

Purification and Characterization of a Calmodulin-dependent Protein Kinase That Is Highly Concentrated in Brain*

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A calcium and calmodulin-dependent protein kinase has been purified from rat brain. It was monitored during the purification by its ability to phosphorylate the synaptic vesicle-associated protein, synapsin I. A 300-fold purification was sufficient to produce kinase that is 90–95% pure as determined by scans of stained sodium dodecyl sulfate-polyacrylamide gels and has a specific activity of 2.9 μmol of ^{32}P transferred per min/mg of protein. Thus, the kinase is a relatively abundant brain enzyme, perhaps comprising as much as 0.3% of the total brain protein.

The Stokes radius (95 Å) and sedimentation coefficient (16.4 S) of the kinase indicate a holoenzyme molecular weight of approximately 650,000. The holoenzyme is composed of three subunits as judged by their co-migration with kinase activity during the purification steps and co-precipitation with kinase activity by a specific anti-kinase monoclonal antibody. The three subunits have molecular weights of 50,000, 58,000, and 60,000, and have been termed α , β' , and β , respectively. The α - and β -subunits are distinct peptides, however, β' may have been generated from β by proteolysis. All three of these subunits bind calmodulin in the presence of calcium and are autophosphorylated under conditions in which the kinase is active. The subunits are present in a ratio of about 3 α -subunits to 1 β/β' -subunit. We therefore postulate that the 650,000-Da holoenzyme consists of approximately 9 α -subunits and 3 β/β' -subunits.

The abundance of this calmodulin-dependent protein kinase indicates that its activation is likely to be an important biochemical response to increases in calcium ion concentration in neuronal tissue.

The functions of specialized cell types within different tissues are coordinated by a variety of extracellular agents. Each of these triggers a series of intracellular regulatory events that ultimately alter the functional state of the cell. The first step in the action of many regulatory agents is to increase the intracellular concentration of a second messenger, such as a cyclic nucleotide or calcium ion (1–3). A complete understanding of the general principles of cellular regulation will require a description of the molecular events triggered by each of these second messengers.

We are interested in the mechanisms of action of calcium

ion in nervous tissue. A number of important neuronal processes are regulated by changes in the intracellular concentration of calcium (4–7). These changes are brought about both by the actions of specific extracellular agents such as neurotransmitters and neurohormones, and by electrical activity which activates voltage-sensitive calcium channels (8). Changes in calcium concentration are important in synaptic processes such as post-tetanic potentiation and long-term potentiation that involve modulation of the quantity of transmitter released per impulse (9–11). In certain neurons, the conductances of specific potassium (12) or calcium (13) membrane channels are regulated by intracellular calcium ion. Such transient changes in the strength of specific synapses, or in the electrical properties of individual neurons can modulate the flow of information through complex neuronal circuits (14). Information about the properties and distributions in different types of neurons of proteins that are regulated by physiological changes in calcium concentration will be necessary to understand, at the molecular level, the responses of various neurons, and of individual synaptic terminals, to changes in calcium flux.

It is generally accepted that the cyclic nucleotides regulate cell physiology primarily, if not exclusively, by activating protein kinases (15, 16). Distinct kinases that are activated by either cyclic AMP or cyclic GMP have been purified and characterized (17, 18). In contrast, the mechanisms by which calcium alters cellular functions appear to be quite diverse (19). Nevertheless, the importance of calcium-regulated protein kinases has recently been recognized (5, 20). Initial characterization of this distinct class of kinases has revealed that, unlike the cyclic AMP-dependent protein kinases, there are a number of calcium-regulated protein kinases that differ in several characteristics, including mechanism of regulation by calcium, substrate specificity, and tissue distribution (21–29).

Calcium and calmodulin-dependent protein kinase activities have been observed in crude brain homogenates and in various neuronal subcellular fractions (26, 27, 29–34). Although two well characterized calmodulin-dependent protein kinases from non-neuronal tissues, myosin light chain kinase, and phosphorylase kinase, are present in brain, they do not account for a large portion of brain calmodulin-dependent protein kinase activity (26, 27). This paper reports the purification and subunit structure of a distinct calmodulin-dependent protein kinase that is highly concentrated in brain. The enzyme was first observed in brain homogenates as a calmodulin-dependent kinase that phosphorylated synapsin I, a protein associated with synaptic vesicles (26, 35). For clarity, we continue to refer to the enzyme as "calmodulin-dependent synapsin I kinase," although we believe that it is likely to be involved in the phosphorylation and regulation of a number of other brain proteins.

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The structure of the synapsin I kinase is different from either myosin light chain kinase or phosphorylase kinase (22–25). However, it is similar but not identical to a glycogen synthase kinase recently purified from liver (36, 37), and another calmodulin-dependent protein kinase recently purified from rat brain (38). Thus, it may be one of a class of related calmodulin-dependent protein kinases.

EXPERIMENTAL PROCEDURES

Materials—ATP, 3',5'-cAMP, dithiothreitol, imidazole, EDTA, PMSF,¹ fast green FCF, Coomassie brilliant blue R, fibrinogen, bovine serum albumin, ovalbumin, phosphorylase b, carbonic anhydrase, soybean trypsin inhibitor, actin, alcohol dehydrogenase, glycogen synthase, trypsin, casein, phosphovitin, arginine-rich histone, Type II hemoglobin, insulin, and transferrin were purchased from Sigma. Thyroglobulin, ferritin, catalase, Protein A and Sepharose 4B were purchased from Pharmacia Fine Chemicals. Leupeptin was purchased from Peninsula Laboratories, Inc. Myosin was purchased from Bethesda Research Laboratories. Lactoperoxidase was purchased from Calbiochem. Chymotrypsinogen was purchased from Worthington. [γ -³²P]ATP was purchased from ICN Nutritional Biochemicals. Na¹²⁵I and [³H]3',5'-cAMP were purchased from New England Nuclear. Trifluoperazine was obtained from Smith, Kline, and French. Ultrapure sucrose was purchased from Schwarz/Mann. DEAE-cellulose (DE-52) was purchased from Whatman. Nitrocellulose membranes (BA85, 0.45 μ m) were purchased from Schleicher and Schuell. RPMI 1640 tissue culture medium, glutamine, penicillin, and streptomycin were purchased from Gibco Laboratories. Newborn calf serum was purchased from Irvine Scientific. Selenous acid (Specpure) was purchased from Johnson Matthey, Inc., Synapsin I was purified from bovine brain by a modification of the procedure of Ueda and Greengard (39), as described in Huttner *et al.* (40). The modifications were as follows. A crude brain particulate fraction was used at step 2, rather than the M-1 fraction. The pH 6 supernatant was adjusted to pH 8 and subjected to chromatography on CM-cellulose, hydroxylapatite and Sephadex G-100. The final purified fractions were concentrated by ammonium sulfate precipitation and dialyzed against 25 mM Tris-HCl, pH 7.0. Calmodulin was purified from bovine brain by the method of Watterson *et al.* (41). Microtubule protein prepared from rat brain by the method of Shelanski *et al.* (42), was a gift of James Soha of our laboratory. Gizzard myosin light chain was a gift of Dr. Robert Adelstein of the National Institutes of Health. Affinity-purified rabbit anti-calceineurin was a gift of Dr. Claude Klee of the National Institutes of Health. Calmodulin-Sepharose was prepared by the method of March *et al.* (43). Simonsen albino rats (140–160 g males) were purchased from Simonsen Laboratories.

Assay for Calmodulin-dependent Synapsin I Kinase Activity—Calmodulin-dependent synapsin I kinase was assayed, with minor modifications, as previously described (26, 35) at 30 °C in a reaction mixture (final volume, 100 μ l) containing 50 mM Tris (pH 8.0), 10 mM MgCl₂, 5 mM 2-mercaptoethanol, 1 μ g of calmodulin, 50 μ M [γ -³²P]ATP (0.5–2 \times 10³ cpm/pmol), varying amounts of enzyme, either 0.4 mM EGTA (minus calcium) or 0.4 mM EGTA/0.7 mM CaCl₂ (plus calcium), and either no synapsin I, or 10 μ g of synapsin I. After preincubation for 1 min, the reaction was initiated by the addition of [γ -³²P]ATP, and terminated after 30 s by the addition of 50 μ l of a "stop solution" containing 0.3 M EDTA and 2 mg/ml of bovine serum albumin (as a carrier) followed immediately by 1 ml of ice-cold 10% trichloroacetic acid. After 10 min at 4 °C, the reaction mixture was centrifuged in a Beckman microfuge B for 2 min, and the supernatant was removed by aspiration. The pellet was redissolved in 100 μ l of 0.1 N NaOH and immediately reprecipitated with 1 ml of 10% trichloroacetic acid. After dissolving the final pellet in 100 μ l of 0.1 N NaOH, 1 ml of Aquasol (New England Nuclear) was added and the radioactivity was quantitated by liquid scintillation spectrometry. Phosphorylation of synapsin I by the calmodulin-dependent kinase was measured as the difference between calcium-stimulated incorporation of ³²P into trichloroacetic acid-insoluble material in the absence and presence of synapsin I. In the later steps of the purification (after DEAE-cellulose chromatography), phosphorylation of synapsin I in the absence of calcium was minimal, and incorporation of ³²P

into trichloroacetic acid-insoluble material was measured only in the presence of calcium. A previous report presented evidence that brain homogenates contained two calmodulin-dependent synapsin I kinase activities that recognized different sites in the synapsin I molecule. The kinase activities were named "30-kDa region" kinase and "10-kDa region" kinase, after the *Staphylococcus aureus* V8 protease peptides that contained the respective sites (26). In the present report, phosphorylation of synapsin I was measured at pH 8, rather than at pH 7. At this pH, 95% or more of the radioactive phosphate incorporated into synapsin I in the presence of calcium was present in the site recovered in the 30-kDa fragment, even in crude brain homogenates. Thus, this assay condition eliminated the need to subtract phosphorylation by the 10-kDa region kinase.

Phosphorylation of the Kinase Subunits—Incorporation of ³²P into the kinase subunits was measured under the conditions used to measure synapsin I kinase activity except that synapsin I was omitted from the assay, [γ -³²P]ATP was used at a lower concentration (10 μ M) and higher specific activity (2–4 \times 10⁴ cpm/pmol), and the reaction was terminated after varying lengths of time by the addition of 50 μ l of a stop solution containing 9% SDS, 6% (v/v) 2-mercaptoethanol, 15% (w/v) glycerol, 0.186 M Tris-HCl (pH 6.7) and a trace of bromophenol blue. The solution was then boiled for 2 min and 120 μ l was subjected to SDS/PAGE. The gel was dried and the ³²P-labeled proteins were localized by autoradiography (26). When quantitation was necessary, the labeled bands were cut out of the dried gel, placed in liquid scintillation fluid, and subjected to liquid scintillation spectrometry.

Substrate Specificity—Incorporation of phosphate into various substrate proteins was measured under standard assay conditions using 60 ng of purified kinase and varying amounts of possible substrate proteins. The reaction was terminated after 30 s by addition of 50 μ l of SDS stop solution. The samples were boiled for 2 min and subjected to SDS/PAGE. After the gels were stained and dried, ³²P-labeled proteins were located by autoradiography. For quantitation, labeled bands were cut out of the dried gel, placed in liquid scintillation fluid and subjected to liquid scintillation spectrometry.

Immunoprecipitation of Kinase Activity—Hybridomas that secrete monoclonal antibodies specific for the synapsin I kinase were selected from cells formed by fusion of NS1/SP2 myeloma cells with spleen cells from mice that had been immunized with kinase purified through the calmodulin-Sepharose step. Details of the preparation and selection of these hybridomas, including the one used in this study (VIE9, anti-kinase) will be published separately.² For immunoprecipitation experiments, VIE9 and a control hybridoma (VF3, anti-sodium channel, kindly supplied by Dr. Larry Fritz of Caltech) were grown to 2 \times 10⁵ cells/ml in RPMI 1640 medium supplemented with 20% calf serum, 2 mM glutamine, 1 mM pyruvate, 0.5 mM oxaloacetic acid, and 1 mg/ml of penicillin-streptomycin. The cells were harvested by centrifugation and washed once in an equal volume of the same medium without calf serum and with 5 μ g/ml of insulin, 0.1 mg/ml of transferrin, 20 μ M ethanolamine, and 0.4 μ g/ml of H₂SeO₃ (selenium) (44). They were then resuspended in the supplemented serum-free medium and kept for 24 h at 37 °C in a CO₂-controlled incubator. Cells were removed by centrifugation from the medium which contained secreted antibodies. The antibodies were concentrated 100-fold by precipitation with 50% ammonium sulfate, and dialyzed against 40 mM Tris-HCl (pH 7.5).

For precipitation experiments, purified synapsin I kinase (15 μ g) was added to 40 μ g of either VIE9 anti-kinase antibody, control monoclonal antibody, or 40 mM Tris-HCl (pH 7.5) in a volume of 0.1 ml and incubated overnight on ice. The resulting immune complexes were pelleted by centrifugation in a Beckman microfuge B for 2.5 min and 10- μ l aliquots of the supernatants were assayed for synapsin I kinase activity. The protein in the remaining supernatants was concentrated by precipitation with 10% trichloroacetic acid, dissolved in 1/3 diluted SDS stop solution, and adjusted to neutral pH with NaOH. Pellets containing the immune complexes were washed twice with phosphate-buffered saline, dissolved in 1/3 diluted SDS stop solution, and, together with the supernatants, subjected to SDS/PAGE.

Densitometric Scans of SDS-Polyacrylamide Gels—The purified kinase was subjected to SDS/PAGE and protein bands were stained with either Coomassie blue (0.075% in 25% isopropanol, 10% acetic acid) or fast green (0.5% in 50% methanol, 10% acetic acid) (45).

¹ The abbreviations used are: PMSF, phenylmethylsulfonyl fluoride; EGTA, ethylene glycol bis(β -aminoethyl ether)-N,N,N',N'-tetraacetic acid; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis.

² N. E. Erondy, M. B. Kennedy, V. Krieger, and C. B. Shapiro, manuscript in preparation.

Lanes were cut from the gels and scanned with a Beckman ACTA III recording spectrophotometer at 560 nm for Coomassie blue and 635 nm for fast green. Peak areas were quantitated using a Tektronix 4052 minicomputer interfaced to a Tektronix 4956 digitizing tablet. The areas of the major peaks were found to be linearly proportional to the amount of protein loaded on the gel over a range of 7.5–30 μ g with the fast green stain. The linear range was more variable with the Coomassie blue stain, but was usually from 2–10 μ g.

Immunoblots—Proteins from SDS gels were transferred onto nitrocellulose paper as described by Towbin *et al.* (46) in a Bio-Rad Trans-Blot Cell. After transfer, the nitrocellulose sheets were incubated with: 1) Buffer D (50 mM Tris (pH 7.4), 0.9% NaCl, 0.1% Na₂S₂O₄) containing 5% hemoglobin (2 h); 2) the appropriate antibody diluted into Buffer D containing 1% hemoglobin (8–12 h); 3) wash buffer (Buffer D containing 0.5% hemoglobin) (1 h with 3 changes); 4) affinity-purified rabbit anti-mouse IgG diluted 1/500 into wash buffer (2 h); 5) wash buffer (1 h with 3 changes); 6) ¹²⁵I protein A (2–3 \times 10⁶ cpm/ml) in wash buffer (2 h); 7) wash buffer (1 h with 3 changes). The nitrocellulose sheet was then dried, and bands containing ¹²⁵I were detected by autoradiography.

Other Procedures—Polyacrylamide gel electrophoresis in the presence of SDS was performed by the method of Laemmli (47). The stacking gel (2.5 \times 16 cm \times 1.5 mm) contained 3.5% acrylamide/0.09% bisacrylamide and the running gel (14 \times 16 cm \times 1.5 mm) contained 10% acrylamide/0.27% bisacrylamide. Molecular mass standards used in SDS/PAGE were: microtubule-associated protein₂, 300 kDa; phosphorylase b, 94 kDa; bovine serum albumin, 68 kDa; α -tubulin, 56 kDa; β -tubulin, 54 kDa; ovalbumin, 43 kDa; alcohol dehydrogenase, 41 kDa; carbonic anhydrase, 29 kDa; chymotrypsinogen, 25 kDa; soybean trypsin inhibitor, 21.5 kDa. The sedimentation coefficient (*s*_{20,w}) of the kinase was determined by sucrose density gradient centrifugation as described by Martin and Ames (48) with ovalbumin (*s*_{20,w} = 3.5 S), fibrinogen (*s*_{20,w} = 7.9 S), catalase (*s*_{20,w} = 11.3 S) and thyroglobulin (*s*_{20,w} = 19.2 S) as standards. The Stokes radius (Å) of the kinase was determined by gel filtration on Sepharose 4B using the analysis described by Porath (49). Protein standards used for calibration of the gel filtration column were: catalase, Stokes radius = 52 Å; ferritin, Stokes radius = 61 Å; thyroglobulin, Stokes radius = 85 Å; fibrinogen, Stokes radius = 107 Å; myosin, Stokes radius = 200 Å. Protein was determined by a modification of the method of Lowry *et al.* (50) with bovine serum albumin as a standard. Iodinated tryptic peptide maps were prepared by the method of Elder *et al.* (51). Calmodulin-stimulated phosphodiesterase was assayed as described by Rangel-Aldao *et al.* (52).

RESULTS

Purification of Calmodulin-dependent Synapsin I Kinase

The initial steps of the purification (through calmodulin affinity chromatography) were as previously described by Kennedy *et al.* (35) with modifications. In that study it was shown that synapsin I kinase is present in both soluble and particulate fractions of brain homogenates and that kinases partially-purified from each of the two fractions are indistinguishable by several criteria. For practical reasons, we have used as our enzyme source, the soluble fraction alone. Under the conditions described, it contains about 60% of the total synapsin I kinase activity. All purification steps were carried out at 0–4 °C.

Preparation of Crude Extract—Brains (1.4 g each) were removed from 90 rats and homogenized immediately by 12 up-and-down strokes with a Teflon/glass homogenizer at 900 rpm in 10 volumes of Buffer A (20 mM Tris (pH 7.5), 1 mM imidazole, 0.1 mM CaCl₂, 1 mM dithiothreitol, 0.1 mM PMSF) containing 1 mM MgCl₂, 25 mg/liter of soybean trypsin inhibitor and 1 mg/liter of leupeptin. The homogenate was centrifuged at 10,000 \times *g* for 20 min and the resulting supernatant was centrifuged at 170,000 \times *g* for 1 h.

DEAE-cellulose Chromatography—The 170,000 \times *g* supernatant was brought to 1 mM iodoacetate, adjusted to pH 7.5, and loaded onto a DEAE-cellulose column (5 \times 17 cm) previously equilibrated with Buffer A. The column was washed with one column volume of 0.05 M NaCl in Buffer A and was

then developed with a 2-liter linear gradient of 0.05–0.30 M NaCl in Buffer A.

Ammonium Sulfate Fractionation—The DEAE column fractions containing synapsin I kinase activity were pooled, adjusted to 0.1 M Tris (pH 7.5), 1 mM dithiothreitol, 0.1 mM PMSF, and brought to 40% saturation by the addition of solid ammonium sulfate. After 3–12 h, precipitated protein was collected by centrifugation and redissolved in a small volume of Buffer B (40 mM Tris (pH 7.5), 0.2 mM CaCl₂, 1 mM dithiothreitol, 0.1 mM PMSF) containing 0.2 M NaCl.

Calmodulin Affinity Chromatography—The redissolved ammonium sulfate precipitate was cleared by centrifugation for 10 min at 10,000 \times *g*, then applied to a calmodulin-Sepharose affinity column (1.5 \times 7 cm) equilibrated with Buffer B containing 0.2 M NaCl. The column was then washed overnight with Buffer B containing 2 M NaCl (~120 ml), followed by one column volume of Buffer B. Synapsin I kinase was eluted with 40 mM Tris (pH 7.5), 2 mM EGTA, 1 mM dithiothreitol, 0.1 mM PMSF. Recovery of enzyme activity from the column was 50–60% with a 13-fold purification.

Sepharose 4B Gel Filtration—The calmodulin-Sepharose column fractions containing synapsin I kinase activity were pooled and adjusted to 40% ammonium sulfate as described above. The precipitate was redissolved in a small volume of Buffer C (40 mM Tris (pH 7.5), 2 mM EGTA, 0.2 M NaCl, 1 mM dithiothreitol) containing 20% (v/v) glycerol and loaded on a Sepharose 4B column (0.75 \times 120 cm) equilibrated with the same buffer. The column was developed at a flow rate of 1.8 ml/h. The elution profile obtained is shown in Fig. 1. It contains a single peak of kinase activity that coincides with the major protein peak. Analysis of the peak fractions by SDS/PAGE indicated that a major 50 kDa protein and two bands in the 60-kDa region (58 and 60 kDa) co-migrated with the peak of kinase activity (data not shown). This step resulted in a 2-fold purification with 40–50% recovery of activity.

Sucrose Density Gradient Centrifugation—The peak Sepharose 4B fractions were pooled and protein was concentrated by precipitation with 40% ammonium sulfate. The precipitate was redissolved in a small volume of Buffer C and loaded onto two 12-ml linear gradients of 5–20% sucrose in Buffer C. The gradients were subjected to centrifugation at 40,000 rpm for 12 h in a Beckman SW 40 Ti rotor. They were then fractionated with an Isco Model 184 density gradient fractionator. As shown in Fig. 2, the gradients contained a single major peak of both synapsin I kinase activity and protein. The minor peak closer to the bottom of the tube may represent an enzyme dimer. The peak fractions were pooled and concentrated by calmodulin affinity chromatography (to remove sucrose), followed by ammonium sulfate precipitation. The ammonium sulfate precipitate was dissolved in a small volume of Buffer C so that the protein concentration was 1–2 mg/ml. After this step, the kinase showed variable stability when stored at 0 °C, but could be stored at –80 °C for long periods with virtually no loss of activity.

The purification procedure is summarized in Table I. The concentrated sucrose pool represented a 290-fold purification over the homogenate with 1.2% recovery of activity from the homogenate and 2.9% recovery from the 170,000 \times *g* supernatant. After the final step, 90–95% of the protein present was associated with peptides that co-migrated with kinase activity throughout the purification and were co-precipitated with kinase activity by an anti-kinase monoclonal antibody (see below).

One reason for the rather low recovery was a variable and unusually poor recovery after DEAE-cellulose chromato-

FIG. 1. **Sepharose 4B elution profile (purification step 7).** Gel filtration on Sepharose 4B was carried out as described in the text. Fractions of 0.6 ml were collected and assayed for synapsin I kinase activity. Aliquots of each fraction were diluted as required to obtain a linear rate of synapsin I phosphorylation in a 30-s assay. The elution positions of standard proteins were determined in separate runs by absorbance at 280 nm and are indicated by *arrows*. Standard proteins were: 1) myosin (Stokes radius = 200 Å); 2) fibrinogen (107 Å); 3) thyroglobulin (85 Å); 4) ferritin (61 Å); and 5) catalase (52 Å).

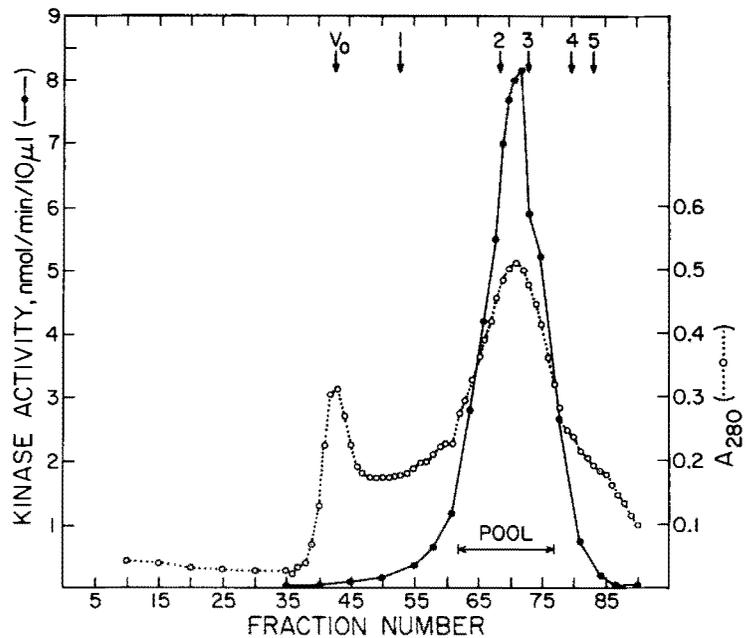


FIG. 2. **Sucrose density gradient sedimentation profile (purification step 8).** Sucrose density gradient centrifugation was carried out as described in the text. Fractions of 0.33 ml were collected (starting from the top of the gradient) and assayed for synapsin I kinase activity. Aliquots of fractions were diluted for assay as described in the legend of Fig. 1. The positions of standard proteins were determined on parallel gradients by absorbance at 280 nm and are indicated by *arrows*. Standard proteins were: 1) ovalbumin ($s_{20,w} = 3.5$ S); 2) fibrinogen (7.9 S); 3) catalase (11.3 S); and 4) thyroglobulin (19.2 S).

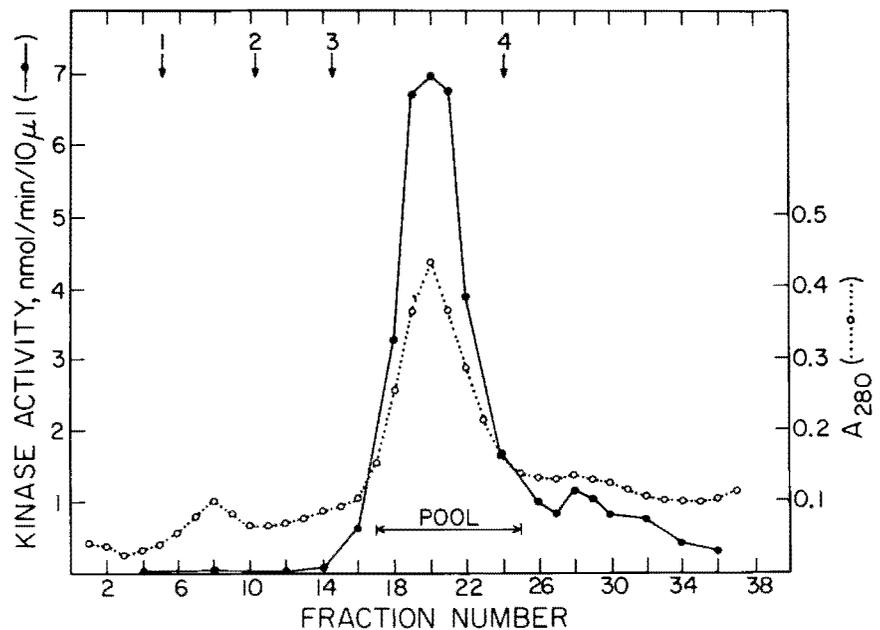


TABLE I
Summary of purification of rat brain calmodulin-dependent synapsin I kinase

Step	Total activity ^a	Total protein ^a	n	Specific activity	Purification	Recovery
	$\mu\text{mol}/\text{min}$	mg		$\mu\text{mol}/\text{min}/\text{mg}$	-fold	%
1. Homogenate	132 ± 27	13,100 ± 1,900	9	0.010	1	100
2. 10,000 × g Supernatant	82.7 ± 23	3,660 ± 755	9	0.023	2.3	63
3. 170,000 × g Supernatant	55.8 ± 16	3,010 ± 747	9	0.019	1.9	42
4. DEAE-cellulose eluate	17.4 ± 6.7	725 ± 84	7	0.024	2.4	13
5. 40% Ammonium sulfate	17.4 ± 5.4	225 ± 45	7	0.077	7.7	13
6. Calmodulin-Sepharose eluate	9.5 ± 3.1	9.6 ± 3.1	7	0.99	99	7.2
7. Sepharose 4B eluate	3.8 ± 0.8	2.1 ± 0.5	5	1.81	181	2.9
8. Sucrose gradient pool	1.6 ± 0.7	0.55 ± 0.2	2	2.90	290	1.2

^a Values shown are mean ± S.D. of *n* experiments.

phy. In large scale preparations, recovery at this step has varied from 18 to 40%. These recoveries are lower than those first reported for smaller scale preparations (35). This may be due to denaturation of the enzyme with time on the column. We have tried recombining the enzyme fraction with other

pooled column fractions and with proteins stripped from the column by 2 M salt and have seen no stimulation of activity. Thus, we have no evidence that we have lost a factor required for maximal activity. Substitution of other DEAE-resins has not improved the recovery. Recoveries for subsequent purifi-

cation steps, as reported in Table I, include small losses during concentration of pooled fractions. Recoveries during the chromatography and centrifugation steps themselves were generally 50% or greater. In recent preparations, we have eliminated the calmodulin-Sepharose concentration step following sucrose density gradient centrifugation, and this has improved recovery.

Structure and Properties of the Calmodulin-dependent Synapsin I Kinase

Identification of the Kinase Subunits—The peptide composition of the enzyme fractions at each stage of the purification is shown in Fig. 3. The purified kinase fraction contained a major peptide band at 50 kDa, a less prominent band at 60 kDa, and minor bands at 58 and 45 kDa. Two lines of evidence indicate that the 50-, 58-, and 60-kDa peptides (termed α , β' , and β respectively) are associated with enzyme activity and are present in a holoenzyme complex.

The amount of these three proteins peaked in the same fractions as kinase activity during gel filtration and sucrose density gradient centrifugation. An example is shown in Fig. 4. In this experiment, the purified kinase was subjected to analytical sucrose density gradient centrifugation. Fractions were assayed for synapsin I kinase activity and subjected to SDS/PAGE. The gels were stained with Coomassie blue and the amount of protein in each band was determined from the area of the optical density peaks on densitometric scans. Although the β and β' -bands were separated on the gel, the optical density peaks on the scans were not well resolved (see Fig. 6). The ratio of the β' "shoulder" to the β peak was constant, so that β and β' -bands were grouped together and treated as one "band" for this analysis. They will be referred to as the β/β' -band. As shown in Fig. 4, both the α and β/β' -

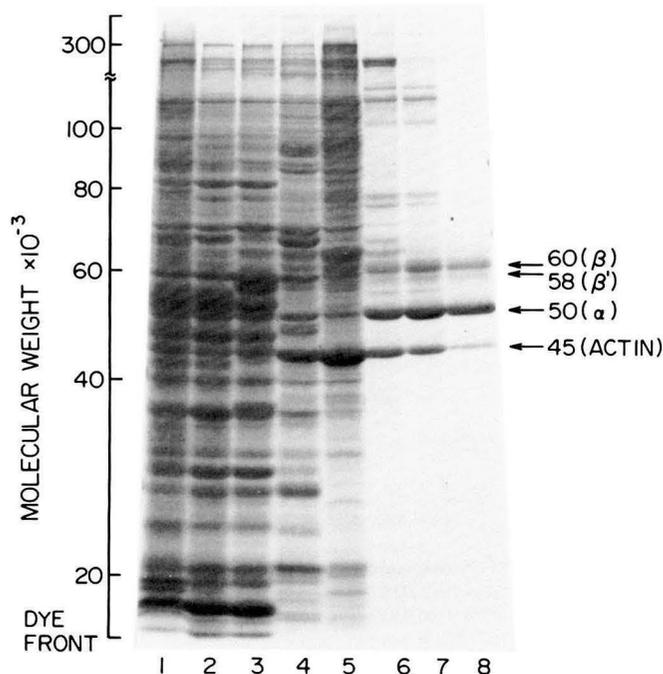


FIG. 3. Purification of synapsin I kinase as monitored by SDS/PAGE. Samples from each step of the purification were subjected to SDS/PAGE and stained with Coomassie blue. The purification step and amount of protein corresponding to each lane are as follows: lane 1, homogenate, 100 μ g; lane 2, 10,000 \times g supernatant, 100 μ g; lane 3, 170,000 \times g supernatant, 100 μ g; lane 4, DEAE-cellulose eluate, 75 μ g; lane 5, 40% ammonium sulfate precipitate, 75 μ g; lane 6, calmodulin-Sepharose eluate, 15 μ g; lane 7, Sepharose 4B eluate, 10 μ g; lane 8, sucrose gradient pool, 7.5 μ g.

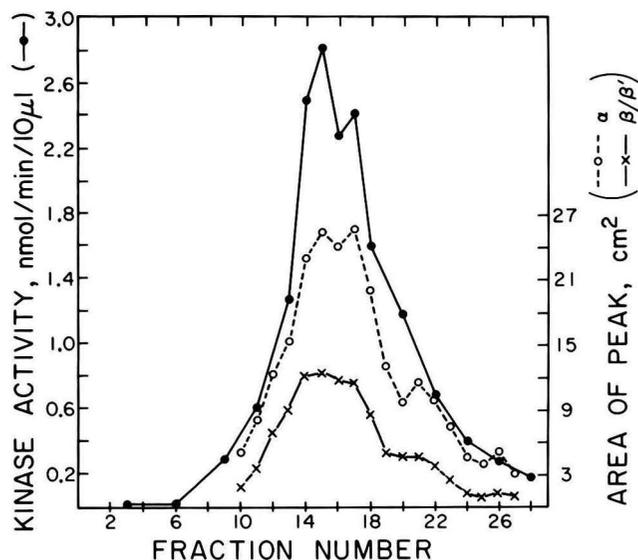


FIG. 4. Co-migration of α and β/β' peptides with kinase activity during sucrose density gradient centrifugation. A portion of the concentrated sucrose pool from purification step 8 (600 μ g) was loaded on a 4.8-ml linear gradient of 5–20% sucrose in Buffer C. The gradient was subjected to centrifugation at 65,000 rpm for 4 h in a Beckman SW 65 Ti rotor. Fractions of 0.18 ml were collected and an aliquot of each was diluted and assayed for kinase activity as described in the legend of Fig. 1. A 75- μ l aliquot of each fraction was subjected to SDS/PAGE. The gel was stained with Coomassie blue and the amount of α and β/β' peptide in each fraction was quantitated by densitometric scans.

bands peak in the same fraction as enzyme activity, and both of the peaks are asymmetric, tailing toward the bottom of the gradient. The exact ratio of the α to β/β' -bands across the gradient was slightly variable, but this probably reflects variability in the staining with Coomassie blue (see below), rather than true differences in the ratio of the bands.

Further evidence that these bands are indeed subunits of the kinase comes from the use of a monoclonal antibody raised against the partially purified kinase. Incubation of the kinase with an appropriate amount of this antibody, as described under "Experimental Procedures," resulted in the precipitation of 92–94% of the enzyme activity along with the α , β , and β' protein bands (Fig. 5). These same three proteins were also specifically precipitated from less pure enzyme fractions that contained several other proteins (data not shown). Incubation without antibody or with a control monoclonal antibody did not precipitate the enzyme activity or the protein bands. The results of the sucrose gradient experiment (Fig. 4) and the immunoprecipitation experiment (Fig. 5) indicate that the α , β , and β' proteins are contained in a complex that has calmodulin-dependent protein kinase activity.

The α and β enzyme subunits appear to be distinct peptides by two criteria. First, phosphopeptide maps of the phosphorylated forms of the two subunits are different and do not support the notion that, for example, the α -subunit was generated from the β -subunit by proteolysis (35). Second, two monoclonal antibodies that react with the α -subunit on immunoblots, do not react with the β or β' -subunits.² On the other hand, β' may have been generated from β by proteolysis, since phosphopeptide maps of these two subunits are consistent with this possibility (35). Further structural studies will be necessary to clarify the relationship of the two β -subunits.

The 45-kDa protein band contained in the purified kinase has been identified as actin by the following criteria (data not shown): 1) It co-migrated with skeletal muscle actin during

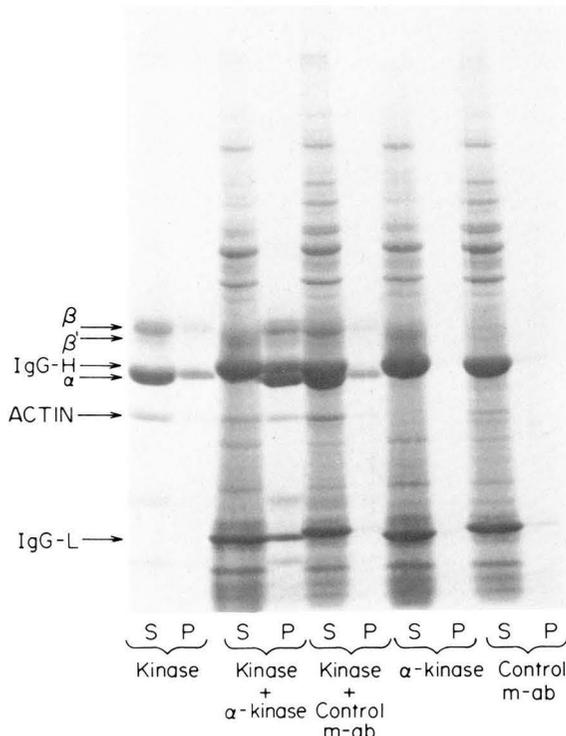


FIG. 5. Immunoprecipitation of kinase activity. Immunoprecipitation of kinase activity was carried out as described under "Experimental Procedures." The protein staining patterns of the supernatant (S) and pellet (P) from incubations containing kinase alone, kinase plus either anti-kinase antibody (α -kinase) or control monoclonal antibody (*m-ab*), and the two antibodies alone are shown. Kinase activity remaining in the supernatant after incubation with α -kinase antibody was 7.8% of the activity in the kinase control and 5.6% of the activity remaining in the supernatant after incubation with the control antibody. The lightly staining, low molecular weight bands seen in the kinase supernatant and in the immunoprecipitate are breakdown products which gradually appear after prolonged storage. These breakdown products are recognized by the α -kinase antibody on immunoblots and are precipitated with the enzyme activity (*kinase + α -kinase pellet*). *IgG-H* and *IgG-L* refer to the heavy and light chains of mouse IgG, respectively.

SDS/PAGE. 2) Radioiodination of the 45-kDa and skeletal muscle actin bands followed by digestion with trypsin produced essentially identical iodinated tryptic peptides. 3) A monoclonal antibody that recognizes only the 45-kDa protein on immunoblots, also recognized skeletal muscle actin. The amount of actin in the purified enzyme fractions has varied from 2 to 5% as measured by densitometry of stained gels (see Fig. 6). It appeared to be in the form of heterogeneous F-actin since it was distributed uniformly throughout both the gel filtration and sucrose gradient fractions. Because the amount of actin present was variable, and decreased in proportion to protein kinase with each purification step (see Fig. 1), we have concluded that it is a persistent contaminant and is not a stoichiometric part of the holoenzyme complex. However, we cannot rule out that the kinase has a specific but low affinity actin-binding site that results in the co-purification of actin. It is of interest that a portion of the actin was precipitated with enzyme activity by the anti-kinase monoclonal antibody (Fig. 5). This may have been the result of a specific interaction between actin and the enzyme, or could simply have been caused by trapping of F-actin in the antibody-enzyme matrix.

Two other low molecular weight protein bands appear slowly in the purified kinase when it is stored at 0 °C. These can be seen in Fig. 5, one slightly above and one below the

light chain of IgG. These are not present in freshly purified kinase (Fig. 6), thus we have concluded that they are breakdown products of the kinase.

Determination of the Subunit Composition of the Holoenzyme—Densitometric scans of gels stained with both Coomassie blue and fast green were used to determine the molar ratio of the synapsin I kinase subunits (Fig. 6). As in the experiment of Fig. 4, the β and β' -subunits were treated as a single subunit for this analysis. The areas of each of the optical density peaks were normalized by dividing the area of the peak by the apparent molecular weight of the protein band. The molar ratio of the subunits was then calculated as the ratio of the normalized peak areas. Results from the fast green-stained gel (Fig. 6A) indicated a molar α to β/β' ratio of 3:1, whereas those from the Coomassie blue-stained gel (Fig. 6B) showed a ratio of 5:1. Coomassie blue-stained gels have shown variable subunit ratios (ranging from 2.5:1 to 5:1), while fast green-stained gels have consistently shown a 3:1 ratio. For this reason, we believe the 3:1 ratio is more reliable. Determination of the exact subunit ratio will await the results of protein sequencing experiments.

The approximate molecular weight of the kinase holoenzyme was determined from its hydrodynamic properties. The Stokes radius of the enzyme, as determined by gel filtration on Sepharose 4B (Fig. 1), was 95 Å, and the sedimentation coefficient ($s_{20,w}$), as determined by sucrose density gradient centrifugation (Fig. 2), was 16.4 S. From these values, a molecular weight of 650,000 and a frictional ratio of 1.67 was calculated for the kinase holoenzyme as described in Table II.

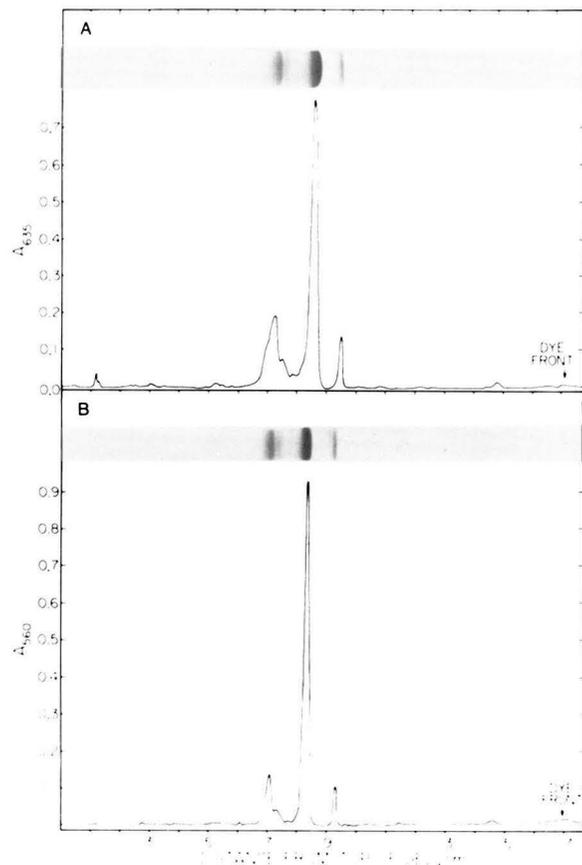


FIG. 6. Densitometric scans of fast green and Coomassie blue-stained gels of the purified kinase. A, fast green-stained gel (22.5 μ g of purified kinase) and densitometric scan at 635 nm. B, Coomassie blue-stained gel (7.5 μ g, of purified kinase) and densitometric scan at 560 nm.

TABLE II

Physical properties of rat brain calmodulin-dependent protein kinase

Property	Method of determination	Value
Stokes radius (a) ^a	Gel filtration	$94.7 \pm 1.2 \text{ \AA}$
Sedimentation coefficient ($S_{20,w}$) ^a	Sucrose density gradient	$16.4 \pm 0.7 \text{ S}$
Molecular weight (M_r) ^b	Stokes radius and sedimentation coefficient	650,000
Frictional ratio (f/f_0) ^c	Stokes radius and sedimentation coefficient	1.67
Subunit structure ($\alpha:\beta'/\beta$)	Molecular weight and subunit ratio	9:3 ($M_r = 654,000$) 10:2 ($M_r = 644,000$)

^a Values shown are mean \pm S.D. of five separate experiments.^b Calculated according to the equation

$$M_r = 6\pi\eta_{20,w} \cdot s_{20,w} \cdot a \cdot N / (1 - \bar{v}\rho_{20,w})$$

where N = Avogadro's number, $\eta_{20,w}$ = viscosity of water at 20 °C, $\rho_{20,w}$ = density of water at 20 °C, and \bar{v} = partial specific volume, for which a value of 0.725 ml/g was assumed (53).

^c Calculated according to the equation (53)

$$f/f_0 = a(4\pi N/3\bar{v}M_r)^{1/3}$$

These numbers indicate that the kinase is a large, somewhat elongated molecule made up of multiple subunits.

From the molecular weight of the enzyme and the ratio of its subunits, we can deduce the subunit structure of the holoenzyme. A subunit ratio ($\alpha:\beta/\beta'$) of 3:1 would indicate a holoenzyme consisting of nine α -subunits and three β/β' -subunits, whereas a molar ratio of 5:1 would indicate a holoenzyme consisting of 10 α -subunits and two β/β' -subunits. The physical properties of the kinase are summarized in Table II.

Identification of the Calmodulin-binding Subunits—Calmodulin-binding proteins in the purified kinase were identified by a calmodulin SDS/PAGE overlay technique (54). ^{125}I calmodulin bound specifically to the α , β , and β' -subunits (Fig. 7). The binding was nearly completely inhibited by trifluoperazine. Calmodulin did not bind to actin or to bovine serum albumin included in gels as a control (data not shown). The binding of ^{125}I calmodulin at the dye front may have been due to interaction with small breakdown products of the kinase. The amount of ^{125}I calmodulin bound to the α and β/β' -subunits was determined by γ -emission spectrometry of excised gel pieces. The ratio of ^{125}I bound to the α -subunit to that bound to the β/β' -subunit was 2.9:1. This result is consistent with a 3:1 subunit ratio, if each subunit has one calmodulin binding site/molecule.

Autophosphorylation of the Subunits—Incubation of the purified kinase under standard assay conditions resulted in phosphorylation of both the α - and β -subunits. The time course of this autophosphorylation is shown in Fig. 8. The incorporation of phosphate was both rapid and stoichiometrically significant. After 1 min, each subunit incorporated at least 1 mol of [^{32}P]phosphate/mol of protein. After 10 min, the α -subunit contained 2 mol of phosphate and the β -subunits 3 mol/mol of protein. As phosphorylation proceeded, labeled protein bands with apparent molecular weights of 64,000 and 54,000 gradually appeared. The appearance of

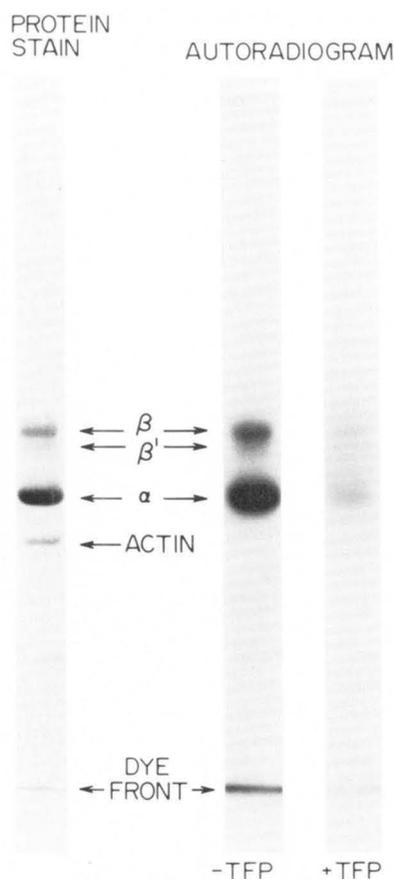


FIG. 7. ^{125}I Calmodulin binding to kinase subunits. Purified calmodulin was iodinated by the lactoperoxidase method to a specific activity of 3.6×10^5 cpm/ μg as described by Carlin *et al.* (54). ^{125}I calmodulin co-migrated with unlabeled calmodulin during SDS/PAGE and was able to activate the purified synapsin I kinase. Purified kinase (6 μg /lane) was subjected to SDS/PAGE. The binding of ^{125}I calmodulin to peptides in the gel was carried out as described by Carlin *et al.* (54) in the absence (-TFP) or presence (+TFP) of 1.0 mM trifluoperazine. Following ^{125}I calmodulin binding, the gels were stained with Coomassie blue, and labeled bands were detected by autoradiography.

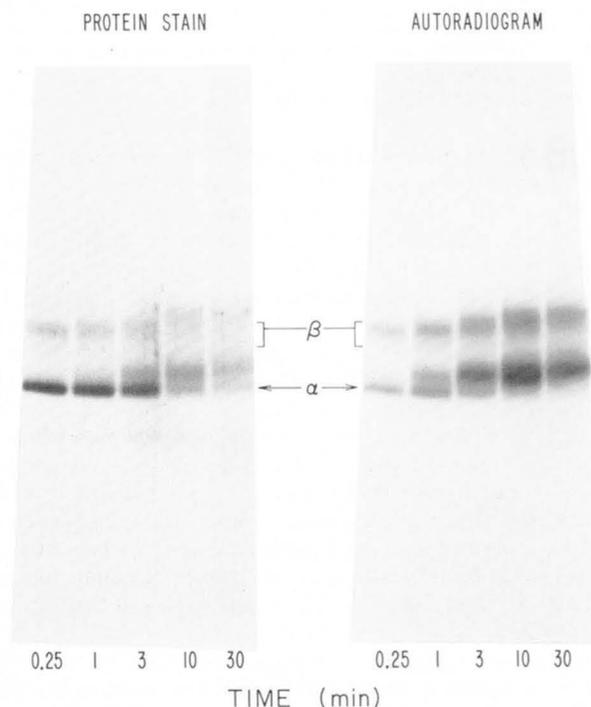


FIG. 8. Autophosphorylation of kinase subunits. Purified kinase (3.0 μg) was endogenously phosphorylated as described under "Experimental Procedures" for varying lengths of time. The enzyme subunits were separated by SDS/PAGE and stained with Coomassie blue. ^{32}P incorporation was detected by autoradiography.

TABLE III
Substrate specificity of rat brain calmodulin-dependent kinase

Substrate	Concentration in assay	Rate of calcium-stimulated phosphorylation of substrate
	mg/ml	%
Synapsin I	0.1	100 ^a
Microtubule protein ^b	0.4	35
Smooth muscle myosin light chain	0.4	34
Arginine-rich histone ^c	0.1	18
Phosvitin	0.4	15
Casein	0.4	4
Phosphorylase b	0.4	0
Glycogen synthase	0.1	0

^a The rate of calcium-stimulated phosphorylation of synapsin I was 57 pmol/min.

^b The microtubule protein sample consisted of approximately 95% tubulin and 5% microtubule-associated protein (MAP). Only MAP₂ was phosphorylated.

^c The arginine-rich histone sample consisted of approximately 30% histone H3, which was the only protein phosphorylated.

these bands was correlated with a decrease in both protein staining and ³²P content in the original α - and β -subunit bands. Our interpretation of these findings is that both subunits can be multiply phosphorylated. Phosphorylation of the first sites has no effect on mobility of the subunits on SDS gels, whereas phosphorylation of subsequent sites results in a considerable shift in mobility.

Substrate Specificity—In order to facilitate comparison of the synapsin I kinase to other protein kinases, the ability of the purified kinase to phosphorylate a number of substrates frequently used for assaying protein kinases was examined. As shown in Table III, microtubule protein, smooth muscle myosin light chain, histone H3, and phosvitin were all phosphorylated at a significant rate, whereas casein, phosphorylase b, and glycogen synthase were phosphorylated poorly or not at all. The microtubule protein consisted of both tubulin (95%) and microtubule-associated proteins (MAPs, 5%). Only MAP₂ was phosphorylated. The small amount of MAP₂ present in the assay and its relatively high incorporation of phosphate suggests that it is a particularly good substrate for the purified kinase. As a control, each of the substrates was included in an assay with synapsin I to determine if any component of the substrate was inhibiting kinase activity. The glycogen synthase (as purchased) had to be dialyzed against 40 mM Tris (pH 7.5) to remove an inhibitory component before being tested as a substrate. Ten times the usual concentration of calmodulin had to be used in the histone assays.

DISCUSSION

We have reported the purification to near homogeneity of a calcium and calmodulin-dependent protein kinase from rat brain. The kinase is a multisubunit complex with a molecular weight of about 650,000. It contains three subunits; α (50 kDa), β (60 kDa), and β' (58 kDa). The α - and β -subunits are distinct peptides, however β' may have been generated from β by proteolysis. All three of these peptides co-migrate with kinase activity during the purification steps and are co-precipitated with kinase activity by a specific anti-kinase monoclonal antibody. They all can bind calmodulin in the presence of calcium and are autophosphorylated under conditions in which the kinase is active. From the ratio of the subunits, and the holoenzyme molecular weight, we postulate a subunit composition for the kinase of approximately nine α -subunits and three β/β' -subunits.

The recovery of purified synapsin I kinase suggests that it is a relatively abundant brain enzyme constituting approximately 0.3% of the total brain protein. This estimate assumes that the losses of catalytic activity during each purification step were due to losses of enzyme protein rather than to a decrease in the activity of individual enzyme molecules. However, two additional lines of argument suggest that the estimate is essentially correct. First, the specific activity of the purified kinase is as high or higher than that of many other purified protein kinases (36–38, 55, 56). Thus, it seems unlikely that there has been a dramatic decrease in the activity of individual enzyme molecules during the purification. Second, a similarly high concentration of the α -subunit in brain homogenates has been measured by a radioimmunoassay utilizing the anti-kinase monoclonal antibodies.²

In an earlier paper (35), we suggested, on the basis of studies carried out with partially purified synapsin I kinase, that three, prominent “substrate” proteins for a calmodulin-dependent protein kinase in brain homogenates might actually be autophosphorylated subunits of the calmodulin-dependent synapsin I kinase itself. The characterization of the subunits of the purified kinase presented in this study confirms that all three of these substrate proteins are in fact part of the synapsin I kinase holoenzyme. Grab *et al.* (34) and Cohen *et al.* (57) have shown that a similar set of three substrate proteins are present in purified cerebral postsynaptic densities, fibrous structures that are located on the cytoplasmic surface of postsynaptic cells, in the region underlying the presynaptic terminal. One of these substrates is a prominent postsynaptic density protein termed the “major 52K PSD protein” by Kelly and Cotman (58). In a separate study, we have shown by biochemical and immunochemical criteria that the α -subunit of the synapsin I kinase is identical to this major 52K PSD protein (70). This suggests that the kinase may be concentrated in postsynaptic densities *in vivo* and thus may be involved in the regulation of postsynaptic as well as presynaptic processes. The presence of the kinase in postsynaptic densities could account for a portion of the synapsin I kinase that is associated with the particulate fraction of brain homogenates (35).

The α - and β -subunits of the kinase each incorporate at least two mol of phosphate/mol of protein in the presence of calcium, calmodulin, magnesium, and ATP. Incorporation of phosphate into the first site does not affect the mobility of the kinase subunits on SDS gels, whereas phosphorylation of additional sites causes a shift in their mobility (Fig. 8). Preliminary experiments have not yet revealed any large effect of this autophosphorylation on the kinetic or structural properties of the purified kinase. Another possible function of the autophosphorylation is the regulation of the association of the kinase with subcellular structures *in vivo*. We have previously noted that the synapsin I kinase is distributed between the soluble and particulate fractions in brain homogenates and that the kinases purified from each of the two fractions appear identical by several criteria (35). Recent experiments in which we examined the autophosphorylation of the kinase subunits in crude soluble and in crude particulate fractions suggested that the soluble form of the kinase, but not the particulate form, may already contain endogenous phosphate at one or more of the autophosphorylatable sites (data not shown). This supports the notion that autophosphorylation may regulate the intracellular location of the kinase.

The calmodulin-dependent synapsin I kinase is different in subunit composition and holoenzyme molecular weight from muscle myosin light chain kinase (23–25) and phosphorylase kinase (22), however, it is similar to two other recently char-

acterized calmodulin-dependent protein kinases (36–38). One of these is a calmodulin-dependent protein kinase from rat brain that phosphorylates smooth muscle myosin light chain and also other endogenous substrates. It was purified by Fukunaga *et al.* (38) and reported to be a 640-kDa holoenzyme composed of 11 to 14 identical 49 kDa subunits. It did not contain the 58- and 60-kDa proteins that we have termed β -subunits. The 49-kDa subunit was autophosphorylated in the presence of calcium, calmodulin, magnesium, and ATP. The recovery of this enzyme suggested that it could constitute as much as 0.1% of the total brain protein. The substrate specificity of this kinase resembles that of the synapsin I kinase. Both enzymes phosphorylate smooth muscle myosin light chain, microtubule-associated protein and arginine-rich histone. However, the kinase of Fukunaga *et al.* (38) phosphorylates casein well and phosphovitin poorly, whereas the synapsin I kinase phosphorylates phosphovitin at a moderate rate, and does not phosphorylate casein (Table III). Because of the close similarity of this protein kinase to the synapsin I kinase described in this report, we have made a comparison between the results of Fukunaga *et al.* (38) and our own, to determine whether the two enzymes might be related.

The first purification step used by Fukunaga *et al.* (38) was precipitation of protein at pH 6.1. This step left 99% of the calmodulin-dependent myosin light chain kinase activity in the supernatant. In contrast, we have found that precipitation at pH 6.1 leaves only about 10% of the calmodulin-dependent synapsin I kinase activity in the supernatant. The rest is irreversibly inactivated. This difference in recovery could mean either that the two protein kinase activities are properties of distinct molecules or that there is some difference in the methods of assay used by each group. Fukunaga *et al.* (38) used an incubation time of 10 min in their standard assay. We have found that the rate of phosphorylation of synapsin I falls off rapidly after about 30 s. Thus, the total incorporation of phosphate into synapsin I after 10 min is not a reflection of the initial enzymatic rate, and does not accurately measure the amount of enzyme present. In order to see whether the same situation might hold for phosphorylation of myosin light chain in brain homogenates, we determined the recovery of calmodulin-dependent myosin light chain kinase activity after pH 6.1 precipitation using both 30-s and 10-min assays. The assays were performed as described by Fukunaga *et al.* (38) with gizzard myosin light chains as substrate. We found an 80% recovery using a 10-min assay, and a 20–25% recovery using a 30-s assay (data not shown). Thus, it is possible that Fukunaga *et al.* (38) overestimated the recovery of kinase activity during their purification.

The major difference between the purification procedure used by Fukunaga *et al.* (38) and our own was this pH 6.1 precipitation step. Their other steps included chromatography methods similar to our own. We think that it is possible that their protein kinase contains α -subunits identical to those we have described, but has lost the β -subunits, perhaps at the acid precipitation step. Although a molecular weight of 640,000 was estimated for their enzyme from gel filtration data, its hydrodynamic properties indicate that it is actually smaller than the synapsin I kinase described in this report. Its Stokes radius, determined by gel filtration, is 81 Å and its sedimentation coefficient, determined by sucrose density gradient centrifugation, is 15.3 S (38), whereas the comparable values for the synapsin I kinase are 95 Å and 16.4 S. The molecular weight of the Fukunaga kinase, calculated from both its Stokes radius and its sedimentation coefficient as described in Table II, would be ~512,000, 140,000 less than the value of 650,000 calculated for the synapsin I kinase. This

apparent difference in size is consistent with the notion that the difference between the two kinases could be the presence or absence of the β -subunits.

The evidence that the β -subunits are a part of the complex that contains synapsin I kinase activity comes from two distinct experiments and is quite strong (see “Results” and previous “Discussion”). However, there have been reports that under some circumstances, protein kinases can form multienzyme complexes with other functionally related molecules (59, 60). We therefore sought to determine whether the β -subunits might be one of two previously described calmodulin-binding proteins with molecular weights of about 60,000, calcineurin A, or calmodulin-dependent cyclic nucleotide phosphodiesterase. Calcineurin is an abundant brain protein thought to have protein phosphatase activity (61). Calcineurin A, its larger, calmodulin-binding subunit, has a molecular weight of 61,000 (62). We obtained polyclonal affinity-purified rabbit anti-calcineurin from Dr. Claude Klee and tested by the immunoblot procedure (see “Methods”) for the presence of calcineurin at various stages in the purification of the kinase. Calcineurin was easily detected in the 170,000 \times g brain supernatant but was not detectable in the final purified kinase preparation (data not shown). Calmodulin-dependent cyclic nucleotide phosphodiesterase is a dimer of two identical calmodulin-binding 59-kDa subunits (63, 64). When purified from brain, it has a specific activity of 160–300 $\mu\text{mol}/\text{min}/\text{mg}$ and is quite stable. We tested for the presence of this protein at various stages in the purification of the kinase by measuring phosphodiesterase activity according to the method of Rangel-Aldao *et al.* (52). We found a level of calcium-activated phosphodiesterase activity in the 170,000 \times g supernatant that was consistent with that reported by other laboratories (63, 64). However, the specific activity in the purified kinase was less than 0.008 $\mu\text{mol}/\text{min}/\text{mg}$, the limit of detection in this assay (data not shown). Thus, the β -subunits do not appear to be either calcineurin A or calmodulin-dependent phosphodiesterase. It should be emphasized, however, that we do not know the specific functions within the kinase holoenzyme of the α - or the β -subunits. Thus, the possibility remains that one of the subunits may have a function that is not directly involved in catalysis of protein phosphorylation.

The second, recently characterized, calmodulin-dependent protein kinase that resembles synapsin I kinase is glycogen synthase kinase. It is a holoenzyme of about 300,000 Da, composed of roughly equal amounts of autophosphorylatable subunits of 50 and 53 kDa (36, 37). The association of autophosphorylatable, 50–53 kDa subunits into a multimeric holoenzyme is reminiscent of the structure of synapsin I kinase. However, there appear to be significant differences in the substrate specificities of the two enzymes. For example, although both of them phosphorylate smooth muscle myosin light chain (36, 37), synapsin I kinase does not phosphorylate glycogen synthase (see Table III). In addition, casein is a poor substrate for synapsin I kinase (Table III), but a good substrate for glycogen synthase kinase (Ref. 36, but see Ref. 37). Nevertheless, the structural similarities of these two kinases suggest that they may be members of a family of related calmodulin-dependent protein kinases that have evolved from a common precursor.

Although the physiological role of the calmodulin-dependent synapsin I kinase is not yet known, several of its properties suggest that it is involved in the regulation of both pre- and postsynaptic functions. For example, one of its brain substrate proteins, synapsin I, is located primarily in synaptic terminals (65, 66) where it is specifically associated with synaptic vesicles (67). Phosphorylation of synapsin I by the calmodulin-

dependent synapsin I kinase can be triggered both *in vivo* and *in vitro* by depolarization of terminals in the presence of calcium (68, 69). In addition, we have recently shown that the synapsin I kinase itself may be a prominent constituent of certain brain postsynaptic densities based on the identity of one of its subunits with the "major 52K postsynaptic density protein" first described by Kelly and Cotman (58, 70). The molecular characterization of synapsin I kinase presented in this report, and the generation of antibodies that specifically recognize it,² will aid in the elucidation of its full range of substrate proteins, and of its distribution in different parts of the nervous system and within individual nerve cells. Such information should be useful in the development of a molecular model for synaptic function and its regulation.

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