Purification and Characterization of a Ca^{2+}/Calmodulin-Dependent Protein Kinase from Rat Brain†

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ABSTRACT: A soluble Ca^{2+}/calmodulin-dependent protein kinase has been purified from rat brain to near homogeneity by using casein as substrate. The enzyme was purified by using hydroxylapatite adsorption chromatography, phosphocellulose ion-exchange chromatography, Sepharose 6B gel filtration, affinity chromatography using calmodulin–Sepharose 4B, and ammonium sulfate precipitation. On sodium dodecyl sulfate ($NaDodSO_4$)–polyacrylamide gels, the purified enzyme consists of three protein bands: a single polypeptide of 51 000 daltons and a doublet of 60 000 daltons. Measurements of the Stokes radius by gel filtration (81.3 ± 3.7 Å) and the sedimentation coefficient by sucrose density sedimentation (13.7 ± 0.7 S) were used to calculate a native molecular mass of 460 000 ± 29 000 daltons. The kinase autophosphorylated both the 51 000-dalton polypeptide and the 60 000-dalton doublet, resulting in a decreased mobility in $NaDodSO_4$ gels. Comparison of the phosphopeptides produced by partial proteolysis of autophosphorylated enzyme reveals substantial similarities between subunits. These patterns, however, suggest that the 51 000-dalton subunit is not a proteolytic fragment of the 60 000-dalton doublet. Purified Ca^{2+}/calmodulin-dependent casein kinase activity was dependent upon Ca^{2+}, calmodulin, and ATP·Mg^{2+} or ATP·Mn^{2+} when measured under saturating casein concentrations. Co^{2+}, Mn^{2+}, and La^{3+} could substitute for Ca^{2+} in the presence of Mg^{2+} and saturating calmodulin concentrations. In addition to casein, the purified enzyme displayed a broad substrate specificity which suggests that it may be a "general" protein kinase with the potential for mediating numerous processes in brain and possibly other tissues.

Adenosine cyclic 3′,5′-phosphate (cAMP)[1] has been recognized as a ubiquitous second messenger in eukaryotic cells (Robison et al., 1971). Once elevated in response to an extracellular stimulus, cAMP functions to activate a specific protein kinase. The cAMP-dependent protein kinase and its isozymes appear to be the sole receptors for cAMP, functioning as the transducer for the cAMP message (Nimmo & Cohen, 1977; Greengard, 1978). These enzymes are "general" protein kinases in the sense that they will phosphorylate a wide range of protein substrates while maintaining a degree of selectivity. In this scheme, the selectivity of the cAMP effect is achieved by the presence or absence of substrates in various tissues for the cAMP-dependent protein kinase.

Extracellular signals can also give rise to increased intracellular calcium levels. Intracellular Ca^{2+} concentrations normally ranging from 10 to 100 nM can increase to 1–10 μM upon stimulation (Kretsinger, 1979). It has been proposed, by analogy to the cAMP system, that intracellular Ca^{2+} fluxes act as a second messenger and that some of the actions of calcium are mediated by protein phosphorylation (Greengard, 1978; Schulman, 1982). This notion is based on the finding of Ca^{2+}/calmodulin-dependent protein kinases and endogenous substrates in membrane preparations from brain and other rat tissues (Schulman & Greengard, 1978a,b). More recent studies demonstrate that numerous soluble proteins can be phosphorylated in situ in a variety of systems (Schulman, 1982). For example, addition of α-agonists, vasopressin, or angiotensin II results in the Ca^{2+}-dependent phosphorylation of a minimum of nine cytosolic proteins in isolated hepatocytes (Garrison & Wagner, 1982; Garrison et al., 1984). The phosphorylation of five of these cannot be accounted for by the action of the cAMP-dependent protein kinase, phosphorylase kinase, or protein kinase C. Stimulation of exocrine pancreas with secretagogues leads to a Ca^{2+}-dependent phosphorylation of ribosomal protein S6 (Freedman & Jamieson, 1983), and stimulation of adrenal chromaffin cells by acetylcholine leads to the Ca^{2+}-dependent phosphorylation of tyrosine hydroxylase (Haycock et al., 1983).

The possibility that various tissues contain a soluble counterpart of the membrane-bound Ca^{2+}/calmodulin-dependent protein kinase led us to search for its presence in brain. Prior to the initiation of these studies, two Ca^{2+}/calmodulin-dependent protein kinases were purified. They are phosphorylase kinase (Cohen et al., 1978) and myosin light chain kinase (Pires & Perry, 1977; Hathaway & Adelstein, 1979). The substrate specificity of each of these enzymes is rather narrow; therefore, they are unlikely to serve the multifunctional role postulated for a general protein kinase.

Since the substrate specificity of the hypothesized general Ca^{2+}/calmodulin-dependent protein kinase is not known, we reasoned that casein, which is phosphorylated by many known protein kinases, would enable us to identify and purify the major Ca^{2+}/calmodulin-dependent protein kinase. We report here the purification and characterization of a Ca^{2+}/calmodulin-dependent protein kinase from rat brain using casein as substrate. The enzyme is also capable of phosphorylating glycogen synthase and numerous other substrate proteins and thus has the potential to be a multifunctional protein kinase.

Experimental Procedures

Materials

Proteins. Thyroglobulin, catalase, and aldolase were pur-

† From the Department of Pharmacology, Stanford University School of Medicine, Stanford, California 94305. *Received March 22, 1984.* This investigation was supported by a grant from the National Institutes of Health (GM 30179), a Basil O'Connor Starter Research Grant (5-383) from the March of Dimes Foundation, and a Biomedical Research Support Grant (RR5353) from the National Institutes of Health. J.K. was supported by an NIH Predoctoral Training Grant (GM 07149).

[1] Abbreviations: cAMP, adenosine cyclic 3′,5′-phosphate; cGMP, guanosine cyclic 3′,5′-phosphate; BSA, bovine serum albumin; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; $NaDodSO_4$, sodium dodecyl sulfate; PEG, poly(ethylene glycol); $PhCH_2SO_2F$, phenylmethanesulfonyl fluoride; Pipes, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid); Tris, tris(hydroxymethyl)aminomethane; GABA, 4-aminobutyric acid.

0006-2960/84/0423-5495$01.50/0

chased from Pharmacia Fine Chemicals. BSA, leupeptin, phosvitin, chymotrypsin, hydrolyzed casein (5%, dephosphorylated), phosphorylase *b*, ovalbumin, trypsinogen, β-lactoglobin, and lysozyme were obtained from Sigma Chemical Co. Bovine serum γ-globulin was obtained from Bio-Rad Laboratories. *Staphylococcus aureus* V8 protease was obtained from Miles Biologicals. Calmodulin was prepared from bovine brain as previously described (Schulman & Greengard, 1978b). The catalytic subunit of the cAMP-dependent protein kinase was prepared from bovine brain by published procedures (Beavo et al., 1974). Rabbit liver glycogen synthase was kindly provided by Dr. T. R. Soderling. Smooth muscle myosin light chains were generously provided by Dr. R. Adelstein.

Column Chromatography. Hydroxylapatite (Bio-Gel HTP) was purchased from Bio-Rad Laboratories, phosphocellulose (P11) was from Whatman, and Sepharoses 4B and 6B were from Pharmacia Fine Chemicals. Calmodulin-substituted Sepharose 4B was prepared by published procedures (Klee & Krinks, 1978).

Miscellaneous Information. [γ-^{32}P]ATP (6000 Ci/mmol) was purchased from ICN. Na^{125}I (17 Ci/mg, carrier free in 0.1 M NaOH) was purchased from New England Nuclear. ATP, GTP, and $PhCH_2SO_2F$ were obtained from Sigma. Protein assay reagent dye was obtained from Bio-Rad. Sprague-Dawley rats were obtained from Simonsen Laboratories. Glass fiber filters were purchased from Whatman.

Methods

Phosphorylation Assays. During enzyme purification, Ca^{2+}/calmodulin-dependent casein kinase activity was assayed by a modification of the procedure described for casein kinase II (Glover et al., 1983). Under standard conditions, the reaction mixture contained 50 mM Pipes, pH 6.8, 1 mg/mL BSA, 20 μg/mL calmodulin, 15 mM $MgCl_2$, 1.25 mg/mL casein, and either 200 μM EGTA (minus calcium) or 200 μM EGTA and 500 μM $CaCl_2$ (plus calcium). Assays were started by the addition of 20 μM [γ-^{32}P]ATP (2.5 Ci/mmol) to a final volume of 100 μL. Incubations were conducted at 30 °C for 3 min in duplicate unless otherwise specified. Assays were stopped by the addition of 10 μL of 300 mM EDTA, after which the entire reaction mix was spotted on a glass fiber filter (24-mm diameter). Filters were rinsed in four changes of 10% trichloroacetic acid and 100 mM sodium pyrophosphate, followed by one rinse each of 70% and 100% ethanol (5 min for each rinse; 4–10 mL/filter). Treated filters were then dried under a heat lamp, and Cerenkov radiation was counted in a scintillation spectrometer. Enzyme was diluted so that less than 5% of the ATP substrate was utilized. Characterization of the purified kinase was performed with the same assay; however, ^{32}P incorporation resulting from autophosphorylation was determined by assaying in the absence of casein, and this value was subtracted from ^{32}P incorporation obtained in the presence of casein.

Ca^{2+}/calmodulin-dependent protein kinase activity toward substrates other than casein was determined by using a NaDodSO$_4$–polyacrylamide gel assay. Reactions were made up and started as above, omitting BSA. Assays were stopped by the addition of 50 μL of "stop" solution, containing 9% NaDodSO$_4$, 190 mM Tris-HCl, pH 6.8, 25% glycerol, and trace amounts of the tracking dye bromphenol blue. Samples were boiled for 2 min and then analyzed by 7, 9, or 12% NaDodSO$_4$–polyacrylamide gel electrophoresis as described (Nose & Schulman, 1982). Protein was visualized by Coomassie brilliant blue or by silver staining (Morrissey, 1981). Gels were calibrated by using phosphorylase *b* (M_r 94 000), BSA (M_r 68 000), ovalbumin (M_r 45 000), trypsinogen (M_r 24 000), β-lactoglobin (M_r 18 400), and lysozyme (M_r 14 300) as standards. ^{32}P incorporation was visualized by autoradiography on Kodak BB-1 X-ray film utilizing a Cronex lightning plus intensifying screen. Radiolabeled bands were quantitated by measuring Cerenkov radiation in a scintillation spectrometer.

Purification of Ca^{2+}/Calmodulin-Dependent Casein Kinase. (*1*) *Homogenization.* Brains (1.5 g each) from 50 male rats were rapidly removed and homogenized in groups of 10 with 10 volumes (v/w) of 50 mM $NaKHPO_4$, pH 7.0, 1 mM EDTA, 400 μM $PhCH_2SO_2F$, 1 mg/L leupeptin, and 0.5 mM DTT (homogenization buffer) for three 30-s bursts in a Waring blender at 4 °C. Each burst was separated by about 2 min of rest. All subsequent procedures were at 4 °C. Pooled homogenates were centrifuged at 50000*g* for 60 min in a Beckman type 19 rotor. The pellet was reextracted with 3 volumes of homogenization buffer and recentrifuged as above. The two supernatants were then pooled and clarified with a final centrifugation at 50000*g* for 60 min in the type 19 rotor.

(*2*) *Hydroxylapatite Chromatography.* Fresh extract was made 100 mM in $NaKHPO_4$ by the addition of 2 M $NaKHPO_4$ with stirring at 4 °C. Hydroxylapatite (200 mL) preequilibrated in 100 mM $NaKHPO_4$, pH 7.0, 1 mM EDTA, 400 μM $PhCH_2SO_2F$, 1 mg/L leupeptin, and 0.5 mM DTT (buffer A) was then added to fraction 1 and stirred for 1 h. The solution was decanted after the resin settled and rinsed 3 times with 1 L of buffer A (15 min each). The slurry was finally resuspended in 500 mL of buffer A and poured into a 5-cm-diameter column, and protein was eluted with a linear gradient (1800 mL) of 100–450 mM $NaKHPO_4$ in buffer A. The Ca^{2+}-dependent casein kinase activity eluting as a broad peak between 160 and 260 mM $NaKHPO_4$ was pooled.

(*3*) *$(NH_4)_2SO_4$ Precipitation.* Solid $(NH_4)_2SO_4$ (40% saturation) was added to the hydroxylapatite pool with stirring for 1 h. Enzyme was recovered following centrifugation for 15 min at 20000*g* in a Sorvall SS-34 rotor and resuspended in 15 mL of buffer A. The solution was hand-homogenized in a Teflon–glass homogenizer and clarified by a 15-min centrifugation as above.

(*4*) *Phosphocellulose Chromatography.* Fraction 3 enzyme was applied to a 1.4 × 15 cm column of phosphocellulose equilibrated in buffer A. The column was rinsed with 2 bed volumes (45 mL) of buffer A and enzyme eluted with a 250-mL linear gradient of 50–400 mM $NaKHPO_4$. A minor peak, representing approximately 5% of the recovered activity, was eluted at 50 mM $NaKHPO_4$. This activity may represent a degradation product of the enzyme or a distinct but minor Ca^{2+}-dependent kinase activity. The major peak of activity eluting between 125 and 175 mM $NaKHPO_4$ was pooled.

(*5*) *Second $(NH_4)_2SO_4$ Precipitation.* Solid $(NH_4)_2SO_4$ (60% saturation) was added to fraction 4 with stirring for 30 min. Enzyme was collected by centrifugation as above and resuspended in 3 mL of 175 mM $NaKHPO_4$, pH 7.0, 1 mM EDTA, 400 μM $PhCH_2SO_2F$, 1 mg/L leupeptin, 0.5 mM DTT, and 1% PEG 300 (buffer B) by hand-homogenization in a Teflon–glass homogenizer.

(*6*) *Sepharose 6B Chromatography.* Fraction 5 was applied to 1.6 × 81 cm column of Sepharose 6B equilibrated in buffer B and chromatographed at a flow rate of 10 mL/h. Activity eluted as a symmetrical peak in the included volume (K_{av} of 0.43) and was pooled as fraction 6.

(*7*) *Calmodulin–Sepharose 4B Chromatography.* Fraction 6 was made 1.2 mM in $CaCl_2$ by addition of 100 mM $CaCl_2$ and applied to a 0.7 × 8 cm column of calmodulin–Sepharose

equilibrated in buffer B plus 1.2 mM $CaCl_2$. The column was rinsed sequentially with 2 bed volumes of buffer B plus 1.2 mM $CaCl_2$ and 10 bed volumes of calcium-containing buffer B without $PhCH_2SO_2F$ and leupeptin. Activity was eluted with 150 mM $NaKHPO_4$, pH 7.0, 1 mM EGTA, 0.5 mM DTT, 1% PEG 300, and 10% glycerol, pooled, and stored at −70 °C. This material is referred to as purified enzyme.

Velocity Sedimentation. Sixteen micrograms of the purified enzyme was diluted to 100 μL with water and layered onto preformed 3.6-mL 5–20% sucrose gradients containing 5% glycerol, 50 mM Pipes, pH 6.8, 225 mM NaCl, 0.5 mM DTT, and 1 mM EDTA in 11 × 60 mm tubes. Standards were diluted in 100 μL and applied to identical gradients. All tubes were centrifuged at 36000*g* in a Beckman SW 56 rotor for 9–14 h at 5 °C. Fractions (125 μL) were collected from the bottom of the tubes and assayed for casein kinase or protein as described. Standards utilized were porcine thyroglobulin ($s_{20,w}$ = 19.4 S), bovine catalase ($s_{20,w}$ = 11.3 S), rabbit muscle phosphorylase *b* ($s_{20,w}$ = 8.4 S), and BSA ($s_{20,w}$ = 4.6 S).

Iodination of Calmodulin. Purified calmodulin (200 μg) was iodinated (0.16 mL total volume) with 1,3,4,6-tetrachloro-3α,6α-diphenylglycoluril (2 μg) and $Na^{125}I$ (1 mCi) in 300 mM potassium phosphate buffer, pH 8.0 (Fraker & Speck, 1978). The reaction was stopped after 5 min by the addition of 50 μL of 10 mg/mL tyrosine. Following the addition of BSA carrier (1 mg), the iodinated calmodulin was separated from $Na^{125}I$ by passage through a Sephadex G-25 column (1 × 10 cm) with elution buffer (150 mM NaCl in 40 mM potassium phosphate buffer, pH 7.4). Fractions containing ^{125}I-calmodulin were pooled and stored at −20 °C. The specific activity of the ^{125}I-calmodulin was 28 Ci/mmol as determined by γ emission measured at 71% efficiency.

Binding of ^{125}I-Calmodulin to Proteins in NaDodSO$_4$ Gels. Calmodulin binding proteins were detected in $NaDodSO_4$ gels by a modification of published procedures (Carlin et al., 1981; Blank et al., 1983). Cytosolic extract (130 μg) or purified casein kinase (1.3 μg) was prepared for electrophoresis by heating to 37 °C for 3 min in stop solution and resolved on 9% $NaDodSO_4$ gels as described. The separating gel was cast with 50 μg/mL fibrinogen. After electrophoresis, gels were treated with 20% 2-propanol in 50 mM Tris-HCl, pH 7.0 (4 × 100 mL washes), for 1 h to remove $NaDodSO_4$. The gels were then equilibrated with 200 mL of 500 mM Tris-HCl, pH 7.0, 0.2 N NaCl, and 1 mM 2-mercaptoethanol containing either 1 mM $CaCl_2$ (plus Ca^{2+}) or 1 mM EGTA (minus Ca^{2+}) for 30 min, followed by 50 mL of the same buffers, but containing 2% BSA, for an additional 90 min. Binding of ^{125}I-calmodulin was accomplished by incubating the gels in the BSA-free buffers described above containing 10 μg of ^{125}I-calmodulin (5.1×10^6 cpm/μg) in sealed plastic pouches for 12 h on a rotary shaker at 20 °C. Unbound radioactivity was removed by washing gels in the BSA-free buffers for 27 h using five 200-mL washes in buffers containing either 1 mM $CaCl_2$ or 1 mM EGTA. Gels were then stained with Coomassie brilliant blue and analyzed by autoradiography as usual.

Partial Proteolysis Gels. Samples of 1–5 μg of autophosphorylated kinase were run on 9% $NaDodSO_4$–polyacrylamide gels, stained, and dried, and subunits were localized by autoradiography. Gel slices corresponding to the 51 000- and 60 000-dalton subunits were excised and reswollen in 0.0625 M Tris-HCl, 0.1% $NaDodSO_4$, and 2% 2-mercaptoethanol for 1 h. Swollen gels were loaded into the wells of a 15% $NaDodSO_4$ gel and overlayed with 50 μL of a 0.02–0.20 mg/mL solution of *S. aureus* V8 protease or chymotrypsin containing trace amounts of the tracking dye pyronin Y. Samples were proteolyzed during the 1-h electrophoresis through the stacking gel (2 cm) (50 V). Electrophoresis through the separating gel was performed in the usual manner (180 V). Gels were dried without fixation and autoradiographed to visualize ^{32}P-containing peptides.

Miscellaneous Procedures. Sepharose 6B gel filtration columns were calibrated by using porcine thyroglobulin (M_r 669 000, Stokes radius = 85.0 Å), bovine catalase (M_r 232 000, Stokes radius = 52.2 Å), and aldolase (M_r 158 000, Stokes radius = 48.1 Å) as standards. The void volume (V_0) was estimated by elution of blue dextran 2000. K_{av} values for standards were calculated from their elution volumes (V_e) by the equation $K_{av} = (V_e - V_0)/(V_t - V_0)$, where V_t = the total column volume.

Protein was assayed by the method of Bradford (1976) using bovine plasma γ-globulin as standard.

Results

Purification of Ca^{2+}/Calmodulin-Dependent Casein Kinase. Previous studies from this laboratory demonstrated the presence of a soluble Ca^{2+}/calmodulin-dependent protein phosphorylation system in bovine brain (Nose & Schulman, 1982). In the process of this investigation, we found that various rat tissues contained a similar protein kinase (Schulman et al., 1983; Schulman, 1984a). Since rat brain had a higher specific activity than any other rat tissue and a higher specific activity than bovine brain, we decided to examine the rat brain enzyme. The enzyme was labile post-mortem, and the dissected brain required rapid processing. Brain could not be frozen at −20 or −70 °C for any period of time or even stored at 4 °C for more than 15 min. This may, in fact, be the reason why bovine brain obtained from the slaughterhouse and transported back to the laboratory is inferior as a source of this enzyme. We routinely homogenized rat brains within 3 min of decapitation.

Casein was chosen as a substrate for purification because it was likely to be a substrate for any Ca^{2+}/calmodulin-dependent kinases with broad substrate specificity—the type of enzyme we were trying to identify and purify. In addition, casein kinase assays are simple, rapid, and inexpensive. Disadvantages of the assay include (1) a low rate of phosphorylation with the Ca^{2+}/calmodulin-dependent kinase and (2) a substantial rate of background phosphorylation by Ca^{2+}-independent kinases. These two considerations result in a high blank (minus Ca^{2+}) prior to the hydroxylapatite step of the purification. We therefore report our purification with the hydroxylapatite pool taken as 100% of the starting activity (Table I). Yields of purified enzyme from the hydroxylapatite pool ranged from 2.4% to 5.0% in three complete purifications using casein as substrate. Specific activities in these preparations have ranged from 3 to 45 nmol mg^{-1} min^{-1}. All experiments were performed with the preparation reviewed in Table I. Our purification of the Ca^{2+}/calmodulin-dependent protein kinase can be monitored with substrate proteins other than casein. Although phosvitin is not a convenient substrate, its phosphorylation can be more accurately determined in the cytosolic extract. Using phosvitin as a substrate, we calculate a 330-fold purification of kinase activity from cytosolic extract.

Stability. Once the tissue had been disrupted, the Ca^{2+} kinase activity was quite stable at 4 °C through fraction 5. Instability became a problem following chromatography on Sepharose 6B, with activity having a half-life of 24 h unless 10% glycerol was included at this and all subsequent manipulations. PEG (1%) had a small stabilizing effect on Sepharose 6B chromatography. The activity could be stabilized reproducibly when stored at −70 °C in the presence of 0.5 mM DTT, 10% glycerol, 1 mM EGTA, and 50–150 mM NaKH-

Table I: Purification of Ca^{2+}/Calmodulin-Dependent Protein Kinase Activity from Rat Brain

fraction	volume (mL)	protein (mg)	sp act.[a] (units/mg)	total activity[a] (units)	yield[b] (%)	x-fold purification[b]
(1) cytosol	950	4925	0.39	2000		
(2) hydroxylapatite	560	600	0.64	384	100	1.0
(3) ammonium sulfate	15	161	2.1	343	89	3.3
(4) phosphocellulose	65	29	5.7	165	43	8.9
(5) ammonium sulfate	3	19	6.1	112	29	9.5
(6) gel filtration	19	5.3	7.9	43	11	12.0
(7) affinity chromatography	9	0.58	13.2	8.9	2.4	21.0

[a] One unit of activity is defined as the amount of activity required to incorporate 1 nmol of ^{32}P into casein per min. [b] Calculation is based upon the hydroxylapatite pool as the starting material.

PO_4, pH 7.0, or with other buffers under similar conditions. Stability of freeze–thawing required glycerol and DTT; omission of glycerol results in a half-life of about two thawings.

Structural Studies. (A) Subunit Composition and Autophosphorylation. The protein composition of the fractions obtained during purification of the Ca^{2+}/calmodulin-dependent protein kinase is shown in Figure 1A. Aliquots of each fraction were incubated under phosphorylating conditions without casein or BSA in the absence (odd-numbered lanes) or presence (even-numbered lanes) of Ca^{2+} plus calmodulin. After the first chromatographic step, hydroxylapatite chromatography, the major subunit of the kinase, a protein band of M_r 51 000, becomes prominent on stained $NaDodSO_4$ gels. This is most clearly seen in the ammonium sulfate concentrate of this fraction (Figure 1A, lane 5). Upon Ca^{2+}/calmodulin-dependent phosphorylation, this subunit shifts to a higher apparent molecular weight (compare lane 6 with lane 5). This is due to autophosphorylation of the kinase (discussed below) and facilitates identification of the kinase subunits during purification. The purified enzyme preparation contains two protein bands of M_r 60 000 in addition to the M_r 51 000 peptide (Figure 1A, lane 11). Minor contaminants of M_r 100 000 are also visible in this particular preparation althought they are not consistent components of kinase preparations. The M_r 51 000 peptide and the M_r 60 000 doublet copurified with the kinase activity throughout the purification. Moreover, the three proteins comigrated through every other separation method that was attempted, including blue dextran–agarose and DEAE-cellulose. Densitometric scans of purified enzyme on $NaDodSO_4$ gels ranging from 7% to 15% acrylamide revealed no additional polypeptides of either high or low molecular weight (data not shown). Similar densitometric scans of purified enzyme were performed to assess the ratio of the 51 000-dalton polypeptide to the 60 000-dalton doublet. When 6.8 μg of purified kinase was electrophoresed and stained with Coomassie brilliant blue, the ratio of protein staining was found to be 3.6:1. Since the molecular weight ratio is 0.85:1 (an indication of dye binding capacity), the molar ratio is 4.2:1. Scans of silver-stained gels give a protein staining ratio of 3:1, corresponding to a molar ratio of 3.5:1.

A striking feature of the Ca^{2+}/calmodulin-dependent protein kinase is its autophosphorylation. Autophosphorylated bands, although faint in tissue extracts, can be seen to copurify with kinase activity (Figure 1B). In fact, the kinase activity may be purified by assaying for autophosphorylation. Incubation of purified kinase under Ca^{2+}/calmodulin-dependent phosphorylation conditions results in a mobility shift of the 51 000-dalton polypeptide to 53 000 daltons and of the 60 000-dalton doublet to 64 000 daltons. These shifts correspond to the shifts in protein staining noted above and are likely due to an effect of $NaDodSO_4$ binding to the polypeptides. The apparent decrease in protein staining intensity seen upon autophosphorylation (Figure 1A, compare lanes 11 and 12)

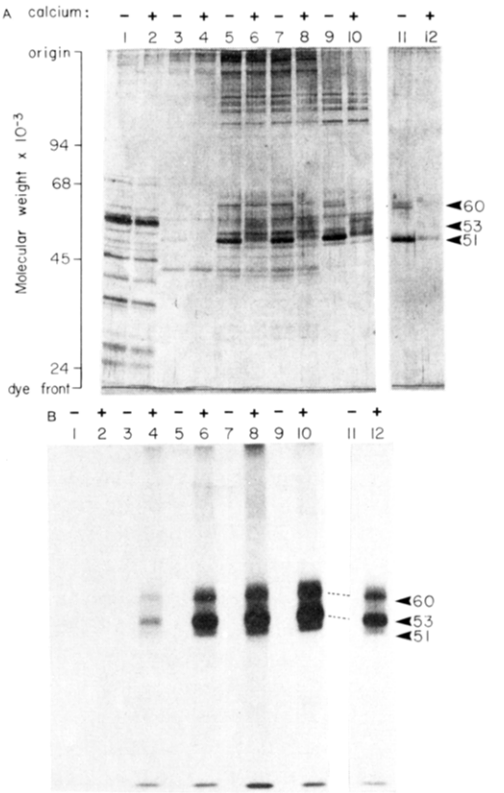

FIGURE 1: Autophosphorylation and $NaDodSO_4$–polyacrylamide gel electrophoresis of column fractions and purified Ca^{2+}/calmodulin-dependent protein kinase. An aliquot of each purification step was incubated in the standard reaction mixture containing 200 μM EGTA (minus calcium) or 200 μM EGTA and 500 μM $CaCl_2$ (plus calcium), 20 μg/mL calmodulin, 15 mM $MgCl_2$, and 20 μM [γ-^{32}P]ATP (1 Ci/mmol) in 50 mM Pipes buffer. Samples were prepared for electrophoresis and the resulting gels silver stained, dried, and autoradiographed as described under Experimental Procedures. Lanes 1 and 2, crude extract (1.1 μg); lanes 3 and 4, hydroxylapatite pool (1.0 μg); lanes 5 and 6, first ammonium sulfate pool (1.0 μg); lanes 7 and 8, phosphocellulose pool (0.8 μg); lanes 9 and 10, Sepharose 6B pool (1.1 μg); lanes 11 and 12, calmodulin–Sepharose pool (0.6 μg). Samples in lanes 11 and 12 were analyzed under identical conditions but on a separate gel from samples 1–10. (A) Silver-stained gel; (B) autoradiograph.

is due primarily to broadening of the shifted protein bands. All protein can be accounted for in densitometric scans of more heavily loaded gels (6.8 μg of protein) stained with Coomassie brilliant blue.

Although $PhCH_2SO_2F$, leupeptin, and EDTA were included in the initial preparation of brain extract, generation of the 51 000-dalton polypeptide or the smaller of the 60 000-dalton doublet by proteolysis of a larger precursor cannot be ruled out. The pattern of autophosphorylation could be seen even at the earliest steps in the purification, suggesting that the 51 000-dalton polypeptide is not a proteolysis product of the

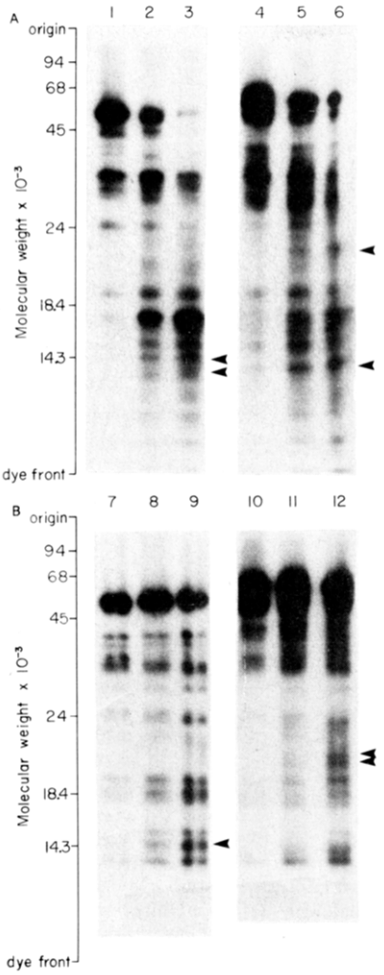

FIGURE 2: Partial proteolysis of autophosphorylated Ca^{2+}/calmodulin-dependent protein kinase subunits. Purified enzyme (0.5 μg) was autophosphorylated by incubation under standard reaction conditions in the absence of substrate and resolved on NaDodSO$_4$ gels, stained, dried, and autoradiographed to localize subunits. Gel pieces containing ^{32}P-labeled kinase subunits were subjected to limited proteolysis as described under Experimental Procedures using varying amounts of (A) *S. aureus* V8 protease or (B) chymotrypsin. Lanes 1–3, M_r 53 000 autophosphorylated subunit with 1, 5, and 10 μg of *S. aureus* V8 protease, respectively; lanes 4–6, M_r 60 000 autophosphorylated subunit with 1, 5, and 10 μg of *S. aureus* V8 protease, respectively; lanes 7–9, M_r 53 000 autophosphorylated subunit with 1, 5, and 10 μg of chymotrypsin; lanes 10–12, M_r 60 000 autophosphorylated subunit with 1, 5, and 10 μg of chymotrypsin. Exposure times for all lanes containing the M_r 60 000 subunit were 2 times longer than those for the M_r 53 000 subunit to facilitate the comparison. Major differences between the subunits are indicated by arrowheads.

60 000-dalton doublet. However, to further probe the possible proteolytic relationship between the kinase subunits, autophosphorylated subunits were compared by the method of partial proteolysis using *S. aureus* V8 protease and chymotrypsin (Cleveland et al., 1977). The phosphopeptide patterns generated are shown in Figure 2. Although the phosphopeptide patterns of the 51 000-dalton and 60 000-dalton subunits are very similar, they are not identical. Numerous differences can be seen in the high molecular weight phosphopeptides. With either protease, however, extensive homology can be seen in phosphopeptides smaller than 24 000 daltons. The differences indicated in Figure 2 suggest that the phosphopeptides from the 51 000-dalton polypeptide are not completely included within the pattern of phosphopeptides from the 60 000-dalton doublet. The 51 000-dalton subunit is therefore not a proteolytic product of the larger subunits.

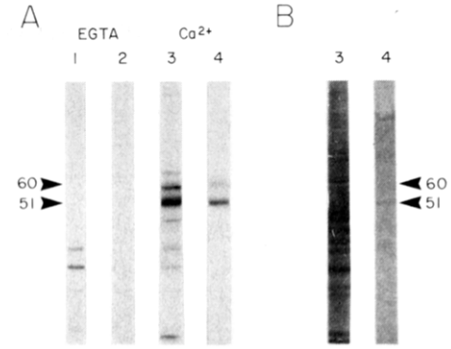

FIGURE 3: Binding of ^{125}I-calmodulin to proteins in NaDodSO$_4$ gels. (A) Cytosolic extracts (130 μg, lanes 1 and 3) and purified Ca^{2+}/calmodulin-dependent protein kinase (1.3 μg, lanes 2 and 4) were run on 9% NaDodSO$_4$ gels, and ^{125}I-calmodulin binding was analyzed as described under Experimental Procedures. Binding was performed in the presence of EGTA (lanes 1 and 2) or $CaCl_2$ (lanes 3 and 4) as described. All samples were run on the same gel. Exposure time for lanes 1 and 2 was 5 times longer than that for lanes 3 and 4 in order to visualize the weak binding seen in the absence of $CaCl_2$. (B) Protein staining of lanes 3 and 4.

In fact, the phosphorylated 51 000-dalton species can be detected and identified in freshly prepared cytosolic extracts on the basis of its characteristic pattern of V8 protease generated phosphopeptides (data not shown). The kinase subunits therefore appear to be distinct though related proteins.

(*B*) *Identification of ^{125}I-Calmodulin Binding Subunits.* ^{125}I-Calmodulin binding to denaturing gels was performed as described under Experimental Procedures in the hope of identifying the kinase subunit which confers the Ca^{2+}/calmodulin dependence to the enzyme. The ^{125}I-calmodulin possessed full calmodulin efficacy in activating the Ca^{2+}/calmodulin-dependent protein kinase (data not shown). Results of ^{125}I-calmodulin binding to both freshly prepared brain cytosol and purified enzyme are shown in Figure 3. In the presence of EGTA, a small amount of ^{125}I-calmodulin bound to two low molecular weight proteins in brain cytosol. No ^{125}I binding to purified enzyme could be detected in the absence of calcium (Figure 3, lane 2). In the presence of calcium, ^{125}I-calmodulin bound to at least 12 cytosolic protein bands of differing molecular weights, including the two bands labeled in the absence of calcium. It appears that the 51 000-dalton polypeptide and the 60 000-dalton doublet in the purified enzyme can each bind ^{125}I-calmodulin independently (Figure 3, lane 4). These results indicate that all kinase subunits have the capacity to participate in calmodulin binding.

(*C*) *Physical Characterization.* A summary of the physical characterization of the Ca^{2+}/calmodulin-dependent protein kinase is given in Table II. Analysis of gel filtration of the ammonium sulfate concentrated phosphocellulose pool on Sepharose 6B during enzyme purification indicated that the holoenzyme was large; the activity consistently eluted just after thyroglobulin. The apparent native molecular weight of the kinase compared to protein standards was 560 000 ± 26 000 (n = 3). A plot of $(-\log K_{av})^{1/2}$ vs. the Stokes radius for the standards gave a value of 81.3 ± 3.7 Å (n = 3) for the Stokes radius of the holoenzyme. From sedimentation velocity experiments on 5–20% sucrose gradients, a value of 13.7 ± 0.6 S (n = 3) was calculated for the sedimentation coefficient (Figure 4). The molecular weight, calculated from Stokes radius and sedimentation coefficient, indicates that the holoenzyme, under the conditions utilized, exists as an elongated molecule with a molecular weight of 464 000 ± 29 200.

Kinetics. All kinetic experiments were conducted with purified enzyme.

Table II: Summary of Ca^{2+}/Calmodulin-Dependent Protein Kinase Characterization

physical characterization	
Stokes radius (Å)	81.3 ± 3.7[a]
$s_{20,w}$ (S)	13.7 ± 0.6[b]
M_r	464 000 ± 29 200[c]
	560 000 ± 26 000[a]
	528 000[d]
frictional ratio (f/f_0)	1.59[c]
kinetic data	
$K_{0.5}$(calmodulin) (nM)	620[e]
	29[f]
$K_{0.5}(Ca^{2+})$ (μM)	0.7[e]
V_{max}[g] (nmol min^{-1} mg^{-1})	13.2
K_m(casein)[g] (mg/mL)	0.33
K_m(ATP)[g] (μM)	22

[a] Measured relative to internal standards by gel filtration on Sepharose 6B. [b] Measured relative to internal standards by velocity sedimentation through 5–20% sucrose gradients. [c] Calculated from the Stokes radius and sedimentation coefficient by assuming a partial specific volume of 0.725 cm^3/g as described by Siegel & Monty (1966). [d] Calculated from the subunit ratio (M_r 51 000:60 000) of 8:2. [e] Calculated by using casein as substrate. [f] Calculated by using phosvitin (0.3 mg/mL) as substrate. [g] Calculated from Figure 5.

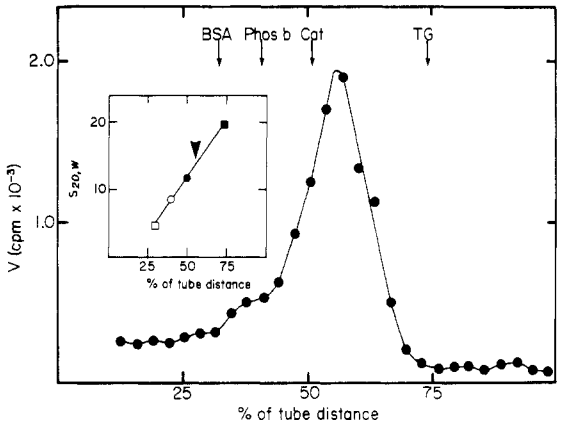

FIGURE 4: Velocity sedimentation. Purified enzyme (8 μg) was applied to a linear 5–20% sucrose gradient as described under Experimental Procedures. The sedimentation coefficient was estimated relative to standard proteins thyroglobulin (TG, ■), catalase (Cat, ●), phosphorylase *b* (Phos b, ○), and bovine serum albumin (BSA, □). Inset: Plot of $s_{20,w}$ vs. R_f (expressed as a percentage of total tube length) for protein standards. The position of kinase is shown by the arrowhead.

(*A*) *pH.* With casein as substrate, the enzyme displays activity from pH 5.5 to 8.5 with a distinct pH optimum at pH 6.6. Other substrates show maximal velocity at more alkaline pHs. For example, the velocity of glycogen synthase phosphorylation was 4-fold greater at pH 7.6 than at pH 6.8.

(*B*) *Mg^{2+} or Mn^{2+} Requirements.* In the presence of Ca^{2+} (0.3 mM free Ca^{2+}), the enzyme exhibited an absolute requirement for divalent metal ion, either Mg^{2+} or Mn^{2+}. Half-maximal stimulation was observed at 5 mM $MgCl_2$ and at 1 mM $MnCl_2$. Although Mn^{2+} was more potent than Mg^{2+}, both divalent metal ions stimulated casein kinase activity to the same maximal level.

(*C*) *Salt Effect.* The purified enzyme was inhibited by NaCl at all concentrations examined, with 50% inhibition occurring at 210 mM. Addition of phosphate was also inhibitory, with 50% inhibition occurring at 28 mM $NaKHPO_4$. This was probably due to the ability of phosphate to chelate Ca^{2+} or to serve as a competitive inhibitor of ATP.

(*D*) *Temperature.* The time course of casein phosphorylation as a function of temperature was examined between 4 and 30 °C. Linearity was observed for only the first 3–5 min of reaction at all temperatures studied. This cannot be accounted for by the intrinsic lability of the kinase and may be a consequence of autophosphorylation.

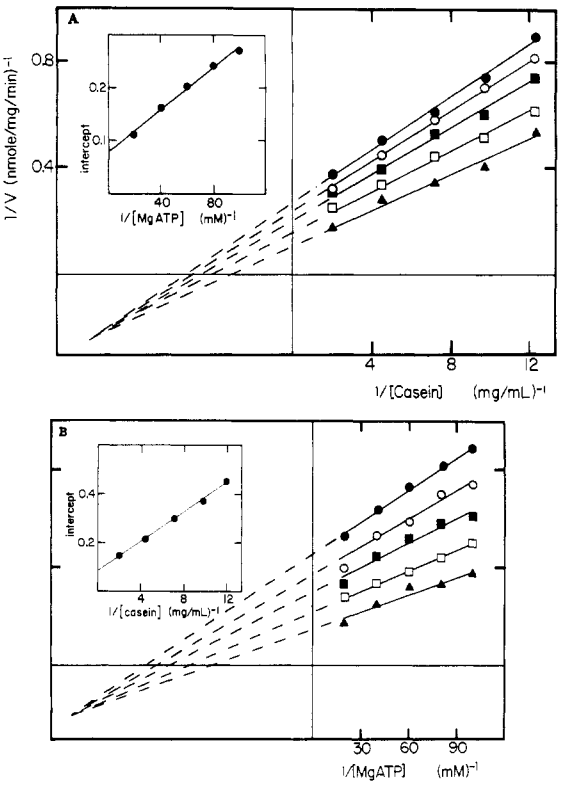

FIGURE 5: Initial velocity studies. All assays were performed in quadruplicate with 0.3 μg of purified enzyme under standard conditions. (A) Double-reciprocal plots of velocity vs. the reciprocal of casein concentration at different fixed ATP concentrations of 10 (●), 12.5 (○), 16.6 (■), 25 (□), and 50 μM (▲). Inset: Replot of $1/V$ intercept vs. $1/[ATP]$. (B) Double-reciprocal plots of velocity vs. [ATP] at different fixed casein concentrations of 2.0 (●), 4.5 (○), 7.1 (■), 9.7 (□), and 12.3 mg/mL (▲). Inset: Replot of $1/V$ intercepts vs. 1/[casein] used to calculate V_{max} and K_m for casein and ATP.

(*E*) *Initial Velocity Studies.* Initial velocity studies were carried out with either ATP or casein as the varied substrate and analyzed on double-reciprocal plots (Figure 5). When $1/V$ vs. $1/[ATP]$ was plotted for different fixed concentrations of casein, a family of lines converging below the abscissa was obtained. A plot of $1/V$ vs. $1/[casein]$ at different fixed concentrations of ATP gave a similar set of lines converging below the abscissa. These results are diagnostic of a sequential reaction mechanism and inconsistent with a ping-pong enzymatic mechanism (Segal, 1975).

Product inhibition studies confirmed these results. When Ca^{2+}/calmodulin-dependent casein kinase activity was assayed in the presence of ADP with varying ATP concentrations at constant, near-saturating casein concentration, a competitive inhibition pattern was observed. A mixed inhibition pattern resulted when ADP challenged enzyme activity under varying casein concentrations at constant ATP concentration (data now shown). Slope replots of the $1/V$ vs. $1/[casein]$ relationship as a function of ADP concentration gave an apparent K_i of 470 μM for ADP in the presence of 100 μM ATP. These results are consistent with a rapid equilibrium random Bi-Bi reaction mechanism (Segal, 1975). Sequential mechanisms have been shown for a *Neurospora* cAMP-independent protein kinase using casein as substrate (Gold & Segal, 1974), for the bovine lung cGMP-dependent protein kinase using a synthetic peptide as substrate (Glass et al., 1981), and for the *Drosophila* casein kinase II using casein as substrate (Glover et al., 1983).

The kinetic constants, K_m and V_{max}, were obtained from replots of $1/V$ intercepts vs. casein or ATP concentration

Table III: Cation Dependence of Ca^{2+}/Calmodulin-Dependent Casein Phosphorylation

cation added[a]	concn (μM)	enzyme activity (%)[b]
Ca^{2+}	10	100
none (Mg^{2+})		2
La^{3+}	10	0
La^{3+}	100[c]	119
Co^{2+}	100	117
Mn^{2+}	10	96
Sr^{2+}	100	5
Sr^{2+}	300	71
Zn^{2+}	100[c]	22
Ba^{2+}	1000	1
Cu^{2+}	1000	1

[a] All velocity measurements were performed in 15 mM $MgCl_2$. [b] Maximum enzyme activity with calcium (6 nmol mg^{-1} min^{-1}) was taken as 100% activity. [c] Higher metal concentrations resulted in precipitation of casein substrate.

(Figure 5, insets) and are summarized in Table II.

Regulation. Enzyme Activation. In the presence of Mg^{2+}, casein kinase activity was strictly dependent upon Ca^{2+} and calmodulin. Half-maximal kinase activation was observed with 620 nM calmodulin in the presence of 300 μM Ca^{2+}. Casein kinase activation was half-maximal with 0.7 μM Ca^{2+} in the presence of 2.2 μM calmodulin. The calmodulin requirement of casein phosphorylation is 1 order of magnitude greater than that for most calmodulin-dependent enzymes studied to date (Klee et al., 1980). We therefore examined the calmodulin dependence of autophosphorylation and kinase activity using phosvitin as substrate. Half-maximal activation of kinase activity with phosvitin (0.3 mg/mL) was observed at 29 nM calmodulin (Table II). This value is the same as that observed with microtubule-associated protein 2 (Schulman, 1984b) as substrate and similar to that observed with autophosphorylation (21 nM, data not shown). The high concentration of calmodulin required to activate casein kinase activity likely results from the binding of calmodulin to the substrate, hydrolyzed casein. Indeed, numerous small proteins have been shown to bind calmodulin (Malencik & Anderson, 1983).

Other metals could substitute for Ca^{2+} in activating the kinase (Table III). In the presence of calmodulin and Mg^{2+}, both Mn^{2+} and Co^{2+} were able to completely activate the kinase at concentrations as low as 10 μM and appeared to be at least as potent as Ca^{2+}. La^{3+}, though inactive at 10 μM, was fully stimulatory above 100 μM. Other metals, such as Sr^{2+}, appeared to be partial activators. Half-maximal activation with Sr^{2+} was observed at 320 μM; however, full activation was never observed, even at 1 mM concentration.

The enzyme was independent of several other second messengers. Neither cAMP nor cGMP had a stimulatory effect when present at concentrations ranging from 1 to 1000 μM. Likewise, Ca^{2+} plus diglyceride and phosphatidylserine, at concentrations that maximally activate the Ca^{2+}/phospholipid-dependent protein kinase from rat brain (Kikkawa et al., 1982), had no stimulatory effect on the enzyme.

Substrate Specificity. Initial Velocity Studies. In cytosolic extracts of brain, numerous proteins appear to be phosphorylated in a Ca^{2+}/calmodulin-dependent manner (Juskevich et al., 1983; Schulman, 1984a). In the purified state, the casein kinase described here is also capable of phosphorylating numerous exogenously added substrates. The substrate specificity of the enzyme was examined in order to characterize this kinase, to serve as a comparison with other protein kinases and as an initial screen of potential physiological substrates of such a protein kinase. Table IV lists several substrate proteins and their apparent kinetic values as determined by double-reciprocal plots using the purified kinase. Glycogen synthase was the best substrate when assayed at pH 7.6 with an apparent V_{max} of 250 nmol mg^{-1} min^{-1}. Other substrates for which a full kinetic analysis was not performed but which were significantly phosphorylated include MAP-2 and the τ proteins (which are microtubule-associated proteins) (Schulman et al., 1983; Schulman, 1984b), vimentin (which is an intermediate filament protein), ribosomal protein S6, synapsin I (which is a synaptic vesicle protein), and GABA–modulin (which is a protein modulator of GABA binding in membrane preparations) (data not shown). The kinase does therefore appear to have the broad substrate specificity expected of a general protein kinase.

Table IV: Kinetic Analysis of Selected Ca^{2+}/Calmodulin-Dependent Kinase Substrates

substrate	$V_{m,app}$ (nmol min^{-1} mg^{-1})	pH	$K_{m,app}$ (mg/mL)
glycogen synthase	240	7.6	2.9
myosin light chain[a] (smooth muscle)	185	7.6	0.87
casein	13	6.8	0.33
phosvitin	10	6.8	1.0
phosphorylase *b*	0	7.6	

[a] Smooth muscle myosin light chains contain 50% phosphorylatable P-light chain.

Discussion

We have described the purification from rat brain of a Ca^{2+}/calmodulin-dependent protein kinase that possesses a broad substrate specificity. This enzyme, purified as a Ca^{2+}/calmodulin-dependent casein kinase, is highly enriched in rat brain, as only a few hundredfold purification is required to achieve apparent homogeneity. Analysis of the purified preparation on $NaDodSO_4$ gels indicates that the enzyme consists of three polypeptides—a major polypeptide with a molecular mass of 51 000 daltons and a doublet at 60 000 daltons. We believe that these are the subunits of a 460 000-dalton holoenzyme. All three subunits bind ^{125}I-calmodulin, suggesting that each of the polypeptides can independently interact with calmodulin. We have not been able to reconstitute activity after separation of the polypeptides and therefore have not been able to determine whether each is catalytically competent as a phosphotransferase. However, each subunit can be labeled with 8-N_3-ATP (data not shown). The 51 000-dalton polypeptide appears structurally similar but distinct from the 60 000-dalton doublet. Thus, it is unlikely that the 51 000-dalton polypeptide is a proteolytic fragment of the larger subunits. At this time, we cannot exclude the possibility that the 60 000-dalton doublet, consistently seen in numerous preparations, is derived by partial proteolysis of the larger of the pair. Because autophosphorylation of the three subunits leads to a shift in their mobility on $NaDodSO_4$ gels, it is possible to detect these subunits in stained gels. They are consistently coeluted from each of the column steps in approximately the same ratio and are not resolved by additional purification steps. The precise stoichiometry of the subunits has not been determined, but the enzyme appears to consist of approximately 6–8 mol of the 51 000-dalton subunit and 1 mol of each of the two 60 000-dalton polypeptides. This would suggest a maximal molecular weight of 528 000, slightly larger than the molecular weight determined from the Stokes radius and sedimentation velocity.

The purified enzyme undergoes autophosphorylation in the presence of Ca^{2+} and calmodulin. All three of the subunits are phosphorylated. Because of the high concentration of this enzyme in brain, autophosphorylation constitutes the pre-

dominant ^{32}P incorporation in assays of endogenous phosphorylation from the earliest column steps. Most protein kinases show some degree of autophosphorylation [e.g., see Maeno et al. (1974), Rosen & Erlichman (1975), deJonge & Rosen (1977), Kikkawa et al. (1982), and Hathaway & Adelstein (1979)]. For three protein kinases, an in vitro effect of autophosphorylation has been documented. Autophosphorylation of the type II cyclic AMP dependent protein kinase, an enzyme activated by dissociation of its catalytic and regulatory subunits, dramatically reduces the rate of reassociation of its subunits and thereby the rate of inactivation of the enzyme (Rangel-Aldao & Rosen, 1976). The apparent binding affinity for cAMP (Beavo et al., 1975) is also reduced. Phosphorylase kinase undergoes autophosphorylation in the presence of Ca^{2+}, resulting in a nearly 100-fold activation (Wang et al., 1976). Autophosphorylation of the heme-regulated eukaryotic initiation factor 2α (eIF-2α) kinase increases its ability to phosphorylate eIF-2α and enhances its inhibition of protein synthesis (Fagard & London, 1981). The effect of autophosphorylation on the Ca^{2+}/calmodulin-dependent casein kinase is not known.

A possible explanation for autophosphorylation of this and some other protein kinases is that the phosphoenzyme is an intermediate of the phosphotransferase reaction. Our observation of a sequential enzymatic mechanism makes it unlikely that autophosphorylation represents an intermediate in catalysis. In addition, the dramatic shift of the 51 000-dalton subunit subsequent to autophosphorylation suggests a nearly stoichiometric incorporation of phosphate. Low levels of phosphorylation of the 51 000-dalton subunit do not shift its mobility, but incorporation of additional phosphate shifts it to an apparent molecular mass of 53 000 daltons. The 60 000-dalton subunits are also shifted to higher molecular mass although this is harder to detect because of their lower concentration. Similar shifts have been seen with several other kinases undergoing autophosphorylation, including the type II cAMP-dependent protein kinase (Rangel-Aldao et al., 1979) and the heme-regulated eIF-2α kinase (London et al., 1981). These shifts may be the result of disruption of the normal interaction of the polypeptides with $NaDodSO_4$ and suggest that the site(s) on the polypeptide responsible for the shift is (are) vacant in the isolated enzyme. This also implies that the same Ca^{2+}/calmodulin-dependent protein kinase isolated with some endogenous phosphate at those sites would appear to have both a 51 000- and a 53 000-dalton subunit. It is possible that the 60 000-dalton doublet may be a single polypeptide chain that contains different degrees of endogenous phosphate.

The Ca^{2+}/calmodulin-dependent casein kinase described here has many features that are similar to those recently reported for other kinases. Fukanaga et al. (1982), Yamauchi & Fujisawa (1983), Goldenring et al. (1983), and Bennett et al. (1983) have purified Ca^{2+}/calmodulin-dependent protein kinases by using myosin light chain, tryptophan hydroxylase, tubulin, and synapsin I as substrates, respectively. It has been suggested that these represent distinct but related enzymes. It is uncertain whether the various differences reported for these enzymes are simply due to analysis by four different laboratories or whether, in fact, the enzymes are a family of related protein kinases. The enzymes show both similarities and differences in substrate specificity. Smooth muscle myosin light chain and MAP-2 are excellent substrates, while casein and phosvitin support moderate phosphorylation. Phosphorylase *b* and the various histones are poor substrates. A direct comparison is difficult to make because the substrates are tested under different conditions and a full kinetic analysis is not provided by these investigators. There are a few glaring differences in their substrate specificities, however. Goldenring et al. (1983) report that tubulin is an excellent substrate and synapsin I the worst substrate for their tubulin kinase, whereas we and Bennett et al. (1983) find synapsin I to be an excellent substrate and tubulin a poor substrate. The lack of tubulin phosphorylation was also reported by Fukunaga et al. (1982) and Yamauchi & Fujisawa (1983). Both Bennett et al. (1983) and Goldenring et al. (1983) indicate that glycogen synthase is a rather poor substrate, whereas we find it to be one of the best substrates for our Ca^{2+}/calmodulin-dependent casein kinase. The inability of the two groups to demonstrate glycogen synthase phosphorylation may be related to their use of crude enzyme from a commercial source. Indeed our attempt to phosphorylate the same commercial preparation was unsuccessful. In addition, differences in the pH optima for the various substrates may drastically affect the rank order of substrates. The subunit composition of the enzyme studied here is similar to the one isolated as synapsin I kinase and possibly the tryptophan hydroxylase kinase (Bennett et al., 1983; Yamauchi & Fujisawa, 1983). The tubulin kinase is reported to have a doublet at both 52 000 and 63 000 daltons (Goldenring et al., 1983). The enzyme purified as the myosin light chain kinase appears to consist of only a single species at 49 000 daltons (Fukunaga et al., 1982). The native molecular weights of the enzymes vary from 460 000 to 650 000. Additional biochemical characterization and direct comparison of the kinases will need to be performed in order to determine whether the kinase described here and the other kinases are identical or members of a family of related Ca^{2+}/calmodulin-dependent kinases.

As indicated earlier, we used casein as a substrate because of its convenience and reliability for purifying and characterizing the Ca^{2+}/calmodulin-dependent protein kinase. Because of the possibility that a closely related family of Ca^{2+}/calmodulin-dependent protein kinases exists in rat brain cytosol and that MAP-2 is the major endogenous substrate in cytosol, we have recently purified the major Ca^{2+}-dependent MAP-2 kinase from rat brain (Schulman, 1984a,b). That enzyme as well as the activity in bovine brain cytosol reported earlier (Nose & Schulman, 1982) has physical properties that are indistinguishable from those for the casein kinase described here. These enzymes, though purified by using different substrates, all appear to have broad substrate specificity, suggesting the enzyme is the major Ca^{2+}/calmodulin-dependent protein kinase in rat brain cytosol. By analogy with the cAMP-dependent phosphorylation system, we have suggested that some of the effects of calcium in both neuronal and nonneuronal membranes and cytosol may be mediated by activation of a common Ca^{2+}/calmodulin-dependent protein kinase (Schulman, 1982). The enzyme described here appears to have a broad substrate specificity and is a good candidate for such a protein kinase.

Several lines of evidence suggest that such a soluble Ca^{2+}/calmodulin-dependent protein kinase may have a widespread distribution. A liver Ca^{2+}/calmodulin-dependent protein kinase originally thought to be a specific glycogen synthase kinase has subsequently been shown to have a broad substrate specificity and can, in fact, phosphorylate MAP-2 and synapsin I (Schworer & Soderling, 1983). It has a native molecular weight of 300 000–350 000, calculated from the sedimentation coefficient and Stokes radius, and consists of 51 000- and 53 000-dalton subunits (Payne et al., 1983; Ahmad et al., 1983). Although not observed in initial preparations,

the liver enzyme from rabbit may also contain a 60 000-dalton subunit (Payne & Soderling, 1983). Synapsin I kinase shows structural similarity to the skeletal muscle glycogen synthase kinase (McGuiness et al., 1983). We carried out a partial purification of a Ca^{2+}/calmodulin-dependent protein kinase from cytosolic extracts of several nonneuronal tissues and complete purification of the kinase from lung. Each preparation was exhibited at autophosphorylation of a 51 000-dalton polypeptide. Moreover, limited proteolysis of this phosphorylated protein from the various tissues generated identical phosphopeptide patterns that were very similar to the pattern of the neuronal 51 000-dalton protein (H. Schulman et al., unpublished results). Thus, a Ca^{2+}/calmodulin-dependent protein kinase similar to or identical with the neuronal casein kinase described here may have a widespread tissue distribution. Such an activity may function to mediate the biochemical and physiological effects of Ca^{2+} acting as a second messenger for a variety of hormones, neurotransmitters, and other regulatory agents.

Acknowledgments

We thank Dr. Maureen Costello, Peter Nose, Leslie Griffith, and Timothy M. Vogel for their assistance and helpful suggestions. We are also grateful to Karen Benight for excellent editorial assistance.

Registry No. ATP, 56-65-5; Ca, 7440-70-2; glycogen synthase, 9014-56-6; phosphorylase *b*, 9012-69-5; protein kinase, 9026-43-1.

References

Ahmad, Z., DePaoli-Roach, A. A., & Roach, P. J. (1982) *J. Biol. Chem. 257*, 8348–8355.

Beavo, J. A., Bechtel, P. J., & Krebs, E. G. (1974) *Methods Enzymol. 38*, 299–308.

Beavo, J. A., Bechtel, P. J., & Krebs, E. G. (1975) *Adv. Cyclic Nucleotide Res. 5*, 241–251.

Bennett, M. K., Erondu, N. E., & Kennedy, M. B. (1983) *J. Biol. Chem. 238*, 12735–12744.

Blank, A., Silber, J. R., Thelen, M. P., & Dekker, C. A. (1983) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 42*, 2148.

Bradford, M. M. (1976) *Anal. Biochem. 72*, 248–254.

Carlin, R. K., Grab, D. J., & Siekevitz, P. (1981) *J. Cell Biol. 89*, 449–455.

Cleveland, D. W., Fischer, S. G., Kirschner, M. W., & Laemmli, U. K. (1977) *J. Biol. Chem. 252*, 1102–1106.

Cohen, P. (1973) *Eur. J. Biochem. 34*, 1–14.

deJonge, H. R., & Rosen, O. M. (1977) *J. Biol. Chem. 252*, 2780–2783.

Fagard, M., & London, I. M. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 866–870.

Flockhart, D. A., & Corbin, J. D. (1982) *CRC Crit. Rev. Biochem. 12*, 133–186.

Fraker, P. J., & Speck, J. C. (1978) *Biochem. Biophys. Res. Commun. 80*, 849–857.

Freedman, S. D., & Jamieson, J. D. (1982) *J. Cell Biol. 95*, 918–923.

Fukunaga, K., Yamamoto, H., Matsui, K., Higashi, K., & Miyamoto, E. (1982) *J. Neurochem. 39*, 1607–1617.

Garrison, J. C., & Wagner, J. D. (1982) *J. Biol. Chem. 257*, 13135–13143.

Garrison, J. C., Johnsen, D. E., & Campanile, C. P. (1984) *J. Biol. Chem. 259*, 3283–3292.

Glass, D. B., McFann, L. J., Miller, M. D., & Zeilig, C. E. (1981) *Cold Spring Harbor Conf. Cell Proliferation 8*, 267–292.

Glover, C. V. C., Shelton, E. R., & Brutlag, D. L. (1983) *J. Biol. Chem. 258*, 3528–3265.

Gold, M. H., & Segel, I. H. (1974) *J. Biol. Chem. 249*, 2417–2423.

Goldenring, J. R., Gonzalez, B., McGuire, J. S., Jr., & DeLorenzo, R. J. (1983) *J. Biol. Chem. 258*, 12632–12640.

Greengard, P. (1978) *Science (Washington, D.C.) 199*, 146–152.

Hathaway, D. R., & Adelstein, R. S. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 1653–1657.

Haycock, J. W., Meligeni, J. A., Bennett, W. F., & Waymire, J. C. (1982) *J. Biol. Chem. 257*, 12691–12698.

Juskevich, J. C., Kuhn, D. M., & Lovenberg, W. (1983) *J. Biol. Chem. 258*, 1950–1953.

Kikkawa, U., Takai, U., Minakuchi, R., Inohara, S., & Nishizuka, Y. (1982) *J. Biol. Chem. 257*, 13341–13348.

Klee, C. B., & Krinks, M. H. (1978) *Biochemistry 17*, 120–126.

Klee, C. B., Crouch, T. H., & Richman, P. G. (1980) *Annu. Rev. Biochem. 49*, 489–515.

Kretsinger, R. H. (1979) *Adv. Cyclic Nucleotide Res. 11*, 1–26.

Laemmli, U. K. (1979) *Nature (London) 227*, 680–685.

London, I. M., Ernest, V., Fagard, R., Leroux, A., Levin, D. H., & Petryshyn, R. (1981) *Cold Spring Harbor Conf. Cell Proliferation 8*, 941–957.

Maeno, H., Reyes, P. L., Ueda, T., Rudolph, S. A., & Greengard, P. (1974) *Arch. Biochem. Biophys. 164*, 551–559.

Malencik, D. A., & Anderson, S. R. (1982) *Biochemistry 21*, 3480–3486.

McGuiness, T. L., Lai, Y., Greengard, P., Woodgett, J. R., & Cohen, P. (1983) *FEBS Lett. 163*, 329–334.

Morrissey, J. H. (1981) *Anal. Biochem. 117*, 307–310.

Nimmo, H. G., & Cohen, P. (1977) *Adv. Cyclic Nucleotide Res. 8*, 145–266.

Nose, P., & Schulman, H. (1982) *Biochem. Biophys. Res. Commun. 107*, 1082–1090.

Payne, M. E., & Soderling, T. R. (1983) *Methods Enzymol. 99*, 299–307.

Payne, M. E., Schworer, C. M., & Soderling, T. R. (1983) *J. Biol. Chem. 258*, 2376–2382.

Pires, E. M. S., & Perry, S. V. (1977) *Biochem. J. 167*, 137–146.

Portzehl, H., Caldwell, P. C., & Reugg, J. C. (1964) *Biochim. Biophys. Acta 79*, 581–591.

Rangel-Aldao, R., & Rosen, O. M. (1976) *J. Biol. Chem. 251*, 3375–3380.

Rangel-Aldao, R., Kuprec, J. W., & Rosen, O. M. (1979) *J. Biol. Chem. 254*, 2499–2508.

Robison, G. A, Butcher, R. W., & Sutherland, E. W. (1971) *Cyclic AMP*, Academic Press, New York.

Rosen, O. M., & Erlichman, J. (1975) *J. Biol. Chem. 250*, 7786–7794.

Schulman, H. (1982) *Handb. Exp. Pharmacol. 58*, 425–478.

Schulman, H. (1984a) *Mol. Cell. Biol. 4*, 1175–1178.

Schulman, H. (1984b) *J. Cell Biol. 99*, 11–19.

Schulman, H., & Greengard, P. (1978a) *Nature (London) 271*, 478–479.

Schulman, H., & Greengard, P. (1978b) *Proc. Natl. Acad. Sci. U.S.A. 75*, 5432–5436.

Schulman, H., Kuret, J. A., & Spitzer, K. A. (1983) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 42*, 2250.

Schworer, C. M., & Soderling, T. R. (1983) *Biochem. Biophys. Res. Commun. 116*, 412–416.

Segel, I. H. (1975) *Enzyme Kinetics*, Wiley, New York.

Sharma, R. K., Wang, T. H., Wirch, E., & Wang, J. H. (1980) *J. Biol. Chem. 255*, 5916–5923.
Siegel, L. M., & Monty, K. J. (1966) *Biochim. Biophys. Acta 112*, 346–362.
Wang, J. H., Stull, J. T., Huang, T.-S., & Krebs, E. G. (1976) *J. Biol. Chem. 251*, 4521–4527.
Yamauchi, T., & Fujisawa, H. (1983) *Eur. J. Biochem. 132*, 15–21.

Influence of an Extrinsic Cross-Link on the Folding Pathway of Ribonuclease A. Conformational and Thermodynamic Analysis of Cross-Linked (Lysine^{7}–Lysine^{41})-Ribonuclease A†

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ABSTRACT: A cross-linked ribonuclease A derivative, Lys^{7}-dinitrophenylene-Lys^{41}-ribonuclease A, has been prepared and characterized for ultimate use in protein-folding experiments. Immunochemical assays and spectroscopic measurements demonstrated that the introduction of this artificial cross-link does not perturb the native conformation of ribonuclease A. The cross-linked protein exhibited a reversible thermal transition with T_m = 53 °C at pH 2.0, which is 25 °C higher than that of unmodified ribonuclease A under the same conditions. The denatured form of the cross-linked ribonuclease A has a conformational chemical potential that is 4.9 kcal/mol higher than that of the denatured form of unmodified ribonuclease A at 40 °C and pH 2.0, assuming that the cross-linked and the unmodified proteins have the same conformational chemical potential in the native conformation. This is in good agreement with a theoretical value of 5.2 kcal/mol, calculated from the reduction of chain entropy of the denatured form upon introduction of the extrinsic cross-link. Thus, it is concluded that the extrinsic cross-link between Lys^{7} and Lys^{41}, formed by the dinitrophenylene group, does not affect the native conformation of ribonuclease A but destabilizes the denatured conformation, probably by decreasing its chain entropy.

Konishi et al. (1982b) proposed two types of pathways for protein folding. One is designated as a growth-type pathway, in which interactions present in the native conformation play significant roles by forming nucleation sites that subsequently direct the folding of other parts of the chain around the nucleation sites. The other is denoted as a rearrangement-type pathway, in which some nonnative interactions are essential for folding, and the disruption of these intermediates or rearrangement to native ones constitutes the rate-limiting step.

Denton et al. (1982) and Lynn et al. (1984) have studied the influences of the α-helix and β-turn-inducing compact structure on the folding pathways. Both the α-helix and the β-turn are stabilized by short- and medium-range interactions. These authors suggested that not all ordered structures in ribonuclease A (RNase A)[1] are equivalent in terms of their influence on the folding pathway; some can play an essential role, but others may not. The α-helix at the N-terminal region of RNase A and one or more bends that establish the compact structure are among those ordered structures that do not play an essential role in the folding of RNase A. [Since the extrinsic cross-link between Lys^{7} and Lys^{41} discussed in this paper (see below) involves two N-terminal α-helices within the loop, we will report a study of the roles of these α-helices in the folding pathways in a subsequent paper.]

In order to study the role of long-range interactions in the folding pathway, Scheraga et al. (1984) proposed that the introduction of an extrinsic cross-link into RNase A would force some nativelike long-range interactions to exist in the denatured and intermediate conformations and hence would shift the folding pathway from a rearrangement-type to a growth-type one. In this and the following papers, we shall examine the consequences of this proposal.

An intramolecular cross-link is one of the sources of distance information about a protein. Furthermore, cross-links enhance the thermodynamic stability of the protein, e.g., cross-linked (Glu^{35}–Trp^{108})-lysozyme has a thermal transition temperature that is 29 °C higher than that of unmodified lysozyme in 1.94 M Gdn·HCl, pH 2.0 (Johnson et al., 1978). Since RNase A is one of the best characterized proteins, various reagents have been designed to form covalent cross-links between residues, especially lysines, in RNase A. Some of the cross-linking reagents that have been used are 1,5-difluoro-2,4-dinitrobenzene (DFDNB) (Marfey et al., 1965a,b), dimethyl adipimidate (Hartman & Wold, 1966, 1967), [2-(*p*-nitrophenyl)allyl]trimethylammonium iodide (ETAC I), and 2-(*p*-nitrophenyl)allyl 4-nitro-3-carboxyphenyl sulfide (ETAC II) (Mitra & Lawton, 1979). Many difficulties, however, have been associated with the use of these cross-linking reagents. Dimethyl adipimidate forms Lys^{7}–Lys^{37} or Lys^{31}–Lys^{37}

† From the Baker Laboratory of Chemistry, Cornell University, Ithaca, New York 14853. *Received March 1, 1984.* This work was supported by research grants from the National Institute of General Medical Sciences (GM-14312), the National Science Foundation (PCM79-20279), and the National Foundation for Cancer Research.

[1] Abbreviations: RNase A, bovine pancreatic ribonuclease A; ^{125}I-RNase A, RNase A labeled with ^{125}I; CL(7–41)-RNase A, cross-linked derivative of RNase A, N^{ϵ},$N^{\epsilon\prime}$-(2,4-dinitrophenylene-1,5)-(lysine^{7}–lysine^{41})-RNase A; DFDNB, 1,5-difluoro-2,4-dinitrobenzene; ETAC I, [2-(*p*-nitrophenyl)allyl]trimethylammonium iodide; ETAC II, 2-(*p*-nitrophenyl)allyl 4-nitro-3-carboxyphenyl sulfide; CD, circular dichroism; NMR, nuclear magnetic resonance; HPLC, high-performance liquid chromatography; CMC, carboxymethylcellulose; C>p, sodium cytidine cyclic 2′,3′-phosphate; Tris, 2-amino-2-(hydroxymethyl)-1,3-propanediol; Gdn·HCl, guanidine hydrochloride; DSS, 4,4-dimethyl-4-silapentane-1-sulfonic acid; DPE-bis-Ac-Lys-NHMe, N^{ϵ},$N^{\epsilon\prime}$-(2,4-dinitrophenylene-1,5)-bis(N^{α}-acetyl-L-lysine methylamide); PBS, phosphate–saline buffer (300 mM NaCl, 63 mM KH_2PO_4, and 120 mM Na_2HPO_4, pH 7.2); BBS/ovalbumin/azide, borate–saline buffer (75 mM NaCl, 100 mM boric acid, and 50 mM sodium tetraborate, pH 8.4) containing 0.1% ovalbumin and 0.02% sodium azide.

0006-2960/84/0423-5504$01.50/0