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Purification and Properties of Novel Calcium-binding Proteins from *Streptomyces coelicolor*

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Two novel calcium-binding proteins, named CAB-I and CAB-II, have been isolated from *Streptomyces coelicolor*. Purification of the calcium-binding proteins involved heat treatment, fractionation with ammonium sulfate, acid treatment, anion exchange and hydrophobic interaction column chromatography, FPLC gel filtration, and preparative isoelectric focusing. A chelex competitive assay and ^{45}Ca autoradiography verified the calcium-binding ability of the proteins. The major band CAB-II has an apparent molecular weight of 26,000 determined by SDS-polyacrylamide gel electrophoresis and 340,000 determined by gel filtration. The isoelectric point of this molecule showed the acidic nature of the molecule. N-terminal amino acid sequence analysis shows homology to rat Ca^{2+}/calmodulin-dependent protein kinase-II (CAB-II) and yeast phosphoprotein phosphatase (CAB-I).

Key words: Calcium-binding protein, phosphoprotein phosphatase (CAB-I), Ca^{2+}/calmodulin-dependent protein kinase-II (CAB-II), *Streptomyces coelicolor*, protein kinase

Ionized calcium (Ca^{2+}) is the most common signal transduction element in cells ranging from bacteria to specialized neurons. Scores of cellular proteins have been adopted to bind Ca^{2+} tightly, in some cases to decrease free Ca^{2+} levels and in others to trigger second-messenger pathways (7). In eukaryotes, Ca^{2+} plays an important role in the regulation of a number of processes such as cell motility, cyclic nucleotide metabolism, protoplasmic streaming, protein phosphorylation, and immune action (23). One of the ways in which Ca^{2+} plays a second messenger role is to activate protein kinases in response to stimuli (2). The receptor and transducer of the calcium signal could be the calcium-binding protein, calmodulin or protein kinase itself. There have been several reports of calcium/calmodulin-dependent protein kinases and calcium/phospholipid-dependent protein kinases (4, 10, 24). A class of calcium/calmodulin-dependent protein kinase II was detected first in rat brain (13, 17) and later in several tissues of both mammalian and nonmammalian origins (1, 3, 19). The best characterized calcium/calmodulin-dependent protein kinase II, the rat brain enzyme, is a heteropolymer with a native molecular weight of 600,000. Protein phosphorylation is known to be an important biochemical mechanism for the regulation of numerous physiological processes (8). Four major types of catalytic subunits of protein serine/threonine phosphatases have been identified and named protein phosphatase 1, 2A, 2B, and 2C (PP1, PP2A, PP2B, and PP2C) (8, 9). Mammalian PP2B, calcium/calmodulin protein phosphatase (also termed calcineurin) is a heterodimer consisting of a 58~59 kDa catalytic subunit combinded to a 19 kDa regulatory subunit which shows 35% identity to calmodulin (15).

Calcium-binding proteins, which are frequently involved in calcium regulation in eukaryotic cells, have recently been reported in some bacteria and have been purified from vegetative cells of *Myxococcus xanthus* (16), *Saccharopolyspora erythraea* (formerly known as *Streptomyces erythreus*) (27), *Bacillus subtilis* (11), and dormant spores of *Bacillus cereus* (26). The presence of calmodulin-like proteins in prokaryotes also has been reported (25).

In this report, we identify two novel calcium-binding proteins from *Streptomyces coelicolor* that may have calcium/calmodulin-dependent protein kinase and phosphoprotein phosphatase activities and describe some of ther characteristics.

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Materials and Methods

Materials

Nitrocellulose membranes (0.2 μm) were purchased from Schleicher and Schuell. Centricon micro-concentrators were purchased from Amicon. P81 phosphocellulose paper was purchased from Whatman. Ammonium sulfate, glycerol, and phosphoric acid were purchased from Junsei Chemical Company (Tokyo, Japan). [γ-^{32}P] ATP and $^{45}CaCl_2$ were purchased from the Amersham Nuclear Co. (Uppsala, Sweden). All other reagents were purchased from Sigma Chemical Co. (St. Louis, USA).

Purification of *Streptomyces coelicolor* calcium-binding proteins

Streptomyces coelicolor was grown at 30°C on YEME medium, harvested and washed with 20 mM Tris-Cl (pH 7.5) containing 2 mM $CaCl_2$. The cell pellet was frozen and stored at −40°C. The thawed cell pellet was glass-homogenized in 3 volumes of buffer A (50 mM Tris-Cl (pH 7.4), 2 mM EDTA, 10% glycerol, 1 mM DTT and 1 mM PMSF). The crude homogenate was centrifuged 12,000 g for 20 min, and the supernatant was treated with streptomycin sulfate to a final concentration of 1.5% (w/v). The resulting precipitate was centrifuged at 12,000 g for 15 min. The supernatant fractions were pooled and heated to 60°C for 10 min and then cooled on ice. The heat-denatured material was removed by centrifugation at 12,000 g for 20 min. Solid ammonium sulfate was added to 55% saturation, and the precipitate was removed by centrifugation. Ammonium sulfate concentration of the supernatant was raised to 80% saturation, and the precipitate was collected by centrifugation. The precipitate was suspended in 10 mM Tris-Cl (pH 7.5) and dialyzed against the same buffer for 10 h at 4°C. The dialysate was centrifuged, and the resulting supernatant was brought to a final concentration of 3.0% (w/v) trichloroacetic acid (TCA) by slow addition of 50% TCA and stirred for 30 min at 4°C. This mixture was centrifuged, the precipitate was collected and dissolved in 10 mM Tris-Cl (pH 7.5), and the solution was adjusted to pH 6.1 by adding NaOH. Insoluble materials were removed by centrifugation, and the supernatant was dialyzed against 20 mM Tris-Cl (pH 7.5). The dialyzed and clarified protein solution was applied to a Q-Sepharose column (2.5×3 cm), which had been equilibrated with 20 mM Tris-Cl (pH 7.5). The column was washed until the A_{280} reached the lowest stable background. The proteins bound to the resin were eluted with a linear gradient of 0~0.5 M NaCl in 20 mM Tris-Cl (pH 7.5) at 40 ml/h while collecting 3-ml fractions. The calcium-binding proteins, determined by the chelex assay (see below), were eluted at 0.12 M NaCl (peak I) and 0.3 M NaCl (peak II).

The calcium-binding protein responsible for the calcium-binding activity peak II was subjected to further purification. Peak II fractions (about 18-ml) were pooled and dialyzed at 4°C against buffer B (50 mM Tris-Cl (pH 7.5), 5 mM $CaCl_2$ and 1M $(NH_4)_2SO_4$). A sample was applied a column of phenyl-agarose (1.5×3.4 cm), which had been equilibrated with buffer B. Following washing with the same buffer, the column was developed with linear gradient of 1~0.1 M $(NH_4)_2SO_4$ in 50 mM Tris-Cl (pH 7.5) with 5 mM $CaCl_2$ and then eluted with 50 mM Tris-Cl (pH 7.5) containing 100 mM $(NH_4)_2SO_4$ and 10 mM EGTA. Pooled active fractions from phenyl-agarose chromatography were concentrated by Centricon, and 200 ml of aliquots were applied to a Superdex 200 fast-flow column. The column was developed with 50 mM Tris-Cl (pH 7.5) containing 0.15 M NaCl at a flow rate of 0.3 ml/min and at 4°C. The native molecular weight was determined by comparison of the elution profile with those of the following standard proteins: catalase (240,000), aldolase (158,000), albumin (45,000), and chymotrypsinogen-A (25,000).

Chelex competition assay

Calcium-binding activity was routinely checked according to the method of Tokuda *et al.* (28) with minor modifications. A slurry of chelex resin was resuspended in 100 mM Tris-Cl (pH 7.5) containing 50 mM NaCl. While stirring, 60 μl of resin was pipetted into a final reaction volume of 270 μl. After 30 min of strong agitation at room temperature, the mixture was centrifuged at 5,000 g for 5 min. A 160 ml aliquot of supernatant was determined for radioactivity. Under this assay condition, a linear curve of c.p.m. (from 10,000 to 50,000) could be obtained by using 2~10 μg of purified bovine brain calmodulin (26).

Electrophoresis and isoelectric focusing

SDS-polyacrylamide gel electrophoresis was carried out in slab gels according to Laemmli (18). Isoelectric focusing was performed with Ampholine, purchased from Amersham Pharmacia Biotech.(Uppsala, Sweden), pH 3.5~10 or pH 4~6 to a final concentration of 5% (v/v). Gels (12×8 cm) were prefocused for 15 min at 300 V at 4°C, with 1 N NaOH as the cathode buffer and 1 N H_3PO_4 as the anode buffer. Samples were focused at constant power for 30 min at 4°C. Gels were stained with 3.5% perchloric acid, 0.025% Coomassie Brilliant Blue G-250.

Detection of calcium-binding proteins by ^{45}Ca autoradiography

Calcium-binding ability was verified by autoradio-

graphy based on the methods of Maruyama *et al.* (20). Proteins were separated on polyacrylamide gels and electrophoretically transferred to nitrocellulose or directly spotted onto the nitrocellulose membrane. The membrane was washed 60 mM KCl, 5 mM $MgCl_2$ and 10 mM Imidazole-Cl (pH 7.1) for 60 min, with the solution being changed three times. After washing, the membrane was incubated in the same solution with $^{45}CaCl_2$ (1 mCi/ml) for 20 min, washed twice with H_2O (2 min each), air-dried, and exposed to X-ray film (Kodak XAR-5) for 24-48 h.

N-terminal amino acid sequencing

The purified samples were resolved by SDS-polyacrylamide gel electrophoresis (peak II) or isoelectric focusing (peak I) as described above. After electrophoretic transfer to a PVDF membrane (5), the protein was stained with Coomassie Blue for 2 min and destained with 50% methanol and 10% acetic acid. The portion of the membrane containing the immobilized protein was excised with a razor blade, rinsed in distilled water, and stored at −40°C until use. The amino-terminal sequencing was carried on a model 475A amino acid analyzer (Applied Biosystems) at the Korea Basic Science Center (Yusung, Korea).

Protein kinase assay

Protein kinase activity was measured in a reaction mixture (20 ml) containing 40 mM HEPES (pH 7.3), 10 mM magnesium acetate, 1 mM DTT, 0.1 mM EGTA, 100 mM [γ-^{32}P] ATP (185 TBq/mmol), and protein samples. When required, 1 mM $CaCl_2$ and 2 mM bovine calmodulin were added. After the mixture was prewarmed to 30°C, the reaction was initiated by the addition of radioactive ATP and terminated after incubation for 20 min at 30°C. Termination and ^{32}P-incorporation were performed by one of the following two methods. Method 1: the reactions were terminated by spotting the mixture onto Whatman P81 paper and washing it in 75 mM phosphoric acid. The radioactivity on the paper was determined by liquid scintillation counting. Method 2: the reactions were terminated by addition of electrophoresis sample buffer. The samples were boiled for 2 min and electrophoresed. The radioactivity on the gel was detected by autoradiography after the gel was stained with Coomassie Brilliant Blue.

Results and Discussion

Purification of *Streptomyces coelicolor* calcium-binding proteins involved several steps including heat treatment, ammonium sulfate fractionation, acid treatment, and ion exchange on Q-Sepharose. Heat treatment (60°C, 10 min) and acid treatment (3% TCA) were very effective in isolating calcium-binding proteins. Two peaks of calcium-binding activity were detected by Q-Sepharose chromatography (Fig. 1). These peaks, eluted at approximately 0.12 and 0.3 M NaCl, were designated peak I (CAB-I) and peak II (CAB-II), respectively. The peak I fraction was further purified to apparent homogeneity using preparative isoelectric focusing. For further purification of peak II fraction, phenyl-agarose chromatography was performed. Some calcium-binding proteins are reported to bind to phenyl-group in the presence of Ca^{2+} and to be released by elution with EGTA (Gopalakrishna, *et al.*, 1982). In contrast, we found that the *Streptomyces* peak II does not bind to the column in the presence of 5 mM $CaCl_2$ and 1 M $(NH_4)_2SO_4$ (22). The flow-through fractions that had contained Ca^{2+}-binding activities were pooled and concentrated by means of ultrafiltration. When the concentrated sample was applied to a Superdex 200 gel filtration column, a single peak of calcium-binding activity was resolved. This fraction contained a homogeneous protein of approximately 26 kDa mdecular mass as determined by SDS-PAGE (Fig. 2). Calcium-binding ability was routinely determined qualitatively during purification by chelex competition assay. Due to the lack of reliable quantitative activity assay for this novel protein, no precise purification data are available. Calcium-binding ability of the purified 26 kDa protein was verified by ^{45}Ca autora-

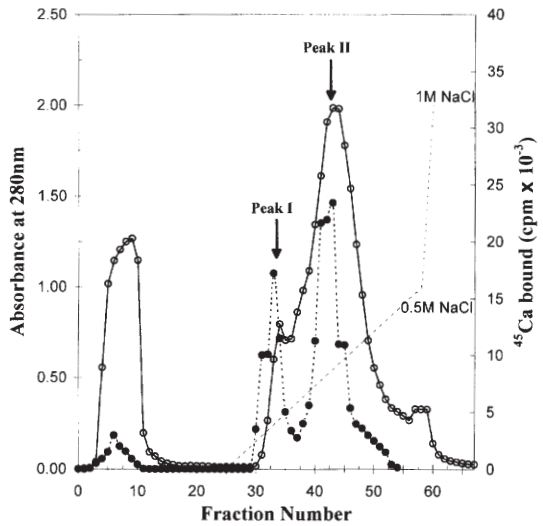

Fig. 1. Separation of two calcium-binding proteins, CAB-I (peak I) and CAB-II (peak II), from *Streptomyces coelicolor* extract on a column of Q-Sepharose. Calcium-binding activity was determined by chelex competition assay as described in the 'Materials and Methods'. (●−●) Calcium-binding activity; (○−○) absorbance at 280 nm, (···) NaCl.

24 Chang *et al.*

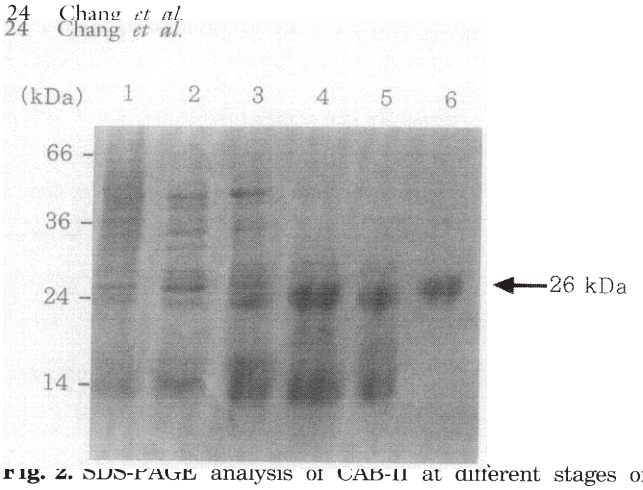

Fig. 2. SDS-PAGE analysis of CAB-II at different stages of purification. Aliquots of pooled active fractions obtained during CAB-II purification were subjected to 5-15 % SDS-PAGE: lane 1, crude extract after heat treatment; lane 2, ammonium sulfate precipitate; lane 3, acid precipitate; lane 4, Q-Sepharose peak II; lane 5, phenyl-agarose; lane 6, Superdex 200. The position and the molecular weight (in kDa) of standard marker proteins are indicated on the left; albumin (66K), glyceraldehyde-3-phosphate dehydrogenase (36K), trypsinogen (24K), and α-lactalbumin (14.2K).

diography in the presence of excess amounts of other cations (Mg^{2+} and K^{+}) (Fig. 3).

N-terminal amino acid sequences of CAB-I and CAB-II were determined and shown in Fig. 4. Searches of the EMBL databases revealed that CAB-II protein showed sequence similarity (73%) to rat calcium/calmodulin-dependent protein kinase II (21) and CAB-I to yeast phosphoprotein phosphatase (6).

CAB-I has an isoelectric point of approximately 5, indicating that the molecule is acidic. The apparent molecular mass determined by SDS-polyacrylamide

	N-terminal Sequence
S. coelicolor CAB-I	NDLL----SGAFRDASM
Yeast protein phosphatase	NDLLRIKLSGVPSDTNY
S. coelicolor CAB-II	GVKLNNVLNVKLAPE
Rat CaMK II	GVKINNKANVVRSPK

Fig. 4. Comparison of N-terminal amino acid sequence of *S. coelicolor* CAB-I with homologous regions of yeast protein phosphatase and CAB-II with homologous regions of rat Ca^{2+}/Calmodulin-dependent Kinase II.

gel electrophoresis is rather small in the range between 10~15 kDa (data not shown).

The purified CAB-II has an apparent molecular mass of about 340 kDa as determined by gel filtration. This result indicates that the native protein exists as a multimer of 26 kDa polypeptides. The isoelectric point of CAB-II is 5.3. Calmodulin and some calcium-binding proteins show an altered electrophoretic mobility on SDS-polyacrylamide gel electrophoresis in the presence or absence of Ca^{2+} (12). In our experiment, electrophoretic shift effect was not observed with CAB-II. The ultraviolet absorption spectrum did not change upon addition of $CaCl_2$, EGTA, or DTT. Calcium-binding activity of CAB-II was found to be very stable; prolonged storage without Ca^{2+} ion at cold temperature (−20°C to 4°C) did not reduce the calcium-binding ability.

Incubation of purified 26 kDa protein (CAB-II) with [γ-^{32}P] ATP indicated that ^{32}P were incorporated into the protein. The autophosphorylation depends on the presence of calcium and bovine calmodulin (Fig. 5). All five substrates tested (Histone H1, Kemptide, Myelin basic protein, Glycogen syn-

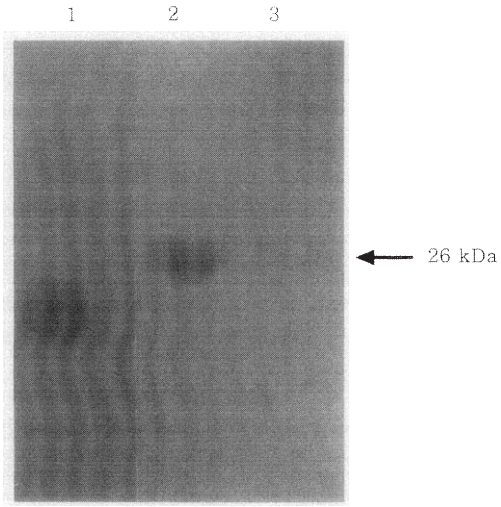

Fig. 3. Autoradiogram showing ^{45}Ca binding to the CAB-II of *S. coelicolor*. lane 1, bovine brain calmodulin (a positive control); lane 2 and 3, CAB-II from *S. coelicolor* (5 mg and 1 mg respectively).

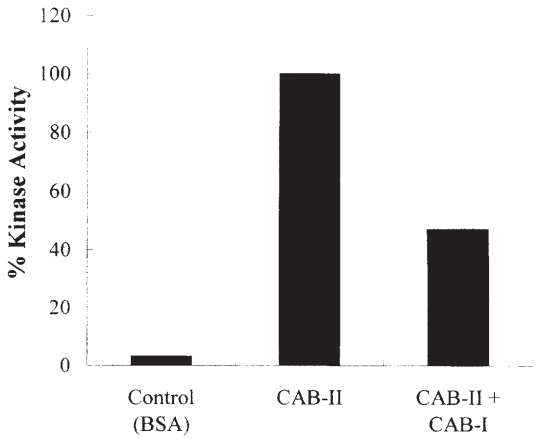

Fig. 5. Autophosphorylation of purified 26 kDa protein of *S. coelicolor*. Purified samples of CAB-I and CAB-II were subjected to phosphorylation using [γ-^{32}P] ATP as described in the 'Materials and Methods'. The amount of ^{32}P radioactivity transferred to CAB-II protein under this condition is set to 100% kinase activity.

thase, and Casein) were not phosphorylated by purified 26 kDa protein. When CAB-I was added to the reaction mixture, a significant decrease of the phosphorylation of CAB-II was observed which supports the phosphoprotein phosphatase nature of CAB-I protein.

At present, the functions of CAB-I and CAB-II are not known. However, based on the N-terminal sequences and the phosphorylation data, we believe that two calcium-binding proteins found in *Streptomyces coelicolor* play a role in the regulation of metabolic pathways which involve protein phosphorylation and dephosphorylation. Although we failed to demonstrate the presence of calmodulin-like protein(s) in *Streptomyces coelicolor*, it is noteworthy to mention that a calcium-dependent but calmodulin-independent protein kinase has been reported in soybean system (2). Incorporation of [γ-^{32}P] into CAB-II is consistent with the hypothesis that two novel calcium-binding proteins may play a role in the regulation of protein phosphorylation/dephosphorylation in *Streptomyces coelicolor*. CAB-II is the first Ca^{2+}/calmodulin-dependent protein kinase that has been reported so far in prokaryotes.

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