Purification of the Multifunctional Calmodulin-Dependent Protein Kinases from Lung and Liver, and the Comparison to the Brain Enzyme

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Abstract—We purified the multifunctional calmodulin-dependent protein kinases (calmodulin-kinase) from rat lung and rabbit liver, and compared the properties of this enzyme with those of the rat brain enzyme. The lung and liver enzymes had molecular weights (Mr's) of 530,000 and 330,000 with main subunits of 52 and 51 kDa, respectively. Although the lung and liver enzymes cross-reacted with antibodies to the brain enzyme, the immunoreactivity of the lung enzyme was weaker. The substrate specificity of the three enzymes showed differences in the relative reaction rate of phosphorylation. The patterns of phosphopeptides of the lung and liver enzymes were similar to each other and only partly common to that of the 60-kDa subunit of the brain enzyme.

Calmodulin-dependent protein kinases include phosphorylase kinase, myosin light chain kinase and the multifunctional calmodulin-dependent protein kinase (calmodulin-kinase). Calmodulin-kinase has been studied extensively in the brain (1–8). The same class of the enzymes were found in many tissues such as the anterior pituitary gland (9), skeletal muscle (10), pancreas (13, 14), heart (15–17), lung (18), the electric organ of Torpedo californica (19), the ganglion of Aplysia (20) and sea urchin eggs (21). The enzymes have broad substrate specificity and therefore may be involved in many of the Ca^{2+}-stimulated functions (22, 23). The monoclonal antibody to the brain calmodulin-kinase cross-reacted with proteins in the spleen, stomach, skeletal muscle, lung, heart and adipose tissue (24). These proteins were considered to be the subunits of the enzymes on the basis of the findings on the purified enzymes.

In the present study, we have developed a simple and conventional purification method for calmodulin-kinases from the lung and liver, and we have compared its physicochemical and kinetic properties with those of the brain enzyme.

Materials and Methods

Materials: DEAE-cellulose (DE-52) was purchased from Whatman; protein A-Sepharose CL-4B, blue dextran 2000, thyroglobulin, ferritin, catalase and aldolase from Pharmacia Fine Chemicals; bovine serum albumin and ovalbumin from Schwarz/Mann; ATP, trypsin inhibitor, phosphorylase b, casein, arginine-rich histone, lysine-rich histone, carbonic anhydrase and Staphylococcus aureus V8 protease (type XVII) from Sigma; r-globulin and horseradish peroxidase-conjugated goat anti-rabbit IgG from ICN; EGTA from Dojin Chemicals; p-APMSF from Wako Pure Chemical Industries; Affi-Gel 15 from Bio-Rad; [32P]orthophosphate from Japan Radioisotope Association. All other reagents were of analytical grade.

Abbreviations: SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; MAP2, microtubule-associated protein 2; p-APMSF, (p-amidino-phenyl)methanesulfonyl fluoride hydrochloride; kDa, kilodalton.
Preparations of proteins: Calmodulin and myelin basic protein were purified from bovine brain by the methods of Gopalakrishna and Anderson (25) and Deibler et al. (26), respectively. Myosin was prepared from chicken gizzard by the method of Nonomura and Ebashi (27). Calmodulin-deficient myosin light chain was obtained from chicken gizzard myosin according to the methods of Perrie and Perry (28) and Matsuda et al. (29) at the final step of the DEAE-cellulose column. MAP2 was prepared by the method of Yamamoto et al. (30). Polyclonal antibodies against rat brain calmodulin-kinase were prepared by immunizing rabbits (K. Fukunaga et al., submitted manuscript). The antibodies were purified on an antigen-affinity column. The specificity and properties of the antibodies will be described elsewhere. The IgG fraction of the antibodies was prepared by ammonium sulfate fractionation and DEAE-cellulose column chromatography.

Purification of calmodulin-kinases from rat lung and rabbit liver: All procedures were carried out at 0–4°C unless otherwise indicated. To purify calmodulin-kinases rapidly, the previously reported procedures (1) were modified.

About 40 g of rat lung, dissected free of large tracheas, was washed with 20 mM Tris-HCl buffer, pH 7.5, 0.32 M sucrose and 10 mM 2-mercaptoethanol; and then the tissues were homogenized with 200 ml of 0.5% Triton X-100, 20 mM Tris-HCl buffer, pH 7.5, 2 mM EDTA, 4 mM EGTA, 10 mM 2-mercaptoethanol, 25 mM p-APMSF and 50 mg/L of trypsin inhibitor at the top speed for 30 sec in an Ultra-turrax homogenizer (Janke & Kunkel Co., FRG). The homogenate was centrifuged at 110,000 g for 40 min. The supernatant was diluted twice with 10% glycerol, 20 mM Tris-HCl buffer, pH 7.5, 0.1 mM EGTA, 10 mM 2-mercaptoethanol, and 20 mM NaCl (buffer A), and then applied to a 5.5x4.2 cm column of DEAE-cellulose, which had been equilibrated with buffer A. The column was washed with 30 ml of buffer B and then 300 ml of buffer B containing 2 M NaCl. After washing the column with a small volume of buffer B, the enzyme was eluted with buffer B containing 200 mM NaCl and 1 mM EGTA instead of 0.2 mM CaCl₂. The active fractions were pooled and concentrated to about 0.2 ml by Amicon ultrafiltration with a PM-10 membrane. The concentrate was layered onto the continuous gradient of 5–25% sucrose and centrifuged as described previously (1). After centrifugation, fractions of 0.2 ml each were collected and assayed for calmodulin-kinase activity. Two peaks of calmodulin-dependent protein kinase activity appeared at the positions corresponding to sedimentation coefficients of 4.5 and 16.0 S (Fig. 1). The active fractions of a higher molecular mass were collected and concentrated.

Calmodulin-kinase was purified from 50 g of rabbit liver essentially as described above. Two peaks of calmodulin-dependent protein kinase activity were obtained by sucrose density gradient centrifugation with sedimentation coefficients of 4.5 and 11.5 S (Fig. 1). The active fractions of a higher molecular mass were collected and concentrated.

Rabbit liver was used as the starting material instead of rat liver, since the content of enzyme activity in rabbit liver (Pi mol/g tissue) was about 7.0 times higher than that of rat liver enzyme. It was often difficult to detect the enzyme activity of rat liver. However, no qualitative and quantitative difference was observed between the enzymes of both species in terms of subunit structure and immunoreactivity, suggesting the absence of species specificity of the enzyme.

Assay of calmodulin-kinase: The standard assay system for the calmodulin-kinase activity was described previously (1): the standard reaction mixture contained in a final volume of 0.2 ml, 20 mM Tris-HCl buffer, pH 7.5, 10 mM MgCl₂, 1 mM dithiothreitol, 0.1 mM CaCl₂, either 0.02 mM (80–230 mCi/
nmol) or 0.5 mM (3.2–9.0 mCi/nmol) [γ-32P]ATP, 40 μg of chicken gizzard myosin light chain as the usual substrate or other substrates as indicated, and the indicated amount of calmodulin-kinase with or without 1 μg of calmodulin. Incubation was carried out at 30°C for 10 min in a shaking water bath. The reaction was terminated and the degree of protein phosphorylation measured as described previously (31, 32).

**Sucrose density gradient centrifugation:** Sucrose density gradient centrifugation was performed as described previously (1). Sedimentation coefficients were determined by the method of Martin and Ames (33) with bovine serum albumin (4.6 S), human γ-globulin (7.0 S), and bovine thyroglobulin (20.3 S) as standards.

**Immunological analysis:** For immunoblotting, proteins were subjected to SDS-PAGE and then transferred electrophoretically to a nitrocellulose membrane. After the membrane was incubated for 1 hr at room temperature with 2.5% (W/V) bovine serum albumin in Tris-buffered saline (TBS) containing 20 mM Tris-HCl buffer, pH 7.5, and 0.15 M NaCl to block non-specific binding sites, it was incubated overnight with polyclonal antibodies diluted 1:200 in TBS containing 2.5% bovine serum albumin. The membrane was further incubated for 1 hr with peroxidase-conjugated goat anti-rabbit IgG. Immunoreactive proteins were detected using 4-chloro-1-naphthol as substrate.

For immunoprecipitation, the calmodulin-kinase of each tissue was incubated with various concentrations of the antibodies for 20 min at 4°C in the reaction mixture containing 10 mM Tris-HCl buffer, pH 7.5, 10 mM 2-mercaptoethanol and 10% glycerol in a total volume of 0.1 ml. After adding 20 μl of a 50% (V/V) suspension of protein A-Sepharose CL-4B, incubation was carried out at 4°C for 20 min. The suspension was precipitated by centrifugation. The kinase activity of the supernatant was assayed with MAP2 as substrate under standard conditions.

**Phosphopeptide mapping:** After phosphorylation, proteins were separated by SDS-PAGE in a 10% acrylamide gel. Protein bands of 49 and 60 kDa for the brain enzyme, 52 kDa for the lung enzyme and 51 kDa for the liver enzyme, stained with Coomassie brilliant blue, were each excised, and they were subjected to digestion with *Staphylococcus aureus* V8 protease on SDS-PAGE by the method of Cleveland et al. (34). Phosphopeptides were separated by SDS-PAGE in a
15% acrylamide, followed by autoradiography.

Other procedures: [γ-32P]ATP was prepared by the method of Post and Sen (35). SDS-PAGE was carried out by the method of Laemmli (36). Autoradiography of the gels was performed as described previously (37). Molecular weights of proteins were calibrated with standards: chymotrypsinogen A (25,000), carbonic anhydrase (29,000), ovalbumin (45,000), bovine serum albumin (68,000), phosphorylase b (94,000) and myosin heavy chain (200,000). Protein was determined by the method of Bradford (38) with bovine serum albumin as a standard.

Results

Purification of calmodulin-kinases from rat lung and rabbit liver: The critical point for the purification of this enzyme is the time required to complete the procedure. The procedures must be completed to the step of the calmodulin-affinity column within 7 hr. All procedures for the enzyme preparation was performed within 36 hr. The lung calmodulin-kinase was purified (2,800-fold) to apparent homogeneity with a 1.1% yield and a specific activity of 170.2 nmol/mg/min using MAP2 as the substrate. The liver enzyme was also purified to near homogeneity at 2,272-fold with a 0.9% yield and the specific activity of 132.5 nmol/mg/min using MAP2 as the substrate.

The brain enzyme was composed of two main subunits with 49 and 60 kDa (Fig. 2A). The lung enzyme showed a major band of 52 kDa and minor bands of 50 and 54 kDa (Fig. 2A). The liver enzyme was composed of a major band of 51 kDa and minor bands of 49, 53 and 54 kDa (Fig. 2A).

These enzymes were autophosphorylated in a Ca2+- and calmodulin-dependent manner (Fig. 2B).
In contrast to the similarity of subunits, the molecular weights of the holoenzymes differed between the brain and lung enzymes and the liver enzyme (Table 1). The liver enzyme also showed smaller values of sedimentation coefficient and Stokes radius (Table 1).

**Immunological analysis:** Antibodies to brain calmodulin-kinase recognized both subunits of 49 and 60 kDa (Fig. 3). The main protein bands of 52 and 51 kDa of the lung and liver enzymes, respectively, and other minor protein bands of each enzyme cross-reacted with the antibodies as shown by immunoblotting (Fig. 3).

To examine the affinity between the antigen and antibodies, the immunoprecipitation method was used. After treatment with IgG from the antibodies and protein A-Sepharose CL-4B, the enzyme activity which remained in the supernatant was determined (Fig. 4). The increasing amount of IgG resulted in the decrease in enzyme activity. The concentrations of IgG required to inhibit about 50% of enzyme activity were 12, 19 and 130 μg/ml for the brain, liver and lung enzymes, respectively, indicating that the liver enzyme had a close affinity for the antibodies to the brain enzyme. In contrast, the lung enzyme had a lower affinity for the antibodies. IgG from the sera of a non-immunized rabbit had no effect on enzyme activity with 1,000 μg/ml being the maximal

### Table 1. Physical properties of calmodulin-kinases from the brain, lung and liver

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Brain</th>
<th>Lung</th>
<th>Liver</th>
</tr>
</thead>
<tbody>
<tr>
<td>Molecular weight</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Holoenzyme</td>
<td>640,000*</td>
<td>590,000*</td>
<td>480,000*</td>
</tr>
<tr>
<td>Subunit</td>
<td>560,000b</td>
<td>530,000b</td>
<td>330,000b</td>
</tr>
<tr>
<td>Sedimentation coefficient (S)</td>
<td>16.5d</td>
<td>16.0d</td>
<td>11.5d</td>
</tr>
<tr>
<td>Stokes radius (nm)</td>
<td>8.1e</td>
<td>7.9e</td>
<td>6.8e</td>
</tr>
<tr>
<td>Frictional ratio</td>
<td>1.49</td>
<td>1.47</td>
<td>1.49</td>
</tr>
</tbody>
</table>

*a Determined by gel filtration on HPLC TSK gel G4000 SW column. *b Calculated from the equation of Siegel and Monty (44). *c Determined by SDS-PAGE. *d Determined by sucrose density gradient centrifugation. *e Determined by gel filtration on HPLC TSK gel G4000 SW column, employing standard proteins: thyroglobulin of 8.5 nm, ferritin of 6.1 nm, catalase of 5.2 nm and aldolase of 4.8 nm. Numbers in parentheses represent minor protein bands of the purified enzymes observed on SDS-PAGE.

Fig. 3. Immunoblotting of calmodulin-kinases from the brain, lung and liver using antibodies to the brain enzyme. The purified calmodulin-kinases from the brain (1.5 μg of protein) (lane 1), lung (1.4 μg of protein) (lane 2) and liver (3.9 μg of protein) (lane 3) were subjected to SDS-PAGE in 9% acrylamide and electrophoretically transferred to a nitrocellulose membrane. The procedures for immunoblotting were as described in Materials and Methods.
The purified calmodulin-kinases from the brain (0.64 μg of protein) (O–O), lung (0.88 μg of protein) (●—●) and liver (2.0 μg of protein) (▲—▲) were immunoprecipitated at 4°C for 20 min with the indicated amount of the IgG fraction from antibodies to the brain enzyme. Calmodulin-kinase activities in the absence of IgG were at the similar level for the enzyme of each tissue.

Table 2. Substrate specificity of calmodulin-kinases from the brain, lung and liver

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Relative rate of phosphorylation (%)</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Brain</td>
</tr>
<tr>
<td>Myosin light chain</td>
<td>100.0</td>
</tr>
<tr>
<td>MAP2</td>
<td>134.5</td>
</tr>
<tr>
<td>Casein</td>
<td>105.2</td>
</tr>
<tr>
<td>Myelin basic protein</td>
<td>93.3</td>
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<tr>
<td>Lysine-rich histone</td>
<td>12.6</td>
</tr>
<tr>
<td>Arginine-rich histone</td>
<td>6.0</td>
</tr>
<tr>
<td>Phosphorylase b</td>
<td>0</td>
</tr>
</tbody>
</table>

Twenty μg of MAP2, 40 μg of myosin light chain and 100 μg of other substrates were each incubated with 0.5 mM [γ-32P]ATP and the purified calmodulin-kinases from the brain (0.92 μg of protein), lung (1.24 μg of protein) and liver (1.54 μg of protein) in the presence of 5 μg of calmodulin, under standard conditions. Values are given as percentages and have been corrected for those determined without substrate.

Substrate specificity: The substrate specificity of calmodulin-kinase were compared under identical conditions (Table 2). Any of the substrates which served as substrates for the brain enzyme was phosphorylated by the lung and liver enzymes. However, the relative rate of phosphorylation differed among the substrates for each enzyme. The substrate specificity of the enzymes was commonly broad. The results may suggest that the differences observed above reflect the structural characteristics of each enzyme.

Phosphopeptide analysis: Main bands of
autophosphorylated subunits of the enzymes were subjected to proteolysis. The resultant phosphopeptides were analyzed by SDS-PAGE, followed by autoradiography (Fig. 5). The patterns of phosphopeptides were distinct between the 49- and 60-kDa subunits of the brain enzyme. The phosphopeptides of 23, 25 and 26 kDa were observed for both the lung and liver enzymes, suggesting that both enzymes have a similarity in their primary structures. The patterns of these phosphopeptides were partly similar to that of the 60-kDa subunit of the brain enzyme, but not to the 49-kDa subunit of the brain enzyme.

**Discussion**

A class of multifunctional calmodulin-dependent protein kinases was reported to occur in many tissues. The level of enzyme activity was highest in the brain (24). The properties of the enzymes from various tissues were similar with respect to the large molecular weight of the holoenzyme, autophosphorylation, broad substrate specificity and immunoreactivity. We reported that the monoclonal antibody to the brain enzyme cross-reacted with proteins in various tissues (24), which were confirmed to be the subunits of the enzymes by the purification of the enzymes from some tissues (17, the present study). So we considered that the subunit structures of the enzymes can be classified into at least three types on SDS-PAGE: 1) brain, 2) spleen, stomach, pancreas, lung and adipose tissue and 3) skeletal muscle and heart (24). The results were further confirmed by polyclonal antibodies to the brain enzyme (K. Fukunaga and E. Miyamoto, unpublished).

Comparisons of the properties of the enzymes were made between skeletal muscle and brain enzymes (39, 40), among the enzymes of the brain, lung, heart, spleen and *Aplysia* ganglia (18), between brain and liver enzymes (41) and among brain, skeletal muscle and liver enzymes (42). In the present study, we attempted to develop a rapid method for the purification of the enzyme and to examine the enzymes from the brain, lung and liver, and then compared them. Schulman et al. (18) reported that the brain and lung
enzymes of the rat have nearly indistinguishable physical and biochemical properties. In contrast, the present study showed that both enzymes apparently differed in subunit structure. Since our results were confirmed by immunoblotting with monoclonal (24) and polyclonal antibodies and autophosphorylation of the purified enzyme (the present study), why there are differences between their results and ours is unclear at present. One explanation is that since their autoradiograph showed the phosphorylation of a main protein at a position between 49 and 60 kDa, minor bands of proteins might be considered to be the subunit of the enzyme. In addition to immunoreactivity, the immunotitrations were compared with antibodies to the brain enzyme. The difference in the affinity to antibodies between the brain and liver enzymes and the lung enzyme may reflect the characteristic structure responsible for antigenicity.

In the present study, the phosphopeptides of the autophosphorylated subunit of the liver enzyme were rather similar in part to the 60-kDa subunit of the brain enzyme. The results differed from those reported by Shenoliker et al., which showed a similarity of the phosphopeptides between the 50-kDa subunit of the brain enzyme and the 51-kDa subunit of the liver enzyme (42). Further studies on the amino acid sequence of the enzyme will be needed to clarify this point. In this sense, it is of interest that the cDNA of the 60-kDa subunit of the brain enzyme was recently obtained and that the amino acid sequence of the subunit was estimated (43).

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References
1298 (1983)


37 Miyamoto, E., Fukunaga, K., Matsui, K. and Iwasa, Y.: Occurrence of two types of Ca2+-dependent protein kinases in the cytosol fraction


