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Preparation and characterization of a single-chain calcineurin–calmodulin complex

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Abstract

Calcineurin (CN), a Ca^{2+}/calmodulin (CaM)-dependent serine/threonine protein phosphatase, is a heterodimer composed of a catalytic subunit (CNA) and a regulatory subunit (CNB). The activity of CNA is under the control of two functionally distinct, but structurally similar Ca^{2+}-regulated proteins, CaM and CNB. The crystal structure of the holoenzyme reveals that the N-terminus and C-terminus of CNB and the N-terminus of CNA each have a long arm not involved in the active site. We constructed a fusion of the genes of CaM, CNB and CNA in that order using linker primers containing six and ten codons of glycine. A single-chain CaM–CNB–CNA (CBA) complex was expressed and purified to near homogeneity. The single-chain complex was fully soluble, and had biochemical properties and kinetic parameters similar to single-chain CNB–CNA (BA) activated by CaM. It was not regulated by CaM and CNB, but was strongly stimulated by Mn^{2+}, Ni^{2+} and Mg^{2+}. Intrinsic fluorescence spectroscopy of the complex showed a change in the environment of tryptophan in the presence of Ca^{2+} and circular dichroism (CD) spectropolarimetry revealed an increase in alpha-helical content. Our findings suggest that fusion of CaM, CNB and CNA does not prevent the structural changes required for their functioning; in particular, CaM within the complex could still interact correctly with CN in the presence of Ca^{2+}.

Keywords: Calcineurin; Calmodulin; Fusion; Single-chain complex; Phosphatase activity; Interaction

1. Introduction

Calcineurin (CN), or protein phosphatase-2B (PP-2B), belongs to a family of serine/threonine phosphatases including protein phosphatase-1 (PP-1) and protein phosphatase-2A (PP-2A), and is the only calmodulin (CaM)-regulated enzyme subject to dual control by two Ca^{2+}-regulated proteins, CaM and the calcineurin B subunit (CNB) [1–3]. The structure of CN is conserved in all eukaryotes, from yeast to man [4]. Biochemical and genetic studies have identified four distinct functional domains in calcineurin A (CNA): a catalytic domain, a CNB binding domain (BBH), a CaM binding domain, and an autoinhibitory (AI) domain [5,6]. CNB binds four calcium ions and has 35% sequence identity with CaM, and it is tightly bound to the enzyme even at low Ca^{2+} concentration [7]. The site of interaction of CaM for CN, as well as for myosin light chain kinase, is highly extended and may include hydrophobic pockets at homologous sites near the carboxy-terminal ends of the two halves of the molecule [8]. In addition, CN requires a divalent metal ion for structural stability and full activity towards either the nonprotein substrate *p*-nitrophenyl phosphate (pNPP) or the phosphoprotein substrates. Mg^{2+} and the transition metals Mn^{2+} and Ni^{2+} are the most potent CN activators [9].

In recent years, several milestones in the determination of the three-dimensional structure have been achieved by X-ray diffraction methods [10–12], and key amino acids affecting the structure and function of CN have been

Abbreviations: CN, calcineurin; CNA, calcineurin A subunit; CNB, calcineurin B subunit; CaM, calmodulin; CBA, CaM–CNB–CNA (single-chain calcineurin–calmodulin complex); BA, CNB–CNA (single-chain calcineurin); pNPP, *p*-nitrophenyl phosphate; FPLC, fast protein liquid chromatography; CD, circular dichroism

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identified by site-directed mutagenesis [13–15]. At present, the three-dimensional structure of CN in complex with CaM remains unknown, and the published crystal structure of CN is also incomplete. Based on previous experimental results [16] and the crystal structure of CN, we have created a fusion of CN and CaM, in which the components retain their interaction sites. Since CaM and CNB are indispensable for investigating the structure and function of CNA, we have asked whether the single-chain complex CaM–CNB–CNA can provide the functions of both CN and CaM? This paper answers that question. In addition, we report that a V314 deletion mutant of single-chain CN significantly alters its phosphatase activity.

Recent advances in protein engineering of a variety of proteins have come from creating multi-functional modular chimeric proteins. The modules are typically joined via an oligopeptide linker, the correct design of which is crucial for activity of the chimeric protein [17]. Fusion proteins have been mainly used to solve problems regarding stability, solubility and membrane anchoring. It is known that CNA is unstable in the absence of CNB [3], and soluble monomers of CN are currently obtained by using double promoters or an SD sequence to coexpress the two subunits, but this is still insufficient to overcome the solubility problem [14]. The construction of a single-chain complex could solve the problem of aggregation of CNA expressed in *E. coli*, and throw light on the aggregation mechanism.

2. Materials and methods

2.1. Construction of expression vectors pETCBA and pETBA

We used the double-PCR to generate DNA products with 5′ *Nde*I and 3′ *Bam*HI sticky ends. The gene for CNB was amplified from the pETB vector using a 5′ primer (5′-CCGCCATATGGGAAATGAGGCGAGTT-3′) tagged with an *Nde*I (underlined) site, and a 3′ primer (5′-***ACCACCACCACCGCCACC***CACATCTACCACCATCTT-3′) tagged with six codons (in italics) for glycine. The genes for CNA and its V314 deletion mutant were amplified from pET vectors containing the corresponding wild-type and mutant gene using a 5′ primer (5′-***GGTGGCGGTGGTGGTGGT***ATGTCCGAGCCCAAGGCG-3′) tagged with six codons (in italics) for glycine, and a 3′ primer (5′-CGCGGGATCCTCACTGAATATTGCTGC-3′) tagged with a *Bam*HI site (underlined). In the first PCR we constructed a full-length fusion from the PCR fragments of CNB and CNA (or its mutant) using the 5′ primer of CNB and the 3′ primer of CNA for amplification. To form the CaM–CNB–CNA complex, we first amplified the fusion of CaM–CNB using a pair of linker primers (5′-***GGTGGTGGTGGCGGTGGTGGTGGCGGTGGT***GGAAATAATGAGGCGAGTTAC-3′;5′-***ACCACCGCCACCACCACCGCCACCACCACC***CTTCGCTGTCATCATCTG-3′) tagged with 10 codons (in italics) for glycine. To ensure the fidelity of the PCR reaction, a pyrobest DNA polymerase was used for all PCR amplifications. The restriction enzyme *Eco*RI whose recognition site is present only in the sequence of CNB was selected to digest the two fusion genes, CaM–CNB and CNB–CNA. When the two fragments, CaM–CNB_N and CNB_C–CNA, were ligated, we obtained the full-length CBA fusion. All the genes of interest and the blank vector pET21a were digested with *Nde*I and *Bam*HI, and positive recombinant colonies harboring the fusion genes were obtained after ligation, transformation and plasmid screening.

2.2. Protein expression

A single colony of *E. coli* strain BL21 (DE3)/pETCBA from a fresh plate (less than 1 week old) was inoculated into 50 mL of LB containing 100 μg/mL ampicillin (amp) and incubated with shaking at 37 °C overnight, then used to inoculate 1 L of TM (amp). When A_{600} reached 1.0, IPTG was added to 0.1 mM, and the culture was shaken continuously at 25 °C for 16 h. The cells were harvested by centrifugation at 2200×*g* for 10 min and washed once with 50 mL of prechilled buffer A (50 mM Tris–HCl, pH 7.4, 1 mM EDTA). All subsequent purification steps were carried out at 4 °C. The cell pellet was weighed, resuspended (at 5 mL/g of wet pellet) in buffer A with 0.2 mM PMSF and 20 mM β-mercaptoethanol, and lysed ultrasonically. The enzymes present in the supernatants of the crude cell lysates obtained by centrifugation at 14,600×*g* for 30 min were then purified.

2.3. Protein purification

Ammonium sulfate powder was added to 45% saturation to a small beaker containing the stirred supernatant. The solution was stirred for another 20 min followed by centrifugation at 14,600×*g* for 30 min. The protein pellet was dissolved in 15 mL of buffer B (50 mM Tris–HCl, pH 7.4, 0.2 mM PMSF, 20 mM β-mercaptoethanol) with 5 mM $CaCl_2$. Fifteen milliliters of the dissolved protein pellet and 15 mL of phenyl-Sepharose resin equilibrated with buffer B and 0.5 mM $CaCl_2$ were mixed in a small beaker and stirred slowly (200 rpm) with a glass rotor for 40 min. The stirred mixture was loaded onto a column and washed with 300 mL of buffer B and 0.5 mM $CaCl_2$ for 2 h. The column was then eluted with 200 mL of buffer C (50 mM Tris–HCl, pH 7.4, 1 mM EGTA, 0.2 mM PMSF, 20 mM β-mercaptoethanol), and 15-mL fractions were collected. After a second ammonium sulfate precipitation, the protein pellet was dissolved in 2 mL of buffer D (20 mM MOPS, pH 7.4, 0.2 mM EDTA, 50 mM NaCl, 0.2 mM PMSF, 20 mM β-mercaptoethanol). Two milliliters of the dissolved protein pellet was loaded onto a superdex 200 prep grade column

equilibrated with buffer D and monitored by FPLC (Pharmacia), and 5-mL fractions collected. The fractions were frozen and dried, and the purity of the proteins was analyzed by 12% SDS-PAGE. The enzyme was stored at −20 °C. BA and its V314 deletion mutant were also purified as described by Qin et al. [16].

2.4. Assay of phosphatase activity

The assay for activity on *p*-nitrophenyl phosphate as substrate with Mn^{2+} as activator was performed in 50 mM Tris–HCl, pH 7.4, 0.5 mM $MnCl_2$, 1 mM dithiothreitol (DTT), 1 mM $CaCl_2$, 0.2 mg/mL bovine serum albumin (BSA), and 20 mM pNPP. When Ni^{2+} was used as activator, $MnCl_2$ was replaced with $NiCl_2$. Reactions were performed in 0.2-ml volumes at 30 °C for 10 min and terminated by adding 1.8-ml 0.5 M sodium carbonate. The absorbance was read at 410 nm. A molar extinction coefficient of 17,800 M^{-1} cm^{-1} was used to convert absorbance values into molar concentrations, and relative specific activity was converted to nmol/(min · mg). Protein phosphatase activity was assayed by release of ^{32}Pi from ^{32}P-labeled RII peptide (DLDVPIPGRFDRRVSVAAE) labeled with [γ-^{32}P] ATP as described by Perrino et al. [18]. The standard assay mixture (20 μL) was the same as for the *p*-nitrophenyl phosphate assay except that 20 mM of pNPP was replaced by 20 μM ^{32}P-RII. Units of activity were nanomoles of [^{32}Pi] released per minute per milligram of enzyme.

2.5. CD spectropolarimetry and fluorescence spectroscopy

CD experiments were performed on a CD instrument (Jasco J-720, Japan) calibrated with d_{10}-camphorsulfonic acid. All spectra were recorded at 20 °C with constant N_2 flushing. The scans were recorded using a bandwidth of 1.0 nm and an integration time of 4 s at a scan rate of 50 nm/min. All measurements were performed for 10 min after sample preparation using a 1-mm spectral path length and the spectra were corrected using a buffer blank containing 50 mM Tris–HCl. Fluorescence measurements were performed with a spectrofluorimeter (FluoroMax-2, America) at 20 °C in a thermostated cell holder with a 1-mm path-length quartz cell. Bandwidths of 5 nm were used for excitation and emission beams, and the excitation wavelength was fixed at 280 nm. Emission spectra were recorded from 285 to 450 nm at a scan rate of 60 nm/min. Each maximum emission wavelength (λ_{max}) represents the average of five measurements.

3. Results

3.1. Molecular cloning of CBA and BA cDNAs

The fusion of the CBA genes in the order CaM, CNB and CNA was constructed with two pairs of linker primers containing six and ten codons of glycine, respectively (Fig. 1A). A fusion of BA and its V314 mutant was also constructed, as described in Materials and methods (Fig. 1B). All fusion fragment cDNAs were cloned into the expression vector pET-21a using the same *Nde*I/*Bam*HI restriction site, and transformed into the expression vector *E. coli* BL21 (DE3). All the recombinant plasmids were identified by digestion with *Nde*I and *Bam*HI, and confirmed by DNA sequencing (data not shown).

3.2. Gel filtration analysis of CBA, BA and CNA

We examined the gel filtration behavior of CBA, BA and CNA with a superdex 200 prep grade column. CNA expressed in *E. coli* existed mostly as an aggregate, as judged by the position of the elution peak (Fig. 2A). The gel filtration chromatogram of BA contained two elution peaks (Fig. 2C); the second peak corresponded to the molecular mass of BA, suggesting that it represented a BA monomer. BA without the linker appeared to form the same monomer, but there was a small aggregate peak immediately before the monomer (Fig. 2B), implying that deletion of the linker between CNA and CNB affected the solubility of the enzyme. On gel filtration, CBA behaved entirely as a monomer (Fig. 2D), indicating that the construction of CBA overcomes the aggregation problem.

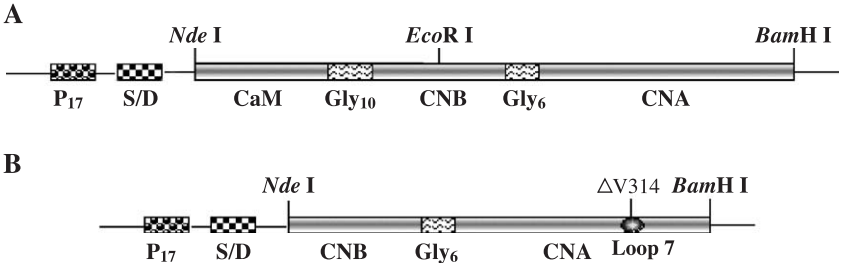

Fig. 1. Molecular cloning of CBA and BA cDNAs. Restriction sites used in vector construction are shown at the top, and the sequence features are indicated below. Abbreviations: P_{T7}, bacteriophage T7 promoter; S/D, Shine/Dalgarno sequence; Gly_{10}, the sequence of 10 codons for glycine; Gly_6, the sequence of six codons for glycine. (A) Structure of the tandem expression construct pETCBA. (B) Structure of the tandem expression construct pETBA.

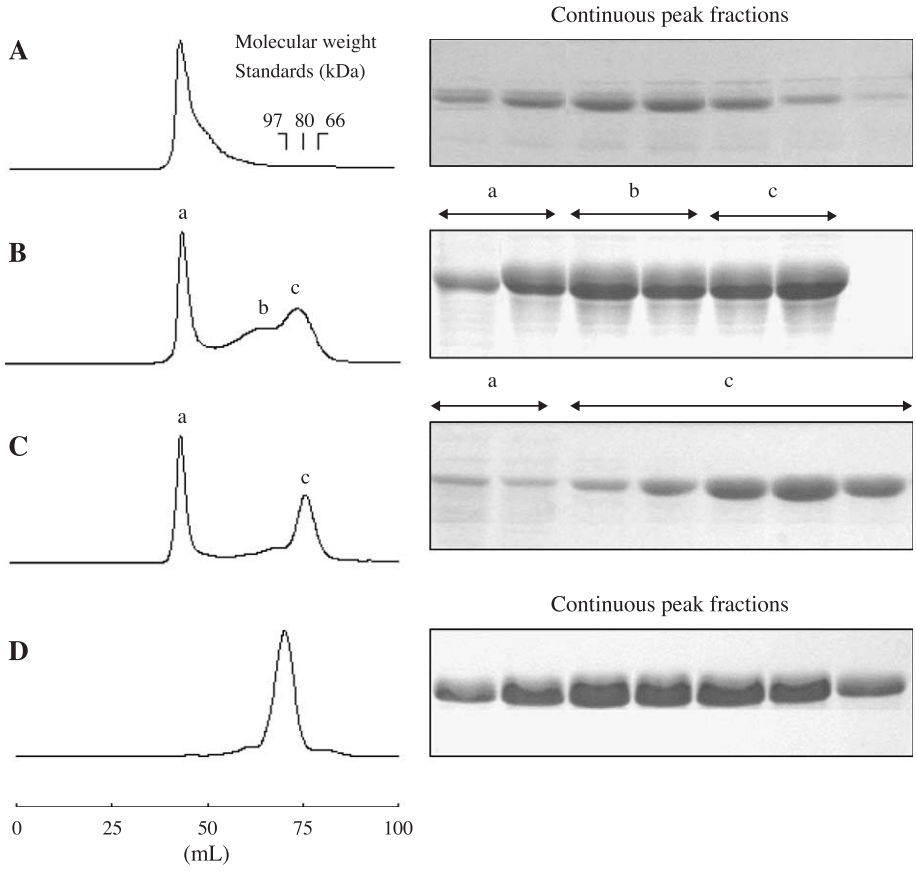

Fig. 2. Gel filtration analysis of CBA, BA and CNA. Traces of the gel filtration chromatograms are shown on the left, and the corresponding gel electrophoreses are shown on the right. (A) CNA; (B) BA without linker: a, void volume peak; b, aggregate peak c; monomer peak; (C) BA: a, void volume peak; c, monomer peak; (D) CBA.

3.3. Purification of single-chain proteins

The proteins in sonic extracts of the various transformants were purified to near-homogeneity by phenyl-Sepharose hydrophobic chromatography (CBA), CaM-Sepharose affinity chromatography (CNA, BA and its V314 mutant) and superdex 200 gel filtration (Fig. 3). Yields of CBA, BA and the mutant were up to 20 mg/l of culture.

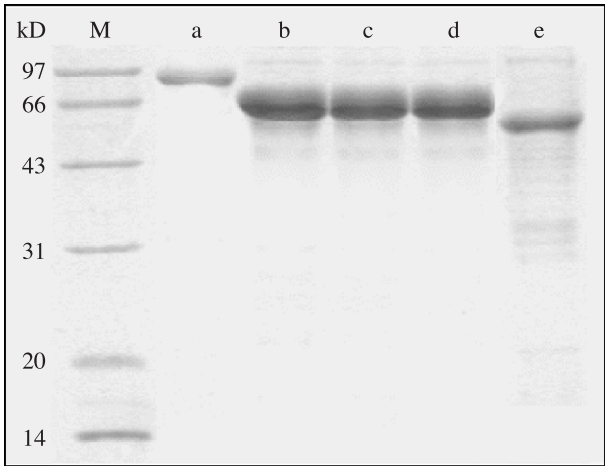

Fig. 3. Purification of single-chain proteins. M, molecular mass markers; a, CBA; b, BA; c, BA without linker; d, a V314 deletion derivative of BA; e, CNA.

3.4. Analysis of the phosphatase activities

The phosphatase activity of CBA was not affected by the addition of CaM or CNB, and its constitutive activity was equivalent to that of BA when activated by CaM (Fig. 4A). The basal activity of the V314 deletion derivative of BA was also significantly elevated and the effect of CaM on it was correspondingly decreased. We also measured the phosphatase activities of the recombinant proteins towards the ^{32}P-RII-peptide. CBA was again not regulated by added CaM (Fig. 4B). The basal activity of the mutant was increased, but the regulatory effect of CaM on it was essentially unchanged.

3.5. Determination of the K_m and V_{max} of CBA

The kinetic constants K_m and V_{max} reflect, respectively, the affinity and catalytic activity of the enzyme for its substrate. We measured the K_m and V_{max} of CBA with various concentrations of pNPP and calculated their values from

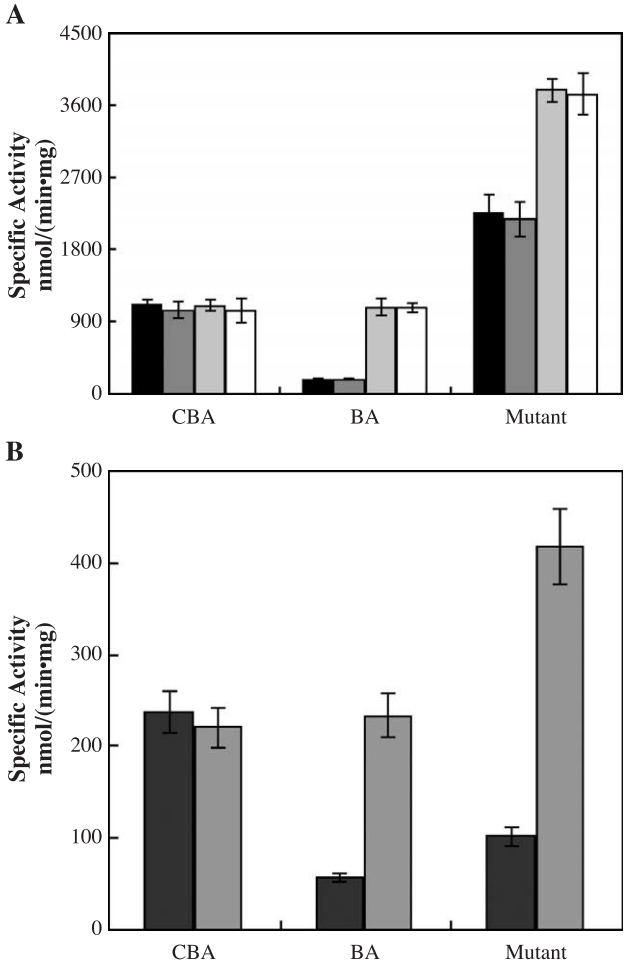

Fig. 4. Phosphatase activities of CBA, BA and its deletion mutant. (A) Activities towards pNPP. (■) Basal activity of enzymes in the absence of CNB and CaM; (▩) activity in the presence of CNB; (▨) activity in the presence of CaM; (□) activity in the presence of CNB and CaM. (B) Activities towards ^{32}P-RII peptide. (■) Basal activity of enzymes in the absence of CNB and CaM; (▩) activity in the presence of CaM.

double reciprocal plots (Fig. 5). The K_m was 32.5 mM and V_{max} 2500 nmol/(min · mg).

3.6. Effect of divalent metal ions

Calcineurin is a metalloenzyme and Mn^{2+}, Ni^{2+} and Mg^{2+} are important activators. We used final concentrations of $MnCl_2$ and $NiCl_2$ from 0 to 10 mM, and of Mg^{2+} from 0 to 100 mM to determine the effects of the divalent metal ions on CBA. Activity increased markedly over these concentration ranges reaching maxima at 1, 5, and 100 mM, respectively. The order of effectiveness was: Mg^{2+}>Ni^{2+}>Mn^{2+} (Fig. 6).

3.7. Solution conformation analysis

We examined the fluorescence spectra of CaM and CBA in the presence and absence of Ca^{2+}. Emission was maximal at 304 and 340 nm, respectively, and the

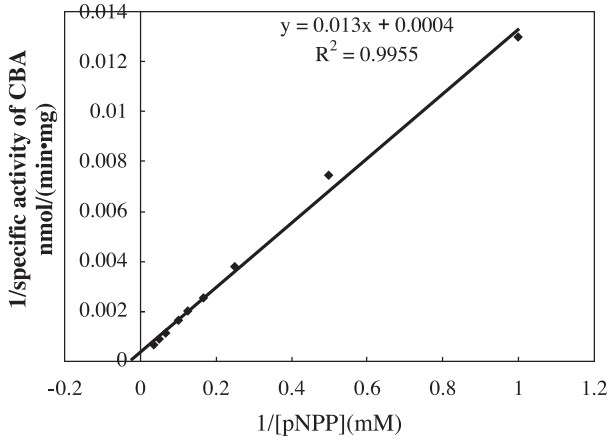

Fig. 5. Dependence of CBA activity on pNPP concentration. Activity was measured at pH 7.4 with 0.5 mM $MnCl_2$ and in the presence of 1 mM $CaCl_2$ and 1 μM CaM.

intrinsic fluorescence of both forms increased strongly in the presence of Ca^{2+} (Fig. 7A and B). The CD spectra of CBA had negative peaks at 209 and 221 nm and alpha-

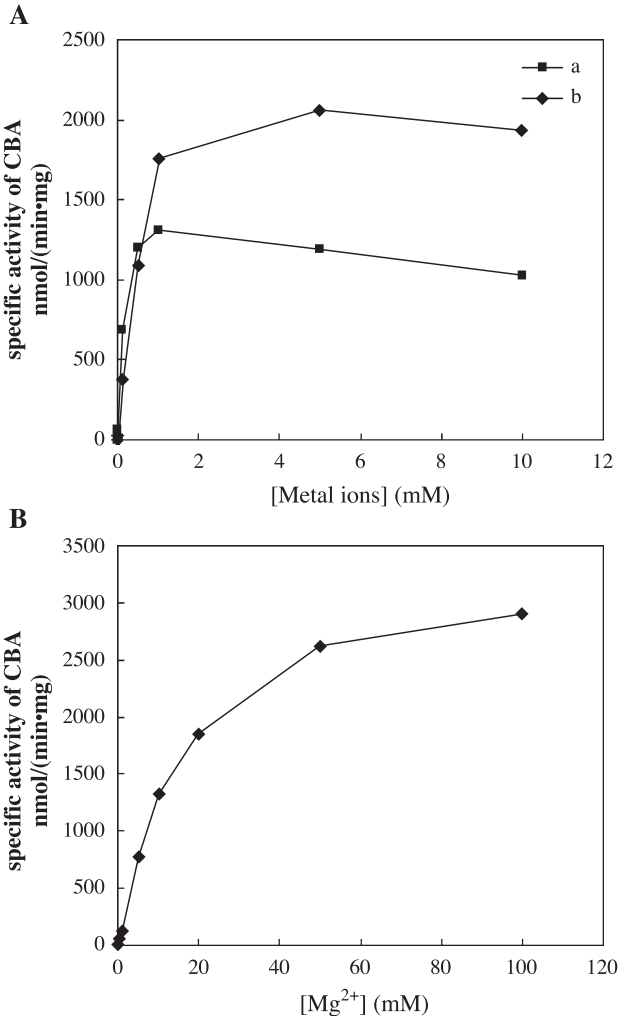

Fig. 6. Effect of ion concentration on CBA activity. (A) Mn^{2+} and Ni^{2+} dependence. (B) Mg^{2+} dependence.

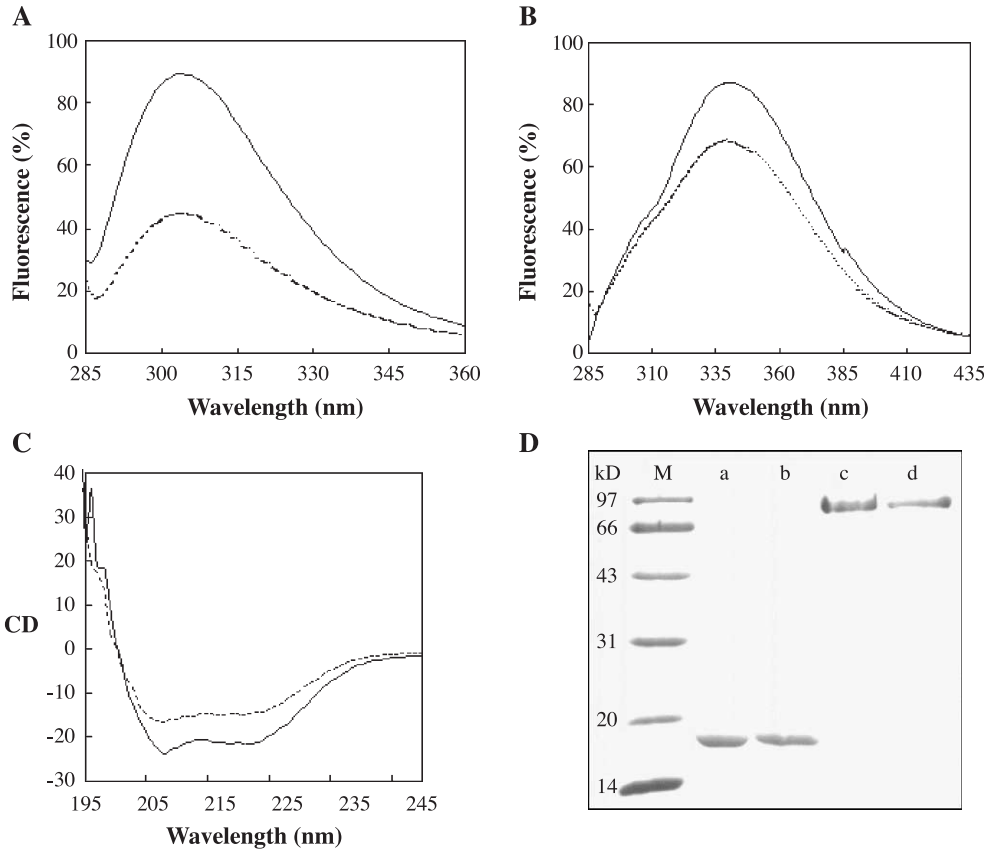

Fig. 7. Solution conformation of CBA. (A) Fluorescence spectra of CaM in the absence (dotted line) and presence (solid line) of Ca^{2+}. (B) Fluorescence spectra of CBA in the presence (solid line) and absence (dotted line) of Ca^{2+}. (C) CD spectra of CBA in the presence (solid line) and absence (dotted line) of Ca^{2+}. (D) Gel electrophoretic behavior of CBA and CaM in the presence of Ca^{2+} and EDTA. M, molecular mass markers; a, CaM with Ca^{2+}; b, CaM with EDTA; c, CBA with Ca^{2+}; d, CBA with EDTA.

helical content increased sharply in the presence of Ca^{2+} (Fig. 7C). Like CaM, gel mobility shift of Ca^{2+}-loaded CBA was more than that with EDTA (Fig. 7D).

4. Discussion

Many cellular processes involve proteins with multiple domains. The linkers between domains play an essential role in maintaining cooperative inter-domain interactions and stabilizing the tertiary structure of the proteins [19]. Argos [20] carried out a statistical analysis of natural linkers with the aim of designing linkers for gene fusions with a low likelihood of disrupting the folding of the flanking domains. However, few successful cases of fusion have been reported for proteins with a quaternary structure, or for pairs of proteins with spatial interaction sites. Based on information from the crystal structure of CN and previous experimental results, we constructed a single-chain calcineurin–calmodulin complex. The complex was not regulated by additional CaM or CNB, but was still strongly stimulated by Mg^{2+}, Mn^{2+} and Ni^{2+}; it was functionally equivalent to the linked enzyme BA with added CaM. This showed that fusion of CaM, CNB and CNA did not affect their folding or the structural changes required for their functioning. CD and fluorescence spectra showed that the conformation of CBA in solution changed in the presence of Ca^{2+}, indicating that Ca^{2+} can bind to CaM within the complex, and that the Ca^{2+}-loaded CaM can interact correctly with CN.

An obvious feature by which CN differs from other members of the serine/threonine phosphatase family is the regulation of its activity by CaM. Loop 7 of CNA is positioned where substrates enter the binuclear metal centers, and the V314 residue is in the center of loop 7. We constructed a V314 deletion mutant of BA and found that it had increased phosphatase activity, consistent with the findings of Yan and Wei [15].

We have further validated the technique of forming single-chain proteins. Many studies of the linker peptides of a number of protein families have concluded that linkers lack regular secondary structure, but display various degrees of flexibility to match their particular purpose [21,22]. Since glycine is more flexible than the other amino acids, we designed an oligopeptide containing 10 glycines as linkers between CN and CaM in the hope of providing a larger gap with more flexibility for achieving the correct spatial interaction after the addition of Ca^{2+}. The experimental results justified this approach.

CaM activated BA to similar extents with or without the flexible linkers, and their specific activities were alike, showing that the fusion and linker insertion did not alter the affinity or catalytic activity of the enzyme. However, BA without linkers was strongly aggregated during gel filtration, demonstrating that although the enzymatic activity of BA did not require the linkers, the hydrophobic interaction between CNA and CNB was disturbed. The covalent bond between the two subunits presumably caused a greater traction resistance to their interaction owing to the absence of a flexible linker. Some exposure of hydrophobic regions is likely to be the cause of the severe aggregation. There is overlap (linker region) between the domains of the native protein, and the interaction and information transfer between domains depend on the structure of the linker and the function of the different domains. Therefore, we believe that the selection of a suitable linker is indispensable for creating a desired protein fusion, in order to meet the requirements of the internal mechanism of that protein.

One disadvantage of expressing heterologous proteins in *E. coli* is that the expressed proteins frequently form aggregated folding intermediates, known as inclusion bodies [23]. The individual expression of the two subunits of CN in *E. coli* usually resulted in the formation of inclusion bodies and soluble aggregates, and coexpression of the two subunits was not sufficient to overcome this solubility problem. We analyzed the solubility of the CBA, BA and CNA by gel filtration and found that CNA existed mostly as aggregates, BA formed some monomers, and CBA was completely monomeric. These results suggested that the two hydrophobic sites on CNA, the CNB and CaM binding domains, were essentially responsible for the formation of aggregation. When the two hydrophobic regions were shielded stepwise by constructing BA and CBA, soluble monomers were expressed in *E. coli*.

Maximal activation of CN requires excessive CaM, whereas CBA has a molar ratio of 1:1:1 between CaM, CNB and CAN. Additional CaM did not increase the activity of CBA; instead there was a slight decrease of activity. Presumably in the absence of Ca^{2+}, the CaM module in CBA can only rotate around the holoenzyme molecule in a radius of the 10 glycines, on account of restriction by the covalent bond. The cumulative length of the nine peptide bond planes ensures that CaM and its target module are close enough for effective interaction. This is equivalent to increasing the effective local concentration of CaM. Following the addition of Ca^{2+}, the conformation of CaM changes, and it interacts with its target molecule. The additional CaM may interfere with this interaction, thus causing a slight decrease in activity.

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References

[1] C.B. Klee, P. Cohen, The calmodulin-regulated protein phosphatase, in: C.B. Klee, P. Cohen (Eds.), Molecular Aspects of Cellular Regulation, Calmodulin, vol. 5, Elsevier Press, Amsterdam, 1988, pp. 225–248.
[2] S. Shenolikar, Protein serine/threonine phosphatases—new avenues for cell regulation, Annu. Rev. Cell Dev. Biol. 10 (1994) 55–86.
[3] C.B. Klee, G. Draetta, M.J. Hubbard, Calcineurin, Adv. Enzymol. 61 (1988) 149–200.
[4] R. Kincaid, Calmodulin-dependent protein phosphatases from microorganisms to man. A study in structural conservatism and biological diversity, Adv. Second Messenger Phosphoprot. Res. 27 (1993) 1–23.
[5] Y. Hashimoto, B.A. Perrino, T.R. Soderling, Identification of an autoinhibitory domain in calcineurin, J. Biol. Chem. 265 (1990) 1924–1927.
[6] Y. Watanabe, B.A. Perrino, B.H. Chang, T.R. Soderling, Identification in the calcineurin A subunit of the domain that binds the regulatory B subunit, J. Biol. Chem. 270 (1995) 456–460.
[7] P.M. Stemmer, C.B. Klee, Dual calcium ion regulation of calcineurin by calmodulin and calcineurin B, Biochemistry 33 (1994) 6859–6866.
[8] Q. Wei, A.E. Jackson, S. Pervaiz, K.L. Carraway, E.Y.C. Lee, D. Puett, K. Brew, Effects of interaction with calcineurin on the reactivities of calmodulin lysines, J. Biol. Chem. 263 (1988) 19541–19544.
[9] C.J. Pallen, J.H. Wang, Regulation of calcineurin by metal ions. Mechanism of activation by Ni^{2+} and an enhanced response to Ca^{2+}/calmodulin, J. Biol. Chem. 259 (1984) 6134–6141.
[10] J.P. Griffith, J.L. Kim, E.E. Kim, M.D. Sintchak, J.A. Thomson, M.J. Fitzgibbon, M.A. Fleming, P.R. Caron, K. Hsiao, M.A. Navia, X-Ray structure of calcineurin inhibited by the immunophilin-immunosuppressant FKBP12–FK506 complex, Cell 82 (1995) 507–522.
[11] C.R. Kissinger, H.E. Parge, D.R. Knighton, C.T. Lewis, L.A. Pelletier, A. Tempczyk, V.J. Kalish, K.D. Tucker, R.E. Showalter, E.W. Moomaw, L.N. Gastinel, N. Habuka, X. Chen, F. Maldonado, J.E. Barker, R. Bacquet, J.E. Villafranca, Crystal structures of human calcineurin and the human FKBP12–FK506–calcineurin complex, Nature 378 (1995) 641–644.
[12] L. Jin, S.C. Harrison, Crystal structure of human calcineurin complexed with cyclosporin A and human cyclophilin, Proc. Natl. Acad. Sci. U. S. A. 99 (2002) 13522–13526.
[13] Q. Wei, E.Y.C. Lee, Mutagenesis of the loop7 connecting β strands 12 and 13 of calcineurin: evidence for a structure role in activity changes, Biochemistry 36 (1997) 7418–7424.
[14] A. Mondragon, E.C. Griffith, L. Sun, F. Xiong, C. Armstrong, J.O. Liu, Overexpression and purification of human calcineurin α from *Escherichia coli* and assessment of catalytic functions of residues surrounding the binuclear metal center, Biochemistry 36 (1997) 4934–4942.
[15] L. Yan, Q. Wei, High activity of the calcineurin A subunit with a V314 deletion, Biol. Chem. 380 (1999) 1281–1285.
[16] Y.L. Qin, D.Y. Yu, Q. Wei, Function and structure of recombinant single chain calcineurin, Biochem. Biophys. Res. Commun. 308 (2003) 87–93.
[17] T.P. Boesen, B. Soni, T.W. Schwartz, T. Halkier, Single-chain vascular endothelial growth factor variant with antagonist activity, J. Biol. Chem. 277 (2002) 40335–40341.
[18] B.A. Perrino, Y.L. Fong, D.A. Brickey, Y. Saitoh, Y. Ushio, K. Fukunaga, Characterization of the phosphatase activity of a baculovirus-expressed calcineurin A isoform, J. Biol. Chem. 267 (1992) 15965–15969.

[19] R.S. Gokhale, C. Khosla, Role of linkers in communication between protein modules, Curr. Opin. Chem. Biol. 4 (2000) 22–27.
[20] P. Argos, An investigation of oligopeptides linking domains in protein tertiary structures and possible candidates for general gene fusion, J. Mol. Biol. 211 (1990) 943–958.
[21] C.R. Robinson, R.T. Sauer, Optimizing the stability of single-chain proteins by linker length and composition mutagenesis, Proc. Natl. Acad. Sci. U. S. A. 95 (1998) 5929–5934.
[22] R. Dieckmann, M. Pavela-Vrancic, H. von Dohren, H. Kleinkauf, Probing the domain structure and ligand-induced conformational changes by limited proteolysis of tyrocidine synthetase 1, J. Mol. Biol. 288 (1999) 129–140.
[23] D.C. Paul, R.M. Van Frank, W.L. Muth, J.W. Ross, D.C. Willianms, Immunocytochemical demonstration of human proinsulin chimeric polypeptide within cytoplasmic inclusion bodies of *Escherichia coli*, Eur. J. Cell Biol. 31 (1983) 171–174.