Available online at www.sciencedirect.com
SCIENCE DIRECT
Biochimica et Biophysica Acta 1748 (2005) 157–164
BIOCHIMICA ET BIOPHYSICA ACTA
BBA
http://www.elsevier.com/locate/bba

Calcium-induced conformational changes of the recombinant CBP3 protein from *Dictyostelium discoideum*

Tsogbadrakh Mishig-Ochiriin[a,1], Chang-Hun Lee[b,2], Sun-Young Jeong[a], Beom-Jun Kim[b], Chang-Hoon Choi[b], Hyung-Soon Yim[b,*], Sa-Ouk Kang[a,b,*]

[a]*Laboratory of Biophysics, School of Biological Sciences, Seoul National University, Seoul 151-742, South Korea*
[b]*Institute of Microbiology, Seoul National University, Seoul 151-742, South Korea*

Received 3 September 2004; received in revised form 24 December 2004; accepted 27 December 2004
Available online 26 January 2005

Abstract

Calcium-binding proteins play various and significant roles in biological systems. Conformational changes in their structures are closely related to their physiological functions. To understand the role of calcium-binding protein 3 (CBP3) in *Dictyostelium discoideum*, its recombinant proteins were analyzed using circular dichroism (CD) and fluorescence spectroscopy. Gel mobility shift analysis showed that Ca^{2+} induced a mobility shift of the recombinant CBP3. Far ultra-violet CD spectra and intrinsic fluorescence spectra on CBP3 and its N- and C-terminal domains exhibited that they underwent a conformational rearrangement depending upon Ca^{2+} binding. Measurement of Ca^{2+} dissociation constants demonstrated that CBP3 had high affinity toward Ca^{2+} in the sub-micromolar range and N-terminal domain had higher affinity than C-terminal domain. The changes of fluorescence spectra by an addition of 8-anilino-1-naphthalene sulfonic acid indicated that the hydrophobic patches of CBP3 and its C-terminal domain are likely to be more exposed in the presence of Ca^{2+}. Since the exposure of hydrophobic patches is thermodynamically unfavorable, Ca^{2+}-bound CBP3 may interact with other proteins in vivo. All these data suggest that Ca^{2+} induces CBP3 to be more favorable conformation to interact with target proteins.

Keywords: Calcium-binding protein 3; Conformational rearrangement; *Dictyostelium discoideum*

1. Introduction

Calcium ion (Ca^{2+}) regulates wide variety of cellular processes such as cell-cycle progression, differentiation, muscle contraction, signal transduction, nucleotide metabolism, and enzyme activities. These roles of calcium ion must be mediated by a variety of calcium-binding proteins. Calcium-binding proteins can act as buffers storing and releasing Ca^{2+}, or as sensors detecting Ca^{2+} concentration and transducing the signals [1–5]. These calcium-binding proteins have highly conserved helix-loop-helix structure, EF-hand motifs, with a Ca^{2+} ion bound to the interhelical loop region [6]. In general, a pair of EF-hand helix-loop-helix motifs forms a globular domain. Proteins containing four such motifs will thus have two domains that can be either structurally dependent or independent [7–9]. Upon Ca^{2+} binding, the sensor proteins undergo conformational change and in turn regulate a large number of target proteins. Calmodulin regulates many intracellular target proteins such as calmodulin kinases, myosin light chain kinases and calcineurin, and NMDA receptor [10]. Troponin C acts as a Ca^{2+} sensor in muscle cells [11] and recoverin in retinal rod cells [12].

There were many reports indicating that calcium ion plays a role during the development of *Dictyostelium*

* Corresponding authors. Hyung-Soon Yim is to be contacted at Tel.: +82 2 880 6703; fax: +82 2 888 4911. Sa-Ouk Kang, Tel.: +82 2 875 0506; fax: +82 2 888 4911.
E-mail addresses: wuseok@hotmail.com (H.-S. Yim), kangsaou@snu.ac.kr (S.-O. Kang).
[1] Present address: Department of Biophysics, Faculty of Biology, National University of Mongolia, Ulaanbaatar 210646, Mongolia.
[2] Present address: Center for Cell Signaling Research, Ewha Womans University, Seoul 120-750, Republic of Korea.

1570-9639/$ - see front matter © 2004 Elsevier B.V. All rights reserved.
doi:10.1016/j.bbapap.2004.12.018

discoideum: Ca^{2+} is involved in numerous developmental stages, such as chemotaxis [13–15], cell–cell adhesion [16], cell fate determination [1,17–19], and transduction of starvation signal [20].

Previously, we have reported the cloning and characterization of *cbpC* from *D. discoideum* [21]: calcium-binding protein 3 (CBP3) found in *Dictyostelium* is a small acidic protein, which has four putative EF-hand motifs and is expressed at the developmental stage. Although several small acidic calcium-binding proteins have been also reported in *Dictyostelium* [22–25], the structural properties of the calcium-binding proteins have not been reported yet. To characterize structural properties and calcium affinity features of the CBP3 protein, CBP3 and its N- and C-terminal domains were overexpressed in *Escherichia coli*, respectively, and their conformational properties were characterized. Here, we report the calcium-induced conformational change of the recombinant *Dictyostelium* CBP3.

2. Materials and methods

2.1. Plasmid construction

The expression vectors for N- and C-terminal half domains of the CBP3 protein in *E. coli* were constructed by polymerase chain reaction (PCR), using pET-CBP3 [21] as a template. For N-terminal domain, forward primer (5′-TAT TCA TAT GTT AAC TAA TAA TG-3′) and reverse primer (5′-GGA TCC TCA ATT AGT TTT TTT GCA) were used. For C-terminal domain, forward primer (5′-CAT ATG CAA AAT GCT GAT ATT GCT GCA TTG) and reverse primer (5′-CCC AGG ATC CTT TTA AAC AAT GTG GAC GGC-3′) were used. The PCR products were cloned into *Nde*I and *Bam*HI site of pET15b expression vector (Novagen) and named pET-CBP3N, pET-CBP3C, respectively. DNA sequencing of both strands verified correct ligation and in-frame insertion of the fragments.

2.2. Recombinant CBP3 Protein expression and purification

E. coli BL21 (DE3) pLysS cells harboring pET-CBP3, pET-CBP3N, and pET-CBP3C were grown in Luria–Bertani (LB) medium containing ampicillin (50 μg/ml). When cells were grown up to absorbance 0.5 at 600 nm, CBP3 expression was induced with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) for 3 h. The induced cells were collected and stored at −20 °C. The frozen cells were thawed and solubilized in binding buffer (20 mM Tris–HCl, pH 7.9, containing 5 mM imidazole and 0.5 M NaCl) and were gently sonicated and centrifuged at 12,000 rpm for 15 min. The pellets were solubilized in binding buffer, which contained 6 M urea. The solubilized proteins were purified using nickel-affinity column (Novagen) under the denaturation condition. The eluates were strongly reduced by adding dithiothreitol (DTT) to a final concentration of 20 mM and refolded rapidly by 100-fold volume of refolding buffer (20 mM Tris–HCl, pH 8.5, containing 30 mM L-arginine, 0.5 mM oxidized glutathione, 150 mM NaCl, and 2.5 mM $CaCl_2$). The concentration of the proteins during refolding process was approximately 10 μM. The samples were incubated at room temperature for 2 h and concentrated by ultrafiltration (Amicon). The N-terminal His-tag leader was removed by thrombin. The cleavage of the His-tag leader and the purity of the protein were assessed by sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) [26] or Tricine SDS-PAGE [27]. The concentration of protein was measured using absorbance at 280 nm and D_C Protein assay kit (Bio-Rad). The molar absorption coefficient of the protein was estimated according to the previously proposed method [28]. The Ca^{2+}-free proteins were prepared according to the previously proposed method [29].

2.3. UV-visible absorption spectrum measurement

The UV-visible absorption spectrum of recombinant CBP3 was recorded on Shimadzu UV-160 spectrophotometer at ambient temperature. The final protein concentration was about 70 μM.

2.4. Far-UV circular dichroism (CD) spectra measurements

The far-UV CD spectra were recorded on Jasco J715 spectropolarimeter at ambient temperature. Protein concentration was approximately 8 μM. Measured ellipticities, θ were converted to molar ellipticity, $[\theta]$, by the relation: $[\theta]=\theta/10lc_{MRW}$, where l is path length and c_{MRW} is the protein molar concentration per residue. Quantification of secondary structure contents was carried out with the self-consistent method [30]. Molar ellipticity data corrected for background was analyzed using the program Dicroprot [31].

2.5. Fluorescence emission spectra measurements

Fluorescence emission spectra were recorded on SLM Amino 48000 spectrofluorometer at ambient temperature with excitation at 280 nm. Spectral measurements were carried out at protein concentration of approximately 8 μM in 20 mM 4-morpholinepropanesulfonic acid (MOPS) buffer at pH 7.2. The slit widths for excitation and emission were 5 and 2 nm, respectively.

The calcium titration data were fitted by the following Hill equation: $f=[Ca^{2+}]^n/K^n+[Ca^{2+}]^n$, where f is the fraction of the fluorescence intensity change at a given free Ca^{2+} concentration from that of apo-protein over the maximal change during the titration. The Hill coefficient (n_H) was obtained from the following equation: $\log[f/(1-f)]=a+n_H\log[Ca^{2+}]$.

8-Anilino-1-naphthalene sulfonic acid (ANS) fluorescence spectra were recorded at the of 450–600 nm (2 nm slit

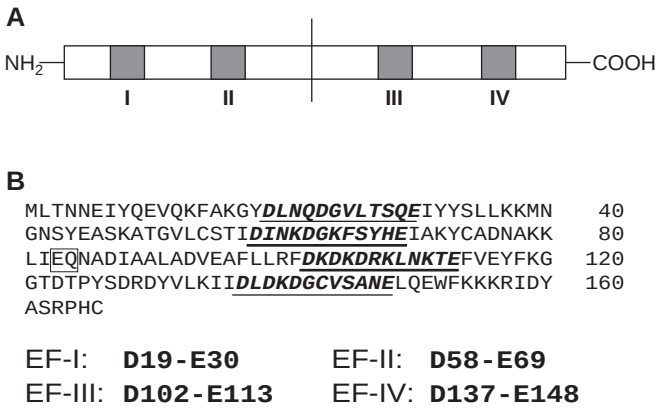

Fig. 1. (A) Graphical representation of CBP3. Four EF-hand motifs are represented with gray box and numbered with I–IV. The vertical line between EF-hand II and III indicates the cleavage site. (B) Sequences of CBP3 and its N- and C-terminal domains. EF-hand motifs were underlined and italicized in bold. The boundary residues between N- and C-terminal domains were Glutamate (E) and Glutamine (Q). The cleaved site between N- and C-terminal domains was boxed.

width) after the excitation at 385 nm (5 nm slit width) and ANS concentration was approximately 60 μM in the presence of 2 mM $CaCl_2$ or 10 mM ethylene glycol-bis(β-aminoethylether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA). Sample buffer was 10 mM Tris–HCl (pH 7.50) containing 1 mM DTT and 150 mM KCl. The final concentration for each protein was approximately 12 μM.

3. Results

3.1. Expression of recombinant CBP3 and its N- and C-terminal domains

CBP3 is one of the several calcium-binding proteins in *D. discoideum* and contains four EF-hand motifs (residues D19-E30, D58-E69, D102-E113, and D137-E148) (Fig. 1A) [21]. By secondary structure prediction, CBP3 is an α-helix rich-protein (63%) and all EF-hand motifs are located in the loop. In case of calmodulin, it is well known that N- (EF-I, II) and C-terminal domains (EF-III, IV) have very similar structure and function independently without any interaction between two domains [4,7,32]. Since the primary structure of CBP3 is quite different from that of calmodulin, it would be important to characterize CBP3 and its N- and C-terminal domains. We constructed recombinant CBP3, N- and C-terminal domains of CBP3 to investigate their Ca^{2+}-binding properties and conformational changes induced by Ca^{2+}.

CBP3 and its N- and C-terminal domains were cloned into *E. coli* expression vector pET15b. N- and C-terminal domains of CBP3 consist of residues 1–83 and 84–160, respectively (Fig. 1B) and each domain contains two EF hand motifs. These recombinant proteins were expressed in *E. coli* strain BL21 (DE3) and the average yields were about 10–25 mg of protein/l of LB medium. The majority of

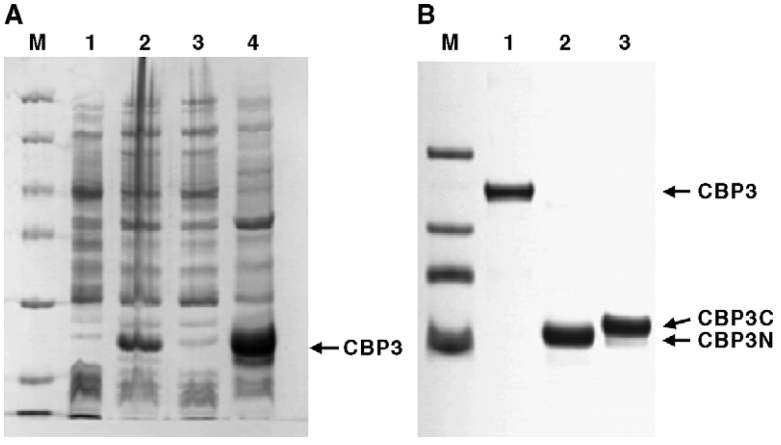

Fig. 2. CBP3 expression in *E. coli*. (A) CBP3 expression profile in *E. coli* BL21 (DE3) pLysS cells harboring pET-CBP3. *E. coli* BL21 cells were induced with 1 mM IPTG and prepared at the indicated time after induction. M, molecular size marker (116, 66, 45, 35, 25, 16.4, and 14.4 kDa from upper band); lane 1, cell lysate at 0 h after induction; lane 2, cell lysate at 3 h after induction; lane 3, soluble fraction of cell lysate at 3 h after induction; lane 4, insoluble fraction of cell lysate at 3 h after induction. (B) Purified CBP3 (lane 1), its N- (lane 2) and C-terminal (land 3) domains after the removal of His-tag leader by thrombin. M, molecular size marker (26.6, 17, 14.4, and 6.5 kDa from upper band).

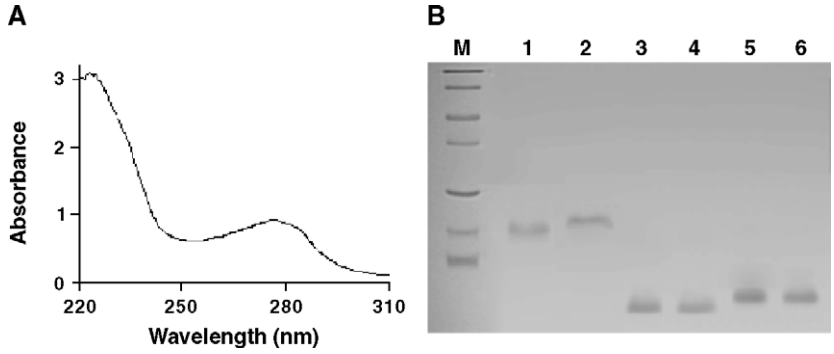

Fig. 3. UV-Vis absorption spectrum of refolding CBP3 protein (A) and calcium-induced gel mobility shift of recombinant CBP3 and its N- and C-terminal domains (B). (A) UV-Vis absorption spectrum of 70 μM purified CBP3 was recorded at ambient temperature. (B) Equal amount of CBP3 with 2 mM $CaCl_2$ (lanes 1, 3, and 5) or 2 mM EGTA (lanes 2, 4, and 6) were loaded on 13% SDS-PAGE. M, molecular size marker (116, 66, 45, 35, 25, 16.4, and 14.4 kDa from upper band); lanes 1 and 2, CBP3; lanes 3 and 4, N-terminal domain; lanes 5 and 6, C-terminal domain.

CBP3 proteins was found in the insoluble fraction, inclusion body (Fig. 2A) and the expression level was increased when 1~2% (w/v) glucose was added in the culture medium (data not shown). To solubilize the recombinant CBP3 proteins, inclusion bodies were dissolved in 6 M urea, and purified by nickel-affinity chromatography under denatured condition, and refolded. The N-terminal His-tag leader was removed by thrombin. The cleavage of the His-tag leader and the purity of the protein were assayed by SDS-PAGE. They appeared as single bands on SDS-PAGE under reducing conditions (Fig. 2B). The purified proteins were dialyzed against appropriate buffer and stored at 4 °C prior to use.

3.2. UV-visible absorption spectrum and calcium induced electrophoretic gel mobility shift of recombinant CBP3

The UV-visible absorption spectrum of CBP3 showed no absorption bands of prosthetic groups or metal ions, except absorption bands of peptide bond and aromatic amino acids (Fig. 3A). The concentration of protein was measured and the molar absorption coefficient of the protein was calculated according to the previously proposed method [28]. The calculated molar absorption coefficient for CBP3 and its N- and C-terminal domains at 280 nm were 22,140, 10,555, and 11,585 M^{-1} cm^{-1}, respectively.

Calcium-binding proteins show different mobility in SDS-PAGE depending on the presence of calcium or EGTA. The refolded recombinant CBP3 protein in SDS-PAGE also migrated slowly in the presence of EGTA (Fig. 3B). This result was in good agreement with the result from CBP3 detected by Western blot analysis in the crude extract of 8 h-developed *Dictyostelium* [21]. These results implied that recombinant CBP3 retained its binding activity to Ca^{2+} and underwent similar conformational change with native CBP3 upon Ca^{2+} binding. Besides the CBP3, N- and C-terminal domains also showed a slight mobility shift in gel (Fig. 3B).

3.3. Far-UV CD spectra and secondary structure contents

The far-UV CD spectra of CBP3 and its N- and C-terminal domains in the absence and presence of calcium were presented in Fig. 4. All spectra showed two minima at 208 and 222 nm, which are characteristics of proteins with a large portion of α-helix. The secondary structure contents and calcium-induced conformational change of CBP3 and its N- and C-terminal domains were predicted (Table 1). In

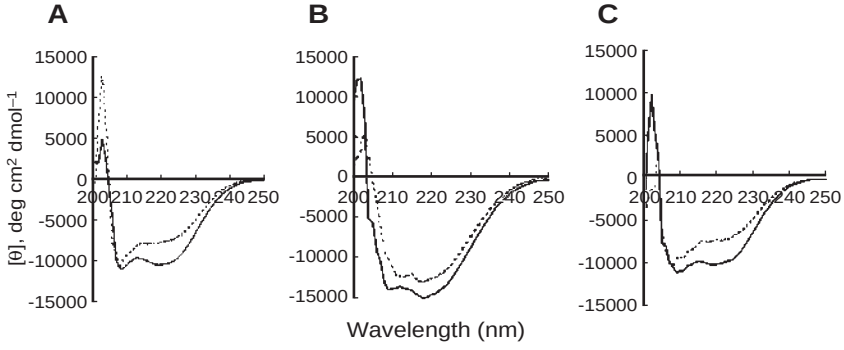

Fig. 4. Far-UV CD spectra of CBP3 (A) and its N- (B) and C-terminal (C) domains. The far-UV CD spectra were recorded on Jasco J715 spectropolarimeter at ambient temperature. Protein concentration was approximately 8 μM. Each protein was in apo- (dotted line) and 2 mM calcium saturated states (solid line) in 20 mM MOPS buffer, pH 7.2. Measured ellipticities were converted to molar ellipticity, [θ] and the quantification of the contents of secondary structure was carried out with the self-consistent method.

Table 1
The secondary structure composition of CBP3 and its domains calculated from CD spectra[a] in the absence or presence of calcium ion[b]

	CBP3		CBP3N		CBP3C	
	$-Ca^{2+}$	$+Ca^{2+}$	$-Ca^{2+}$	$+Ca^{2+}$	$-Ca^{2+}$	$+Ca^{2+}$
α-helix	32.5	31.0	42.9	43.9	21.5	31.8
Turns	27.9	25.8	-	21.7	22.6	25.8
β-sheet and other structure	41.6	46.5	57.1	36.7	60.4	44.7

[a] The secondary structure contents were calculated from Dicroprot [31] program based on the self-consistent method [30].
[b] The concentration of calcium ion was 2 mM.

the apo-state, CBP3 had about 32.5% of α-helix, 27.9% of turns, and 41.6% of β-sheet and another structure. N-terminal domain had 42.9% of α-helical content, which was higher than those of CBP3 and its C-terminal domain. Interestingly, N-terminal domain had no turns and high content of β-sheet and another structure (57.1%). C-terminal domain had lower α-helical content and higher content of β-sheet and another structure than CBP3 and its N-terminal domain. When 2 mM of Ca^{2+} was added, the changes in the CD spectrum indicated that conformational rearrangements occurred due to calcium binding. CBP3 showed slightly decrease in the contents of α-helix and turns and increase in the content of β-sheet and another structure. N-terminal domain showed increase in the content of turns from 0% to 21.7% and decrease in the content of β-sheet and another structure from 57.1% to 36.7%, respectively. C-terminal domain underwent increase of the contents of α-helix and turns from 21.5% to 31.8% and from 22.6% to 25.8%, respectively, while the content of β-sheet and another structure decreased from 60.4% to 44.7%. The Ca^{2+}-induced $\Delta[\theta]_{222}$ value of full-length CBP3 was 2.76. While N-terminal domain had more α-helical content, the Ca^{2+}-induced $\Delta[\theta]_{222}$ value of C-terminal domain was 2.65, which was greater than 1.94 of N-terminal domain. This kind of Ca^{2+}-induced spectral change implies that C-terminal domain may undergo greater conformational change than N-terminal domain after the addition of Ca^{2+}. CD spectrum of full-length CBP3 was not the direct sum of the spectra of two separate domains (Fig. 4), which suggests that there might be some interactions between the N- and C-terminal domains.

3.4. Calcium dissociation constants (K_D) and Hill coefficient

The effect of calcium on the conformation of CBP3 was also investigated by its intrinsic fluorescence. Since excitation wavelength was 280 nm, seven tyrosine residues in N-terminal domain and four tyrosine and one tryptophan residues in C-terminal domain could contribute to their intrinsic fluorescence. As the concentration of calcium ion increased, fluorescence intensities of CBP3 and its N- and C-terminal domains increased (Fig. 5). This result supports the conclusion that calcium-induced conformational change of CBP3 and its domains. Even though fluorescence intensities of three proteins increased after the addition of calcium, CBP3 and its N-terminal domain showed red-shifted λ_{max} (327→337 nm and 320→323 nm, respectively), while C-terminal domain did blue-shifted λ_{max} (354→349 nm). Calcium binding probably changed the environment of fluorophores of CBP3 and its N- and C-terminal domains differently. The spectra in the presence of a denaturating agent, 6M guanidine–HCl indicated that CBP3 and its N- and C-terminal domains had no other chromophores and represented their intrinsic fluorescence due to tyrosine or tryptophan, respectively (Fig. 5). Using changes of intrinsic fluorescence by calcium, K_D value and Hill coefficient of CBP3 and its domains were calculated (Table 2). K_D of CBP3 for calcium was 7.76×10^{-6} M, which means high affinity for Ca^{2+} and the calcium affinities of each domain were relatively lower than that of CBP3. A calculated Hill coefficient of CBP3 was 2.2, while those of N-terminal and C-terminal domains were less than 1. These values obtained

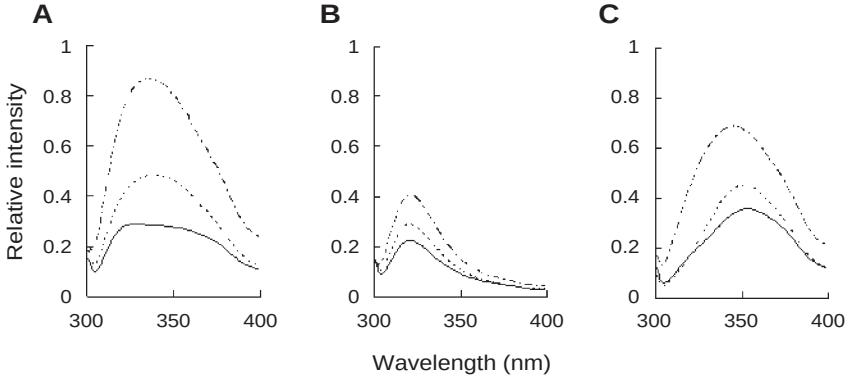

Fig. 5. Changes of fluorescence emission spectra of CBP3 (A) and its N- (B) and C-terminal (C) domains induced by Ca^{2+}. Samples were excited at 280 nm and fluorescence emission spectra were recorded on SLM Amino 48000 spectrofluorophotometer at ambient temperature. The protein concentration was approximately 8 μM in 20 mM MOPS buffer at pH 7.2. Each protein was in the apo- (solid line), calcium-saturated (dotted line) and denaturated state with 6 M guanidine–HCl (dashed line).

Table 2
Hill coefficients and calcium dissociation constants of CBP3 and its domains[a]

	CBP3	CBP3N	CBP3C
n_H	2.2	0.72	0.44
K_D(M)	7.76×10^{-6}	1.20×10^{-4}	7.59×10^{-4}

[a] The Hill coefficient n_H and dissociation constants (K_D) were calculated using Hill equation.

from intrinsic fluorescence suggested full-length protein of CBP3 and its domains responded to Ca^{2+} with different sensitivity and positive cooperativity in calcium binding occurred only in full-length of CBP3 protein and there was a strong interaction between the N- and C-terminal domains.

3.5. Interaction of recombinant CBP3 with ANS

To investigate whether calcium changes the hydrophobicity of recombinant CBP3, ANS fluorescence was measured. ANS fluorescence strongly depends on polarity of the local environment around the fluorophore and is applied to monitor protein conformational changes [29]. The Ca^{2+} forms of CBP3 showed a considerable binding of ANS, an enhancement of fluorescence, and a significant blue shift of λ_{max} (512→502 nm) compared to the apo-state of CBP3 (Fig. 6A). The N-terminal domain of CBP3 also showed the binding of ANS on both apo- and Ca^{2+}-binding forms (Fig. 6B), but the enhancement of fluorescence or blue shift was not observed. The C-terminal domain of CBP3 exhibited a similar fluorescence spectrum to CBP3 with significant blue shift of λ_{max} (515→509 nm) (Fig. 6C). When the same molar protein concentration on each protein was used, the CBP3 fluorescence in calcium-added state was approximately the sum of the fluorescence of N- and C-terminal domains, suggesting the hydrophobicity of each domain does not affect each other. According to these data, hydrophobic regions are exposed on the surface of CBP3 and its domains even in Ca^{2+}-free states (Fig. 6) and calcium ion changed hydrophobicities of CBP3 and C-terminal domain, but did not affect the hydrophobicity of N-terminal domain. Thus, it seems that CBP3 possesses both functions of Ca^{2+} sensor and Ca^{2+} buffer unlike the other calcium-binding proteins [29]. Since the exposure of hydrophobic surfaces is thermodynamically unfavorable, there is a possibility that probably CBP3 interacts with other target proteins. From the difference of the hydrophobicity changes induced by Ca^{2+}, it is suggested that C-terminal domain may participate in calcium-induced target protein interaction, since hydrophobic regions of the C-terminal domain are drastically changed by the addition of Ca^{2+} than those of N-terminal domain and it was suggested that surface hydrophobic regions are involved in the binding to target protein [33].

4. Discussion

To understand functions of calcium-binding proteins, many efforts have been made on structural studies including calcium-induced conformational changes. Although thirteen different genes encoding small calcium-binding proteins in *Dictyostelium* have been identified, their structural characterization has not been carried out yet. Previously, we cloned *cbpC* and found that it expressed differentially during the development of *Dictyostelium*. The results of this work clearly indicated that CBP3 has a high affinity toward Ca^{2+} ion and undergoes Ca^{2+}-induced conformational changes. The effect of Mg^{2+} on conformational change and ANS fluorescence of CBP3 was monitored, but it did not make any difference (data not shown). These results implied that the conformational change due to the binding of Mg^{2+} was much smaller than the binding of Ca^{2+} and CBP3 had high selectivity to Ca^{2+} and may play a specific role in Ca^{2+}-mediated cellular processes. The Ca^{2+}-induced conformational change enables calcium sensor proteins to interact with their target proteins. Besides conformational change, there was a report that hydrophobic residues on the surface play a key role in the interaction with the target protein [33]. In this report, calcium induced the conformational change of CBP3,

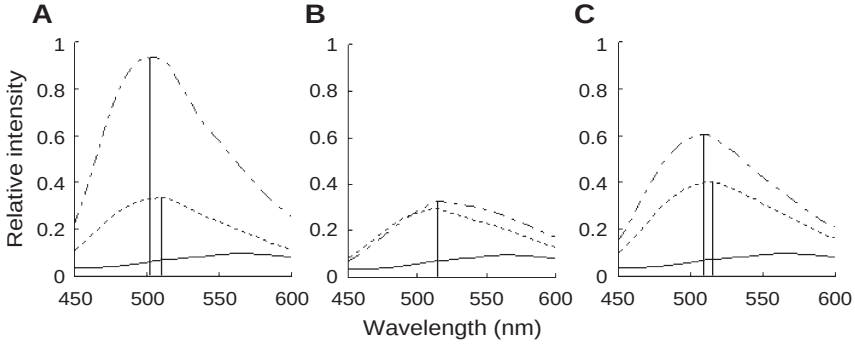

Fig. 6. Fluorescence spectral changes after the binding of ANS to CBP3 (A) and its N- (B) and C-terminal (C) domains. ANS fluorescence spectra were recorded at the range of 450–600 nm after the excitation at 385 nm. The buffer used was 10 mM Tris–HCl (pH 7.5), containing 60 μM ANS, 1 mM DTT, and 150 mM KCl and the final concentration for each protein was approximately 12 μM. Solid line, ANS; dashed line, ANS with apo-protein; and dotted line, ANS with calcium-bound protein.

especially the exposure of hydrophobic residues, suggesting the existence of target protein(s) of CBP3 mediated by calcium. We already found the target protein and now are investigating the interaction between CBP3 and its target protein in vitro and in vivo (unpublished data). In general, Ca^{2+} sensor proteins undergo Ca^{2+}-induced conformational changes leading to the exposure of hydrophobic patch, which offers the binding surface for target proteins, while Ca^{2+} buffer proteins do not expose hydrophobic surface due to the binding of Ca^{2+}, even though they have a higher affinity for calcium than Ca^{2+} sensor. Spectroscopic data indicated CBP3 shares some characteristics of Ca^{2+} sensor protein but lacks the properties of Ca^{2+} buffering. $Calbindin_{28k}$ showed very similar conformational changes with CBP3 in a Ca^{2+}-dependent manner [29] and was recently reported to exhibit properties characteristic of Ca^{2+} sensor [34]. Therefore, CBP3 may interact with target molecule(s) and function in a similar way with $calbindin_{28k}$. Interestingly, although each domain of CBP3 has two EF-hand structural motifs, calcium-induced conformational changes of N-terminal domain were different from those of C-terminal domain. That means that N-terminal domain has more rigid conformation with high affinity for Ca^{2+}. Taken together, it is suggested that when CBP3 interacts with other proteins, N-terminal domain may have a role to sense Ca^{2+} and its C-terminal domain may undergo conformational change and expose its hydrophobic regions, which helps the interaction with its binding partners.

Acknowledgements

We thank to Dr. Hyung-Sik Won and Prof. Bong-Jin Lee for technical assistance of CD spectropolarimeter operation. This work was supported by the Research Fellowship of BK21 project and Korean Research Foundation Grant (KRF-2003-041-C00318).

References

[1] A.B. Cubitt, R.A. Firtel, G. Fischer, L.F. Jaffe, A.L. Miller, Patterns of free calcium in multicellular stages of *Dictyostelium* expressing jellyfish apoaequorin, Development 121 (1995) 2291–2301.
[2] V. Döring, F. Veretout, R. Albrecht, B. Mühlbauer, C. Schlatterer, M. Schleicher, A.A. Noegel, The in vivo role of annexin VII (synexin): Characterization of an annexin VII-deficient *Dictyostelium* mutant indicates an involvement in Ca^{2+}-regulated processes, J. Cell Sci. 108 (1995) 2065–2076.
[3] C. Schlatterer, F. Gollnick, E. Schmidt, R. Meyer, G. Knoll, Challenge with high concentrations of cyclic AMP induces transient changes in the cytosolic free calcium concentration in *Dictyostelium discoideum*, J. Cell Sci. 107 (1994) 2107–2115.
[4] B.E. Finn, J. Evenäs, T. Drakenberg, J.P. Waltho, E. Thulin, S. Forsén, Calcium-induced structural changes and domain autonomy in calmodulin, Nat. Struct. Biol. 2 (1995) 777–783.
[5] H. Kuboniwa, N. Tjandra, S. Grzesiek, H. Ren, C.B. Klee, A. Bax, Solution structure of calcium-free calmodulin, Nat. Struct. Biol. 2 (1995) 768–776.
[6] G. Travé, P.J. Lacombe, M. Pfuhl, A. Saraste, A. Pastore, Molecular mechanism of the calcium-induced conformational change in the spectrin EF-hands, EMBO J. 14 (1995) 4922–4931.
[7] M. Zhang, T. Tanaka, M. Ikura, Calcium-induced conformational transition revealed by the solution structure of apo calmodulin, Nat. Struct. Biol. 2 (1995) 758–767.
[8] P. Mercier, M.X. Li, B.D. Sykes, Role of the structural domain of troponin C in muscle regulation: NMR studies of Ca^{2+} binding and subsequent interactions with regions 1–40 and 96–115 of troponin I, Biochemistry 39 (2000) 2902–2911.
[9] R.H. Kretsinger, C.E. Nockolds, Carp muscle calcium-binding protein: II. Structure determination and general description, J. Biol. Chem. 248 (1973) 3313–3326.
[10] A. Crivici, M. Ikura, Molecular and structural basis of target recognition by calmodulin, Annu. Rev. Biophys. Biomol. Struct. 24 (1995) 85–116.
[11] P.C. Leavis, J. Gergely, Thin filament proteins and thin filament-linked regulation of vertebrate muscle contraction, CRC Crit. Rev. Biochem. 16 (1984) 235–305.
[12] A.M. Dizhoor, C.K. Chen, E. Olshevskaya, V.V. Sinelnikova, P. Phillipov, J.B. Hurley, Role of the acylated amino terminus of recoverin in Ca^{2+}-dependent membrane interaction, Science 259 (1993) 829–832.
[13] D. Malchow, R. Mutzel, C. Schlatterer, On the role of calcium during chemotactic signalling and differentiation of the cellular slime mould *Dictyostelium discoideum*, Int. J. Dev. Biol. 40 (1996) 135–139.
[14] S. Saran, H. Nakao, M. Tasaka, H. Iida, F.I. Tsuji, V. Nanjundiah, I. Takeuchi, Intracellular free calcium level and its response to cAMP stimulation in developing *Dictyostelium* cells transformed with jellyfish apoaequorin cDNA, FEBS Lett. 337 (1994) 43–47.
[15] T. Nebl, P.R. Fisher, Intracellular Ca^{2+} signals in *Dictyostelium* chemotaxis are mediated exclusively by Ca^{2+} influx, J. Cell Sci. 110 (1997) 2845–2853.
[16] S.K. Brar, C.-H. Siu, Characterization of the cell adhesion molecule gp24 in *Dictyostelium discoideum*. Mediation of cell–cell adhesion via a Ca^{2+}-dependent mechanism, J. Biol. Chem. 268 (1993) 24902–24909.
[17] Y. Kubohara, K. Okamoto, Cytoplasmic Ca^{2+} and H^{+} concentrations determine cell fate in *Dictyostelium discoideum*, FASEB J. 8 (1994) 869–874.
[18] M. Azhar, P.S. Manogaran, P.K. Kennady, G. Pande, V. Nanjundiah, A Ca^{2+}-dependent early functional heterogeneity in amoebae of *Dictyostelium discoideum*, revealed by flow cytometry, Exp. Cell Res. 227 (1996) 344–351.
[19] P. Schaap, T. Nebl, P.R. Fisher, A slow sustained increase in cytosolic Ca^{2+} levels mediates stalk gene induction by differentiation inducing factor in *Dictyostelium*, EMBO J. 15 (1996) 5177–5183.
[20] Y. Tanaka, R. Itakura, A. Amagai, Y. Maeda, The signals for starvation response are transduced through elevated $[Ca^{2+}]_i$ in *Dictyostelium* cells, Exp. Cell Res. 240 (1998) 340–348.
[21] Y.-H. Han, S.-O. Kang, Cloning of a cDNA encoding a new calcium-binding protein from *Dictyostelium discoideum* and its developmental regulation, FEBS Lett. 441 (1998) 302–306.
[22] T. Liu, J.G. Williams, M. Clarke, Inducible expression of calmodulin antisense RNA in *Dictyostelium* cells inhibits the completion of cytokinesis, Mol. Biol. Cell 3 (1992) 1403–1413.
[23] F. Abe, Y. Maeda, Specific expression of a gene encoding a novel calcium-binding protein, CAF-1, during transition of *Dictyostelium* cells from growth to differentiation, Dev. Growth Differ. 37 (1995) 39–48.
[24] B. Coukell, J. Moniakis, A. Grinberg, Cloning and expression in *Escherichia coli* of a cDNA encoding a developmentally regulated Ca^{2+}-binding protein from *Dictyostelium discoideum*, FEBS Lett. 362 (1995) 342–346.
[25] B. Andre, A.A. Noegel, M. Schleicher, *Dictyostelium discoideum* contains a family of calmodulin-related EF-hand proteins that are developmentally regulated, FEBS Lett. 382 (1996) 198–202.

[26] U.K. Laemmli, Cleavage of structural proteins during the assembly of the head of bacteriophage T4, Nature 277 (1970) 680–685.
[27] H. Schägger, G. von Jagow, Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100 kDa, Anal. Biochem. 166 (1987) 368–379.
[28] C.N. Pace, F. Vajdos, L. Fee, G. Grimsley, T. Gray, How to measure and predict the molar absorption coefficient of a protein, Protein Sci. 4 (1995) 2411–2423.
[29] T. Berggård, M. Silow, E. Thulin, S. Linse, Ca^{2+}- and H^{+}-dependent conformational changes of calbindin D (28k), Biochemistry 39 (2000) 6864–6873.
[30] N. Sreerama, R.W. Woody, A self-consistent method for the analysis of protein secondary structure from circular dichroism, Anal. Biochem. 209 (1993) 32–44.
[31] G. Deleage, C. Geourjon, An interactive graphic program for calculating the secondary structure content of proteins from circular dichroism spectrum, Comput. Appl. Biosci. 9 (1993) 197–199.
[32] Y.S. Babu, J.S. Sack, T.J. Greenhough, C.E. Bugg, A.R. Means, W.J. Cook, Three-dimensional structure of calmodulin, Nature 315 (1985) 37–40.
[33] K.L. Yap, J.B. Ames, M.B. Swindells, M. Ikura, Diversity of conformational states and changes within the EF-hand protein superfamily, Proteins 37 (1999) 499–507.
[34] T. Berggård, S. Miron, P. Önnerfjord, E. Thulin, K.S. Åkerfeldt, J.J. Enghild, M. Akke, S. Linse, $Calbindin_{28k}$ exhibits properties characteristic of a Ca^{2+} sensor, J. Biol. Chem. 277 (2002) 16662–16672.