fig. 7. Phosphorylation of synapsin I with cyclic-AMP-dependent protein kinase enables us to localize unequivocally the labelled fragments as N-terminal fragments. Furthermore, if we estimate by SDS/polyacrylamide-gel electrophoresis the

apparent molecular mass of the proteolytic fragments obtained, we can tentatively localize the peptides obtained by limited proteolysis, identify the peptide that forms dimers in the presence of Cu^{2+} /phenanthroline and then localize the cysteines implicated. As shown in Fig. 4, and in agreement with previous results (Huttner & Greengard, 1979), after partial hydrolysis by protease V8 of synapsin I phosphorylated by cyclic-AMP-dependent protein kinase, a phosphorylated peptide of about 14 kDa appears simultaneously with a low-molecular-mass non-phosphorylated peptide. As clearly shown, the phosphorylated peptide does not give rise to dimer in the presence of Cu^{2+} /phenanthroline (Fig. 4, autoradiography, lane 1).

After partial digestion of synapsin I by α -chymotrypsin, three ^{32}P -labelled peptides are identified. Similar patterns are observed when hydrolysis is carried out on the synapsin dimer or monomer, and especially the phosphorylated 26 kDa fragment does not form a dimer in the presence of Cu^{2+} /phenanthroline. The 26 kDa fragment (Fig. 5) is more abundant in synapsin I dimer than in synapsin I monomer; this result cannot be explained by a dimerization of the lowest-molecular-mass fragment, since phosphorylated peptide of similar molecular mass obtained by V8-protease hydrolysis does not give rise to dimer; it could be explained if we assume that dimerization partially impedes a further hydrolysis of the 26 kDa fragment to the 13 kDa one. These results obtained with phosphorylated peptides allow us to exclude the participation of cysteine residues located in the 26 kDa N-terminal part of the molecule in the formation of synapsin I dimers induced by Cu^{2+} /phenanthroline.

Partial hydrolysis of synapsin by protease V8 also produces a doublet of apparent molecular mass 35/

Fig. 7. Schematic representation and tentative alignment of synapsin I fragments obtained by limited proteolysis

P, phosphorylation sites. The fragments obtained by hydrolysis with α -chymotrypsin (upper part) or V8 protease (lower part) are aligned. The fragments phosphorylated at site 1 by cyclic-AMP-dependent protein kinase are N-terminal fragments; the collagenase-sensitive fragments are C-terminal fragments. Hatched box: part of the synapsin I molecule where are located the cysteine residues implicated in synapsin I cross-linking.

32 kDa which is not modified by dimerization of synapsin I by Cu^{2+} /phenanthroline. This doublet is unequivocally identified as the C-terminal portion of synapsin Ia and Ib: this part of the molecule is very poor in acidic amino acids, and thus resistant to V8-protease hydrolysis. At least in the rat brain protein, tyrosine residues are absent from this fragment, and under our experimental conditions this doublet is not labelled by ^{125}I (cf. lanes d and e in Fig. 6b) and overall the 35/32 kDa doublet is hydrolysed by purified collagenase (Fig. 6a, lane d) and thus corresponds to the C-terminal collagenase-sensitive part of the molecule. This doublet, which contains the site for kinase II, is clearly different from the 28 kDa peptide also obtained by partial hydrolysis with V8 protease; this latter peptide contains several tyrosine residues, since it is labelled by ^{125}I , and in addition it is not modified after incubation with collagenase, and thus is located in the collagenase-resistant part of the molecule. This peptide is not present in lane 1 of Fig. 4, corresponding to V8-protease hydrolysis products of synapsin dimer. Instead of the 28 kDa peptide, a 57 kDa band is clearly apparent in this lane. Thus we can assume that synapsin dimer formation, which is revealed in the presence of Cu^{2+} /phenanthroline, implies cysteine residues which belong to a peptide (hatched box in Fig. 7) located in the hydrophobic globular head of the molecule, between the longest N-terminal fragment (26 kDa phosphorylated peptide obtained by a α -chymotrypsin hydrolysis) and the 35/32 kDa collagenase-sensitive C-terminal part of the synapsin molecule which do not give rise to polymers in the presence of Cu^{2+} /phenanthroline.

In conclusion, synapsin I seems to comprise different binding domains: its C-terminal collagenase-sensitive domain is involved in synaptic-vesicle binding (Ueda, 1981); the two ends of the synapsin I molecule appear to bind actin (Petrucci *et al.*, 1988); as reported in the present experiments, the central hydrophobic core of the molecule is implicated in synapsin self-association. Thus synapsin I could be viewed as forming bridges between synaptic vesicles and the cytoskeleton. The bundling activity of synapsin does not necessarily require the formation of synapsin polymers, since the observations made by Hirokawa *et al.* (1989) suggest that a single synapsin I molecule can cross-link actin filaments, or microtubules. However, when adjacent microtubules are further apart, those authors describe structures which could correspond to synapsin molecules interacting at the level of their heads. This latter observation is in good agreement with our results, which show that synapsin dimers revealed by chemical cross-linking result from interactions between two synapsin molecules at the level of their hydrophobic globular heads.

Further studies will be necessary to investigate the effects of synapsin I dimerization on its interactions with the various components of the neuronal cell.

After this manuscript was submitted, Bähler *et al.* (1989) have reported results from experiments on cysteine-specific cleavage of bovine brain synapsin I. In good agreement with us, they locate cysteine residues in the hydrophobic collagenase-resistant middle region of the molecule, and suggest that this domain is responsible for the observed self-association properties of intact synapsin I.

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REFERENCES

- Bähler, M. & Greengard, P. (1987) *Nature* (London) **326**, 704–707
- Bähler, M., Benfenati, F., Valtorta, F., Czernik, A. J. & Greengard, P. (1989) *J. Cell Biol.* **108**, 1841–1849
- Baines, A. J. & Bennett, V. (1985) *Nature* (London) **315**, 410–413
- Baines, A. J. & Bennett, V. (1986) *Nature* (London) **319**, 145–147
- Bennett, V., Baines, A. J. & Davis, J. (1986) *Methods Enzymol.* **134**, 55–69
- Carden, M. J. & Eagles, P. A. (1986) *Biochem. J.* **234**, 587–591
- Cleveland, D. W., Fischer, S. G., Kirschner, M. W. & Laemmli, U. K. (1977) *J. Biol. Chem.* **252**, 1102–1106
- Correia, J. J., Welch, M. K. & Williams, R. C., Jr. (1987) *Arch. Biochem. Biophys.* **255**, 244–253
- Czernik, A. J., Pang, D. T. & Greengard, P. (1987) *Proc. Natl. Acad. Sci. U.S.A.* **84**, 7518–7522
- Font, B., Eichenberger, D., Goldschmidt, D. & Vial, C. (1987) *Mol. Cell Biochem.* **78**, 131–140
- Gaffney, B. J. (1985) *Biochim. Biophys. Acta* **822**, 289–317
- Hirokawa, N., Sobue, K., Kanda, K., Harada, A. & Yorifuji, H. (1989) *J. Cell Biol.* **108**, 111–126
- Huttner, W. B. & Greengard, P. (1979) *Proc. Natl. Acad. Sci. U.S.A.* **76**, 5402–5406
- Huttner, W. B., De Gennaro, L. J. & Greengard, P. (1981) *J. Biol. Chem.* **256**, 1482–1488
- Jacobs, E. & Clad, A. (1986) *Anal. Biochem.* **154**, 583–589
- Ji, H. H. (1979) *Biochim. Biophys. Acta* **559**, 39–69
- Johnson, E. M., Ueda, T., Maeno, H. & Greengard, P. (1972) *J. Biol. Chem.* **247**, 5650–5652
- Kobashi, K. (1968) *Biochim. Biophys. Acta* **158**, 239–245
- Krebs, K. E., Prouty, S. M., Zagon, I. S. & Goodman, S. R. (1987a) *Am. J. Physiol.* **253**, C500–C505
- Krebs, K. E., Zagon, I. S. & Goodman, S. R. (1987b) *Brain Res. Bull.* **18**, 793–798
- Laemmli, U. K. (1970) *Nature* (London) **227**, 680–685
- Lynch, W. P., Riseman, V. M. & Bretscher, A. (1987) *J. Biol. Chem.* **262**, 7429–7437
- McCaffery, C. A. & De Gennaro, L. S. (1986) *EMBO J.* **5**, 3167–3173
- Middaugh, C. R., Vanin, E. F. & Ji, T. (1983) *Mol. Cell Biochem.* **50**, 115–141
- Nestler, E. J. & Greengard, P. (1986) *Prog. Brain Res.* **69**, 323–339
- Penefsky, H. S. (1977) *J. Biol. Chem.* **252**, 2891–2899
- Peterson, G. L. (1977) *Anal. Biochem.* **83**, 346–356
- Petrucci, T. C. & Morrow, J. S. (1987) *J. Cell Biol.* **105**, 1355–1363
- Petrucci, T. C., Mooseker, M. S. & Morrow, J. S. (1988) *J. Cell. Biochem.* **36**, 25–35
- Rehm, H., Wiedenmann, B. & Betz, H. (1986) *EMBO J.* **5**, 535–541
- Schiebler, W., Jahn, R., Doucet, J. P., Rothlein, J. & Greengard, P. (1986) *J. Biol. Chem.* **261**, 8383–8390
- Steiner, J. P., Ling, E. & Bennett, V. (1987a) *J. Biol. Chem.* **262**, 905–914
- Steiner, J. P., Gardner, K., Baines, A. & Bennett, V. (1987b) *Brain Res. Bull.* **18**, 777–785
- Ueda, T. (1981) *J. Neurochem.* **36**, 297–300
- Ueda, T. & Greengard, P. (1977) *J. Biol. Chem.* **252**, 5155–5163
- Weber, K. & Osborn, H. (1969) *J. Biol. Chem.* **244**, 4406–4412
- Weber, S., Dölz, R., Timpl, R., Fessler, J. H. & Engel, S. (1988) *Eur. J. Biochem.* **175**, 229–236