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Study on calcium-binding protein from *Escherichia coli* L-form NC7

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A calmodulin-like protein (CaLP) that has been regarded as a Ca^{2+}-dependent modulator protein in *Escherichia coli* L-form NC7 was purified by fluphenazine-sepharose 4B affinity chromatography. The molecular weight of the CaLP was approx. 18,000 kDa on SDS-polyacrylamide gel electrophoresis and isoelectric point of 4.5 was confirmed by electric focusing gel (pH 3 to 10). The CaLP were heat stable and exhibited Ca^{2+}-binding property. Further, the CaLP stimulated bovine hart phosphodiesterase activity. These results suggest the presence of Ca^{2+}-regulatory system in prokaryotes.

Introduction

In eukaryotic cells, the action of Ca^{2+} as a secondary messenger is mediated by Ca^{2+}-binding proteins such as calmodulin. Calmodulin has been recognized in eukaryotic cells as a small, acidic, heat stable, and calcium binding protein and is now known to activate several enzymes of physiological importance. In prokaryotes, calmodulin-like proteins have been reported in *Escherichia coli* (Iwasa *et al.*, 1981), *Bacillus subtilis* (Fry *et al.*, 1986), Mycobacterial species (Falah *et al.*, 1988; Salih *et al.*, 1991), Cyanobacteria (Petterson and Bergman, 1989), and *Myxococcus xanthus* (Inouye *et al.*, 1983). Calmodulin-like proteins and calcium-mediated regulation in a number of bacteria have been critically reviewed by Onek and Smith (1992). Recently, three calmodulin-like proteins has identified in *E. coli* (Laoudj *et al.*, 1994). Previously, we reported that Ca^{2+} is also required for growth of *E. coli* L-form (Onoda *et al.*, 1992). In this study, we present evidence for a calmodulin-like protein in L-form NC7 derived from *E. coli* K12, which require calcium for growth.

Materials and Methods

Organism and growth conditions

Escherichia coli K12 3301 were aerobically grown at 37°C in LB medium containing 1% peptone, 0.5% yeast extract, 0.5% NaCl and 0.2% glucose. L-form NC7 (Onoda, 1986) derived from *E. coli* K12 3301 were grown in NaPY medium containing 1%

peptone, 0.5% yeast extract, 2% NaCl, 1 mM $CaCl_2$ and 0.2% glucose as static culture at 32°C. The pH in media were adjusted to 7.2 with NaOH. 100 unit/ml of penicillin were added to NaPY medium throughout this experiment.

Purification of calmodulin-like protein (CaLP)

L-form cells were grown at 32°C in a LB medium and harvested at the end of logarithmic phase by centrifugation at 5,000 × g for 10 min at 4°C. The cells were washed twice with Buffer I containing 10 mM Tris-HCl (pH 7.5) and 0.4 M NaCl and then stored at −20°C until use. The cell pellet was thawed and resuspended in equal volume of buffer II containing 20 mM Tris-HCl (pH 7.5), 1 mM magnesium acetate, 1 mM imidazole, 0.1 mM EGTA, 1 mM PMSF, 10 mg/ml of DNase I and 10 μg/ml of RNase I. The cell lysate was allowed to stand for 30 min at 4°C, and then mixing and sonicated for 5 min on ice. Cell debris was removed by centrifugation of 15,000 × g for 30 min at 4°C and the supernatant was fractionated with 60% ammonium sulfate precipitation. The fraction was dialyzed for 18 hours against buffer III containing 20 mM Tris-HCl (pH 7.1), 1 mM $CaCl_2$ and centrifuged at 100,000 × g for 1 hour. The supernatant was mixed with equal volume of fluphenazine-sepharose 4B suspended in buffer III (Charbonneau and Cormier, 1979). The mixture was stirred for 1 hour at 4°C and washed twice with 2 volume of buffer III. Then, the gel was packed in a column and was washed with buffer IV containing 20 mM Tris-HCl (pH 7.0) and 0.5 M NaCl. The sample was eluted with buffer V containing 20 mM Tris-HCl (pH 8.0) and 5 mM EGTA and desired fractions were pooled as sample. To the sample was added 0.4 M $CaCl_2$ to final concentration of 6 mM and the sample was concentrated by Centricon 30 [Amicon corp.]. The concentrated sample was heated in a boiling water for 5 min and denatured protein was removed by centrifugation of 548,000 × g for 1 hour at 4°C. The supernatant containing CaLP was stored at −80°C.

Calmodulin assay

The ability of L-form CaLP preparations to stimulate bovine heart phosphodiesterase was tested by an enzyme assay according to the method of Nielsen and Rickenberg (1974) with modifications (Thompson *et al.*, 1979). The reaction mixture contained 10 mM Tris-HCl (pH 7.0), 4 mM $MgSO_4$, 0.2 mM $CaCl_2$, 6×10^{-6} unit bovine heart phosphodiesterase and an appropriate amount of extract in a total volume of 150 μl. The reaction was started by addition of ^{3}H-cAMP (2 mM, 0.5 μCi). After 3 min incubation at 37°C, the reaction was terminated by placing the tubes in a boiling water bath for 1 min, and cooling on ice. Samples were incubated with 5'-nucleotidase (2.5 mg/ml) at 30°C for 15 min. ^{3}H-Adenosine was separted from unreacted substrate using Dowex-1 × 8 anion exchange resin, and centrifuged at 10,000 × g for 5 min to remove resin. The resulting supernatant was assayed for calmodulin activity by its capacity to stimulate cAMP-phosphodiesterase.

Protein determination

Protein was determined by using the Bradford protein assay (Bio-Rad). Bovine albumin was used as a standard.

Chemicals

Peptone was purchased from Kyokuto Pharmaceutical Industrial Co. Yeast extract powder was from Oriental Yeast Industrial Co. Phosphodisterase and fluphenazine were purchased from Sigma. [^{3}H]-cAMP was from Amersham. All other chemicals were reagent grade and obtained from commercial sources.

Results and Discussion

Fig. 1 shows the elution profile on fluphenazine-sepharose affinity chromatography. The peak (fractions 100 through 150) were pooled, concentrated by an Amicon ultrafiltration cell, and heated for 5 min in a boiling water bath to abolished the endogenous activity of phsophodiesterase. Such heat stability is typical of calmodulin (Klee and Vanaman, 1982). These samples were employed for the following studies. The eluted peak appeared as a single protein band of approx. 18 kDa by sodium dodecyl sulfate (SDS)-polycacrylamide gel electrophoresis (PAGE) (Fig. 2). The CaLP from the L-from shows similarities in molecular mass to those of the respective proteins (17.6 and 18.6 kDa) of mammalian (Watterson *et al.*, 1980) and plant (Wegerhoff and

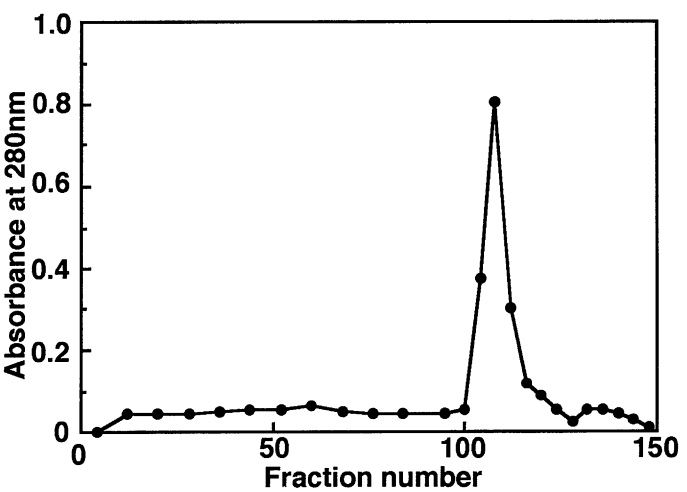

Fig. 1. Fluphenazine-Sepharose affinity chromatography of calmodulin-like protein from *E. coli* L-form. The ammonium sulfate precipitated sample was applied to a fluphenazine-Sepharose column equilibrated with Ca^{2+}-containing buffer. The column was washed with the same buffer. Calmodulin-like protein was eluted with the buffer containing EGTA in place of $CaCl_2$. Fractions of 5 ml were collected.

Wagner, 1992) but appears to be smaller than those of *Bacillus subtilis* (23 to 25 kDa) (Fry *et al.*, 1991) and *Bacillus cereus* (24 kDa) (Shyu and Foegeding, 1991). However, in different cyanobacterial species, the presence of proteins in the range of 16-18 kDa were reported (Onek, 1991; Petterson and Bergman, 1989; Bianchini, *et al.*, 1990). In this study, it was found that the CaLP is an acidic protein; an isoelectric point of 4.5 was estimated by isoelectric focusing gels in the range of pH 3 to 10 (data not shown). The isoelectric point of CaLP from the L-form differs slightly from that of CaLP from *B. subtilis* (4.9 to 5.0) (Fry *et al.*, 1991). The CaLP displayed a no significant Ca^{2+}-induced shift in mobility, which is an important criterion for identification of calmodulin (data not shown). Another important property of calmodulin is the ability to bind calcium. To demonstrate Ca^{2+} binding directly 10 μg of the CaLP was absorbed to nitrocellulose membrane and after washing twice with 10 mM Tris-buffer (pH 7.0), the membrane was immersed in Tris-buffer containing 0.1 mM $^{45}Ca^{2+}$ (100 μCi/ml) and after 5 min, washed twice with same buffer and dried. Radioactivity of dried membrane was estimated by scintillation counter. Table 1 shows that Ca^{2+} was bound to the purified L-form CaLP. In addition, the ability of CaLP preparations to activate bovine heart cAMP phosphodiesterase was tested. The dose-response plot of the activation of

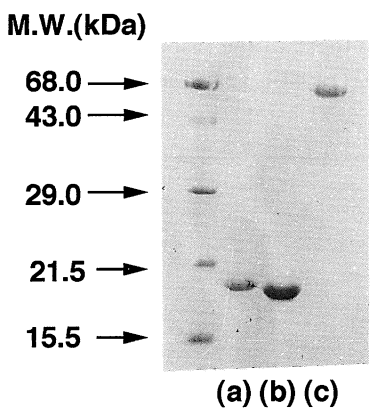

Fig. 2. SDS-PAGE of calmodulin-like protein from *E. coli* L-form. (a) Bovine heart calmodulin. (b) Calmodulin-like protein. (c) Bovine serum ablumin. Molecular mass markers are indicated at the left.

Table 1. Binding of ^{45}Calcium to Calmodulin-like protein

*Sample	DPM
CLP	267.46
**Calmodulin	240.70
***BSA	22.26

* 5 μg protein spotted
** Bovine brain calmodulin
*** Bovine serum albmine

bovine heart phosphodiesterase with the CaLP was shown in Fig. 3. The boiled sample increased the activities of bovine heart phosphodiesterase by 30%. Such heat stability is typical of calmodulin (Klee and Vanaman, 1982). Fig. 4 shows the effect of calcium concentration on bovine heart phosphodiesterase activity, demonstrating that Ca^{2+} was required for stimulation of the phosphodiesterase activity by the CaLP preparation. A recent review (Onek and Smith, 1992) presented a number of data which supported the existence of such proteins in different species of bacteria. Harmon *et al.*, (1985) identified three heat stable proteins (33, 47 and 60 kDa), which bind $^{45}Ca^{2+}$, in crude extracts of *E. coli*. These proteins did not activate the calmodulin-dependent enzyme. Molecular masses of these proteins are larger than that of the CaLP from L-form NC7. Recently, Onek *et al.*, (1994) have reported that a 21 kDa protein which prossesses characteristics of the calmodulin present in eukaryotic cells was isolated from the Cyanobacterium Nostoc sp. This protein activated pea NAD kinase *in vitro*, in a calcium requiring reaction and reacted with polyclonal antibodies raised against spinach calmodulin, but not with those raised against bovine brain calmodulin. Petterson and Bergman (1989) reported that boiled crude extracts caused a Ca^{2+}-dependent stimula-

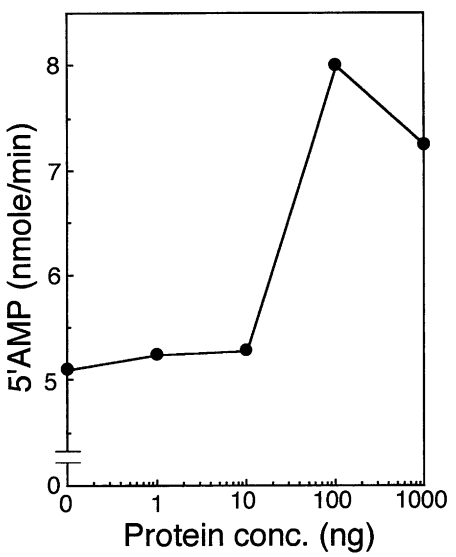

Fig. 3. Activation of bovine heart 3'-5' phosphodiesterase with calmodulin-like protein. 6×10^{-6} units of bovine heart phosphodiesterase was incubated with CaLP in the buffer containing 0.1 mM of $CaCl_2$, ^{3}H-cAMP (2 mM, 0.5 μCi), 10 mM Tris-HCl pH 7.0, 4 mM $MgSO_4$. Reaction conditions are described in the Materials and Methods.

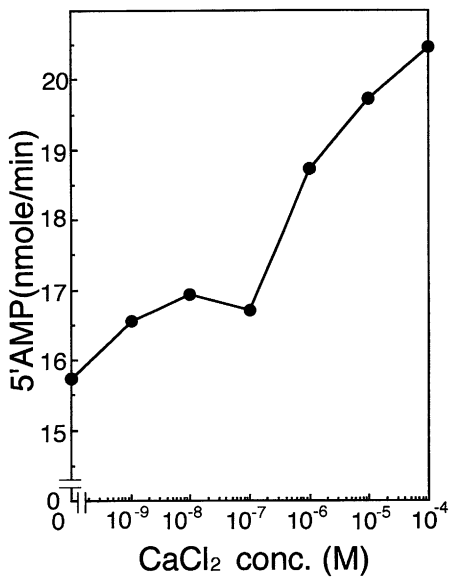

Fig. 4. Calcium-dependent activation of bovine heart 3'-5' cAMP phosphodiesterase activity with calmodulin-like protein. Reaction mixture contained 100 ng of CaLP. Reaction condition was described in the Materials and Methods.

tion of NAD kinase and Western blot analysis using antiserum against eukaryotic spinach calmodulin, revealed the presence of about 17 kDa protein. Laoudi *et al.*, (1994) have been shown that when *E. coli* mutant (verA, dilA) specifically resistant to the Ca^{2+} channel inhibitors verapamil and diltiazem, respectively was treated with EGTA, at least, three proteins with molecular masses of 12, 18 and 34 kDa, which is heat stable and acidic, were induced and these proteins cross-reacted with antibodies to eukaryote calmodulins. Of these proteins, protein with molecular mass of 18 kDa seem to be like CaLP (approx. 18 kDa) from L-form NC7. Calmodulin has been implicated in the Ca^{2+}-dependent regulation of a number of cellular activities. Growth of the L-form NC7 were affected by calmodulin antagonists such as chlorpromazine, W-7 and W-13 (Onoda *et al.*, in preparation). The DNA synthesis of the L-form and ^{32}Pi incorporation into acid insoluble fractions were strongly inhibited by these drugs. Leadlay *et al.*, (1984) have reported that an acidic, 21 kDa protein from *Streptomyces erythreus*, which is capable of binding calcium with high affinity, does not activate 3'5'-cAMP phosphodiesterase. It was proposed (Norris *et al.*, 1988; Holland *et al.*, 1990) that in prokaryotes, as in eukaryotes, calcium could play a predominant role in the control of growth and the cell cycle. The CaLP in L-form NC7 (*E. coli*) support the concept of Ca^{2+} mediated regulation in procaryotic cells. Further work will be required to clarify both the structure and the biological role of this protein.

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