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Function and structure of recombinant single chain calcineurin

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Abstract

Calcineurin (CN) is a Ca^{2+}/calmodulin(CaM)-dependent serine/threonine protein phosphatase which is a heterodimer composed of a 61 kDa catalytic subunit (CNA) and a 19 kDa regulatory subunit (CNB). The enzyme is critical for several important intracellular signal-transducing pathways, including T-cell activation. Its crystal structure reveals that the C-terminal of CNB lies in close vicinity of the N-terminal of CNA and each end has a long arm not involved in the active site. After fusing two subunits, it was determined that folding and function of the protein were not affected by the fusion. We amplified a fused gene of A and B subunits using a pair of linker primers including six codons of glycine. A single chain calcineurin was constructed and purified to near-homogeneity. The recombinant enzyme was fully soluble, displayed high specific activity with substrate, and exhibited biochemical properties and kinetic parameters similar to those of the native enzyme from the bovine brain. It was still activated by Ca^{2+}/calmodulin but was not regulated by extra CNB and was still strongly stimulated by Mn^{2+} and Ni^{2+} divalent metal ions. The solution conformations of both recombinant enzyme and bovine calcineurin were assayed under the same conditions using intrinsic fluorescence spectroscopy and circular dichroism spectropolarimetry, and results showed their graphs are approximately identical. Our findings suggested that the fusion of A and B subunits of calcineurin does not affect their folding pathways and structural changes involved in their function, furthermore, they are bound to the correct binding site.

Keywords: Calcineurin; Fusion; Phosphatase activity; Folding; Binding site

Calcineurin, or protein phosphatase-2B (PP-2B), belongs to a family of serine/threonine phosphatases including PP-1 and PP-2A [1,2]. Biochemical and genetic studies have identified four distinct functional domains of CaNA [3–5]: a catalytic domain, a CNB binding domain (BBH), a calmodulin binding domain, and an auto-inhibitory (AI) domain. CNB binds four calcium ions and has 35% sequence identity with CaM. CN phosphatase activity is stimulated by Ca^{2+} binding to CNB and by Ca^{2+}-induced binding of CaM to CNA. The interaction site on calmodulin for calcineurin, as well as for myosin light chain kinase, is very extended and may include hydrophobic pockets at homologous sites near the carboxyl-terminal ends of the two halves of the molecule [6]. In addition, calcineurin requires a divalent metal ion for structural stability and full activity toward the nonprotein substrate *p*-nitrophenyl phosphate (pNPP) or phosphoprotein substrates. The transition metals Mn^{2+} and Ni^{2+} are the most potent calcineurin activators [7].

Since the initial comparisons of the crystal structure of both bovine calcineurin [8] and human recombinant enzyme [9], people have done a great deal of scientific research in finding key amino acids affecting the structural and functional behaviors of calcineurin. This work has been done using the scanning mutagenesis on the residues surrounding the binuclear metal center [10] and on the residues of loop 7 connecting β strands 12 and 13 [11,12]. Also work has been done using the various truncations of CNA [13] and the salt bridge mutants. Meantime, the crystal structure of calcineurin demonstrates that outside the active center the N-terminal of CNA lies in close vicinity to the C-terminal of CNB and each end has a long arm not involved in the active site. It is feasible for the folding and function to occur without major consequences after the A and B subunits are fused. CNB is composed of two globular domains and

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the C-terminal domain plays a critical role in stimulating the phosphatase activity of CNA. Nuclear magnetic resonance (NMR) indicated BBH was a strong dissolving reagent for CNB [14]. The soluble monomers of calcineurin were obtained using pure biochemical technology to purify from mammalian tissues [15] or adopting double promoters or SD sequence to coexpress two subunits [16]. Is this single chain calcineurin soluble? Do the conformations of both A and B subunits change after fusion? Are they bound on the correct binding site after folding? This paper answers these questions.

Protein fusion technology is widely applied in many fields of basic science and biotechnology. It allows the creation of new proteins, which have the functional properties of the protein domains that are linked together. The fused protein can solve the problems regarding stability, solubility, and anchoring to membranes, which were found in using fusion technology. Recent data suggest that protein domains may accept the insertion of another protein without major effect on the folding and function of both fusion partners [17]. It has been shown that adjacent domains in a protein may be more flexible within the protein than alone [18]. We inserted six glycines between A and B subunits for decreasing the traction in the folding process of two subunits and for achieving the correct spatial molecule docking. The experimental results verified this point.

Materials and methods

Molecular cloning

We applied the double PCR method to generate DNA products with 5′ *Nde*I and 3′ *Bam*HI sticky ends. The gene for CNB was PCR-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) of glycine. The gene for CNA was PCR-amplified from the pETA vector using a 5′ primer (5′-***GGTGGCGGTGGTGGTGGT***ATGTCCGAGCCCAAGGCG-3′) tagged with six codons (in italics) of glycine and a 3′ primer (5′-CGCGGGATCCTCACTGAATATTGCTGC-3′) tagged with an *Bam*HI (underlined) site. We made the PCR fragments of the CNB and CNA mutual templates generate a full-length fusion gene using the 5′ primer of CNB and the 3′ primer of CNA in the first PCR amplifications. To ensure the fidelity of the PCR, a Ex Taq polymerase was used for all PCR amplifications. The fusion gene fragments and the blank vector pET21a were digested with *Nde*I and *Bam*HI, and a positive recombinant colony was obtained after ligation, transformation, and plasmid screening.

Protein purification strategy

A single colony of the *Escherichia coli* strain BL21(DE3)/pETCN from a fresh plate (less than 1 week old) was inoculated into 50 ml LB containing 100 μg/ml ampicillin (amp), incubated by shaking at 37 °C overnight, and used to inoculate 1 L of TM (amp). When A_{600} reached 1.0, IPTG was added to the final concentration of 0.5 mM. The culture was shaken at 25 °C for 16 h continuously. 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 the buffer A with 0.2 mM PMSF and 20 mM β-mercaptoethanol, and lysed by an ultrasonic wave. The crude cell lysate was centrifuged at 14,600*g* for 30 min to yield supernatant which was subjected to the following purification steps.

(a) Ammonium sulfate precipitation. Ammonium sulfate powder was added to a small beaker with the stirred supernatant to 45% saturation. 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, and 20 mM β-mercaptoethanol) with 5 mM $CaCl_2$.

(b) CaM–Sepharose chromatography. Fifteen milliliters of the dissolved protein pellet and 15 ml of the CaM–Sepharose resin equilibrated with buffer B with 0.5 mM $CaCl_2$ were added to a small beaker together and stirred with a glass rotor slowly (200 rpm) for 40 min. The stirred mixture was loaded onto a column and washed with 300 ml of buffer B with 0.5 mM $CaCl_2$ for 2 h. Then the column was eluted with 200 ml of buffer C (50 mM Tris–HCl, pH 7.4, 1 mM EGTA, 0.2 mM PMSF, and 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, and 20 mM β-mercaptoethanol).

(c) Superdex 200 filtration. Two milliliters of the dissolved protein pellet was loaded onto a column with Superdex 200 equilibrated with buffer D and 5 ml fractions were collected. The fractions were frozen and dried while analyzing the purity of the proteins on 12% SDS–PAGE. The enzyme was stored at −20 °C before being used for further characterization.

Assay of phosphatase activity

The assay for activity toward *p*-nitrophenyl phosphate as the substrate using Mn^{2+} as the 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 an activator, $MnCl_2$ was replaced with $NiCl_2$. Reactions were performed in a 0.2 ml volume at 30 °C for 20 min and terminated by the addition of 1.8 ml of 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 the absorbance values into molar concentrations. The relative specific activity was converted to nmol/(min mg). Protein phosphatase activity was assayed by measuring the release of ^{32}Pi from ^{32}P-labeled R_{II} peptide (DLDVPIPGRFDRRV SVAAE). The R_{II} peptide was labeled with [γ-^{32}P]ATP as described by Perrino et al. [19]. The standard assay mixture (20 μl) was the same as that for the *p*-nitrophenyl phosphate described above except that 20 mM pNPP was replaced by 15 μM [^{32}P]R_{II}. Units of activity were defined as nanomoles of ^{32}Pi released per minute per mg enzyme.

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 10 min after sample preparation using a 1 mm spectral path length and the spectra were corrected using a blank buffer containing 50 mM Tris–HCl.

Fluorescence measurements were performed with a FluoroMax-2 spectrofluorimeter (America and France) at 20 °C in a thermostated cell holder using a 1 mm path length quartz cell. Bandwidths of 5 nm

were used for excitation and emission beams. The excitation wavelength was fixed at 295 nm where the contribution of tyrosine residues is negligible. The emission spectra were recorded from 300 to 400 nm at a scan rate of 60 nm/min. Each maximum emission wavelength (λ_{max}) was the average calculated from five emission spectrum measurements.

Results

Construction of the expression vector pETCN

The fusion gene of calcineurin was constructed using a pair of linker primers with six codons of glycine (Fig. 1). The fusion fragment cDNAs were cloned into the expression vector pET-21a and transformed into the expression *E.coli* strain BL21(DE3) cells. The recombinant plasmids were identified by *Nde*I and *Bam*HI and confirmed by DNA sequencing (data not shown).

Purification and characterization of recombinant single chain calcineurin

After the recombinant single chain holoenzyme was expressed, the cells were disrupted using ultrasonic waves. SDS–PAGE gels showed a large clear band at the position of the estimated molecular mass of 80 kDa that appeared to be soluble proteins. After CaM–Sepharose affinity chromatography and Superdex 200 filtration, the enzyme was purified to near homogeneity (Fig. 2A). Yields of the purified calcineurin ranged up to 20 mg/L of the culture. The single chain calcineurin was also monitored by Western blot analysis using a mouse polyclonal antibody against calcineurin A subunit (Fig. 2B).

Analysis of the phosphatase activity and function

We analyzed the activation of calmodulin to the recombinant calcineurin and examined effect of extra B subunit on the enzyme. The relative specific activity is measured using pNPP at a final concentration of 20 mM. The results showed that the phosphatase activity of single chain calcineurin was strongly activated by calmodulin and reached the maximum at a 1 μM concentration (Fig. 3A), but was not affected by extra B subunits and displayed zero-degree reaction curve without calmodulin or with 1 μM calmodulin (Fig. 3B).The phosphatase activities of the recombinant and native protein towards $[^{32}P]R_{II}$ peptide substrate were determined under the identical conditions and both exhibited the same activated trend (Fig. 4). This suggests that the conformation of the CaM-binding domain of CNA was correct, the hydrophobic side of the CNB-binding domain was sealed and the molecule docking was correctly performed between two subunits.

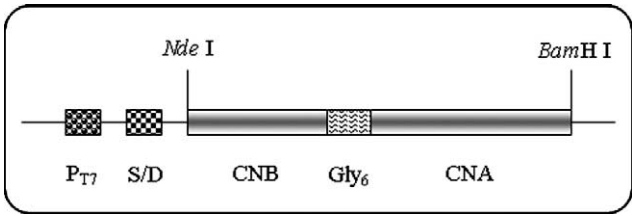

Fig. 1. Configuration of the tandem expression construct pETCN. Restriction sites used in vector construction are shown at the top and the sequence features are indicated below. Abbreviations: P_{T7}, bacteriophage T7 promoter; S/D, Shine/Dalgarno sequence; and Gly_6, the 6× glycine.

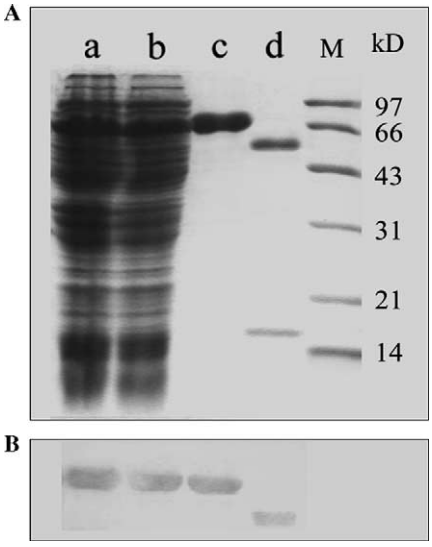

Fig. 2. (A) Purification of recombinant single chain calcineurin. The recombinant enzyme was expressed in *E. coli* and purified as described in Materials and methods. The proteins were run on a 12% acrylamide gel and stained with Coomassie brilliant blue. Lane a, crude lysate from BL21(DE3) cells; lane b, supernatant of lysate from BL21(DE3) cells; lane c, purified single chain calcineurin; lane d, purified bovine enzyme; and lane M, protein marker. (B) Western blot analysis of single chain calcineurin expressed in *E. coli*.

Determination of K_m and V_{max} of the enzyme

Kinetic constants K_m and V_{max} can reflect affinity and catalytic ability of the enzyme for its specific substrate, respectively. K_m and V_{max} of single chain calcineurin and bovine enzyme were measured using various concentrations of pNPP ranging from 1 to 30 mM. The values of K_m and V_{max} of the both were calculated by double reciprocal construction (Figs. 5A and B). They are 37.2 mM and 2000.0 nmol/(min mg) (single chain calcineurin) and 48.3 mM and 1428.6 nmol/(min mg) (bovine enzyme), respectively.

Effect of the divalent metal ions

Calcineurin is a metallic enzyme and both Mn^{2+} and Ni^{2+} are very important activators for it. We use a final concentration ranging from 0 to 10 mM $MnCl_2$ or $NiCl_2$

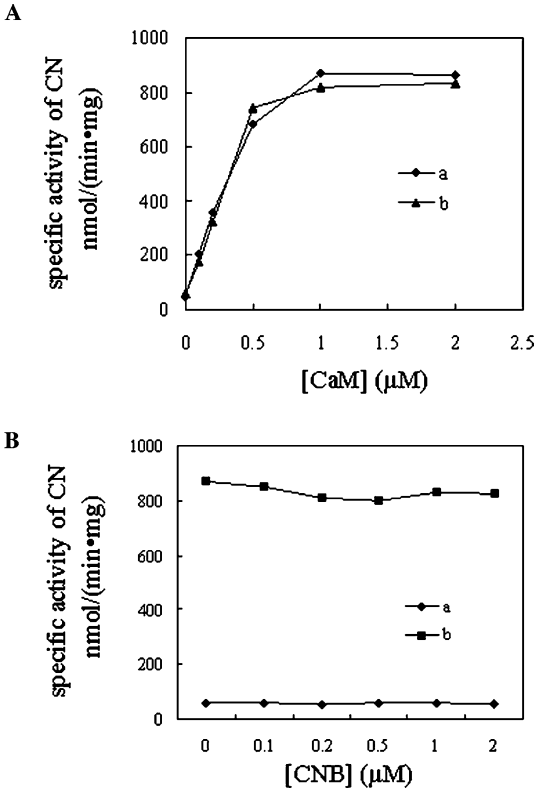

Fig. 3. Phosphatase activities of single chain calcineurin towards *p*-nitrophenyl phosphate. (A) Activation of calmodulin concentrations to the phosphatase activity of single chain calcineurin. a, Without CNB; b, with 1 μM CNB. (B) Effect of extra B subunit concentrations on the phosphatase activity of single chain calcineurin. a, Without calmodulin; b, with 1 μM calmodulin.

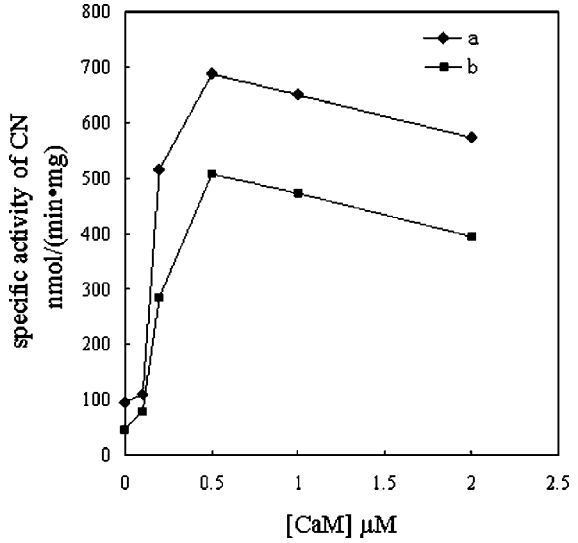

Fig. 4. Phosphatase activities of single chain calcineurin and bovine enzyme towards $[^{32}P]R_{II}$ peptide. a, Single chain calcineurin; b, bovine enzyme.

while analyzing the effects of the divalent metal ions on the recombinant single chain calcineurin. The calmodulin concentrations were 1 μM. The results indicated

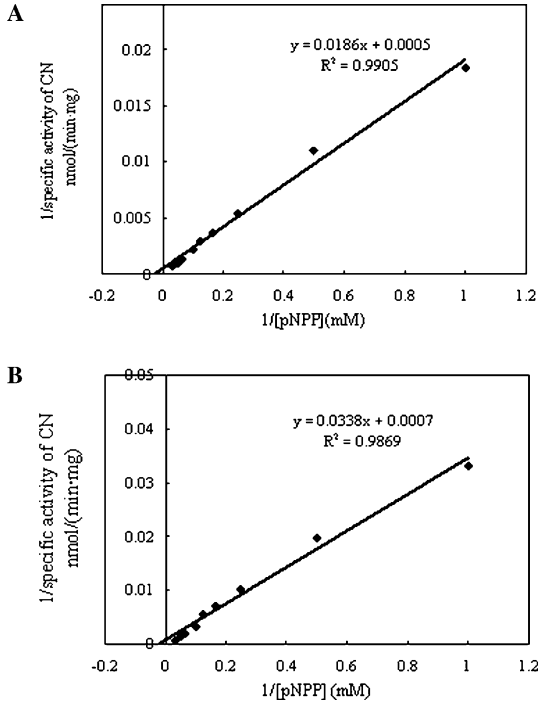

Fig. 5. Kinetic curves of recombinant and native enzyme towards *p*-nitrophenyl phosphate. (A) Single chain calcineurin. (B) Bovine enzyme. The phosphatase was measured at pH 7.4 with 0.5 mM $MnCl_2$ in the presence of 1 mM $CaCl_2$ and 1 μM calmodulin.

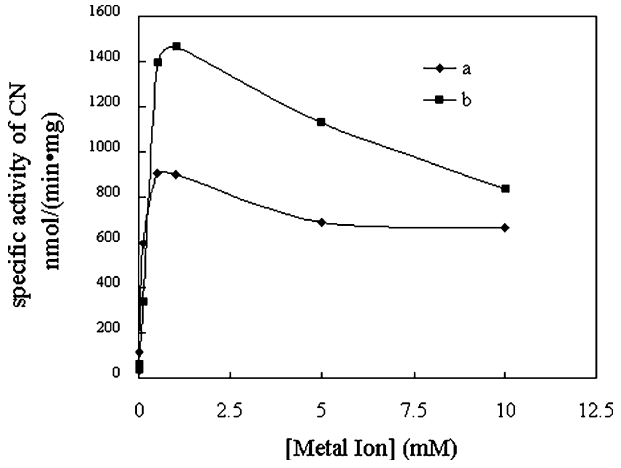

Fig. 6. Effects of $MnCl_2$ and $NiCl_2$ concentrations on the phosphatase activity of single chain calcineurin towards pNPP. a, Effect of various concentrations of $MnCl_2$; b, effect of various concentrations of $NiCl_2$.

that the enzyme activities were sharply increased at concentrations ranging from 0 to 0.5 mM $MnCl_2$ or $NiCl_2$ and reached the maximum at 1 mM, and the activities with Ni^{2+} were much higher than those with Mn^{2+} (Fig. 6), this is consistent with native enzyme.

The optimum pH and temperature

We determined the phosphatase activities of the recombinant enzyme at temperatures ranging from 20 to

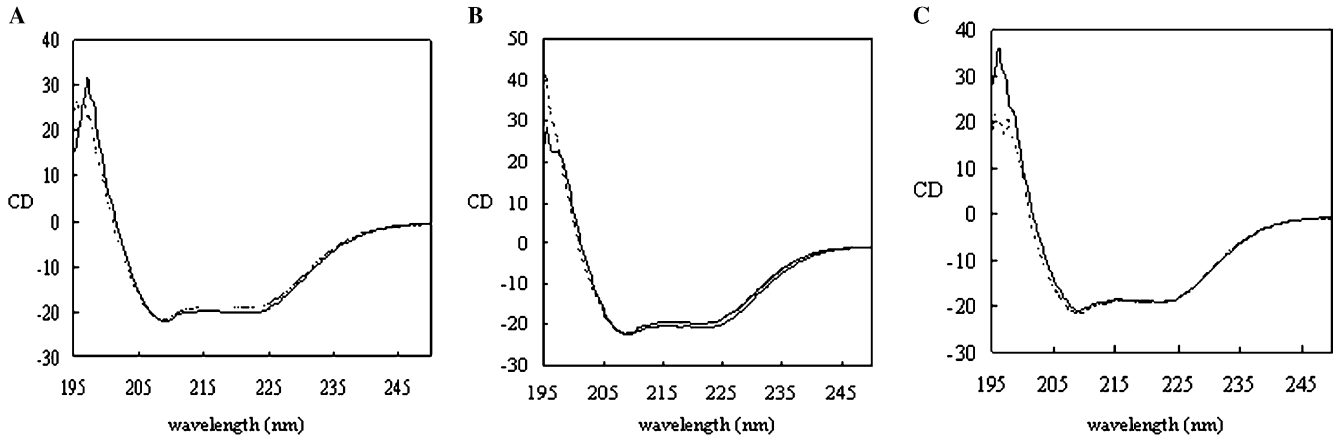

Fig. 7. CD spectra of the single chain calcineurin (dotted line) and the native bovine enzyme (solid line). The enzyme concentration is 4.5 μM. (A) Without divalent metal ion; (B) with 0.5 mM Mn^{2+}; and (C) with 0.5 mM Ni^{2+}.

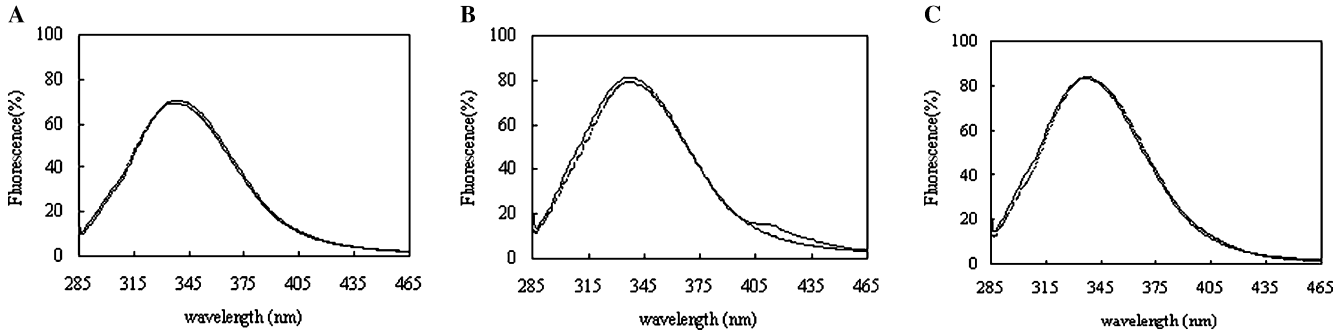

Fig. 8. Fluorescence spectra of the single chain calcineurin (dotted line) and the native bovine enzyme (solid line). The enzyme concentration is 1.5 μM. (A) Without divalent metal ion; (B) with 0.5 mM Mn^{2+}; and (C) with 0.5 mM Ni^{2+}.

80 °C and pH ranging from 4.0 to 10.0. The optimum pH and temperature of the enzyme are 7.0 and 45 °C, respectively.

CD spectra comparison between recombinant calcineurin and native bovine enzyme

CD is an important method of determining the second structure features of a protein in solution. We measured the recombinant enzyme and the bovine calcineurin under the same conditions. It was shown that double negative peaks appeared at 209 and 221 nm and the graphs of both CD spectra were in agreement to a high degree with or without divalent metal ions, especially with Ni^{2+} (Figs. 7A–C), implying that the backbone conformation of the single chain calcineurin was correct.

Intrinsic fluorescence spectra comparison of both recombinant calcineurin and native bovine enzyme

Intrinsic fluorescence mainly assayed partial conformations of the tertiary structure of a protein containing the tryptophans. The tryptophans of subunit A of calcineurin, equally distributed in molecule, can reflect the tertiary structure of whole molecule. It was indicated that the maximum emission appeared at 340 nm and the plots of fluorescence spectra of recombinant and bovine calcineurin were also approximately identical (Figs. 8A–C), suggesting that the tertiary structure of the single chain calcineurin basically matched that of the native holoenzyme.

Discussion

Glycine is more flexible than the other amino acids. We selected six glycines as linkers between the A and B subunits of calcineurin and expected to provide a bigger space with more flexibility for realizing the correct spatial molecule docking after the folding of the A and B subunits. Meantime, the insertion of six glycines gave birth to a covalent bond. The covalent force can further restrict the mobility of B subunit and produce a lessening of physical distance before two subunits bind to each other. This covalent force was also likely to affect the hydrophobic effect between both A and B subunits, but this point was not reflected in our results. Can the

covalent force limit the motion of the CaM-binding domain of subunit A while restricting the movement of subunit B? These suppositions may be proved by the intact crystal structure of calcineurin.

The fusion of domains from unrelated proteins does not affect their folding pathways and the structural changes involved in their function [20]. In contrast, the addition of a single N-terminal residue may destabilize a protein [21,22]. Also, the fusion of two subunits of heterodimer possessing spatial binding site for each other does not even alter the respective conformations, then, are they bound in the correct binding site? At present, few examples have been described in detail. The CNB-binding domain of CNA is a five-turn amphipathic α helix, and CNB consists of two globular Ca^{2+}-binding domains flanked by a long C-terminal β strand and these structures form a 33 Å long hydrophobic groove into which the top half of the BBH is embedded [8]. The single chain calcineurin is still regulated by Ca^{2+}/calmodulin, but is not activated by extra B subunits, suggesting that the conformation of the calmodulin-binding domain of CNA may be correct and the hydrophobic side of the CNB-binding domain is sealed, and the molecule kissing was performed between the two subunits correctly. The CaM binding domain and the autoinhibitory domain together serve as a calcium-sensing "on-and-off" switch for the phosphatase activity of calcineurin [13,23]. Kinetic constants K_m and V_{max} lie in the normal value range, implying that the conformations of the switch and the active site meet the requirements. In addition, its phosphatase activity is still strongly stimulated by the divalent metal ions Mn^{2+} and Ni^{2+}, hinting that the perturbation of the residues relating to the Mn^{2+} and Ni^{2+} binding sites does not occur. These experimental results and the optimum pH and temperature are consistent with those of the native enzyme. The CD and fluorescence spectra indicate the agreement to a high degree between recombinant and bovine calcineurin, further supporting the structural comparability of single chain calcineurin with that of the native enzyme. According to recent data, the fusion of domains, subunits, or proteins does not affect respective folding pathways. Here seemingly exists a general mechanism that both fusion partners do not cause much traction to each other, both can still correctly fold due to internal instruction restrictions. This effect brings us an advantage, that is, we can perform our aims by adopting the virtues of both.

Calcineurin is a metallic enzyme containing the intrinsic metal ions Fe^{3+} and Zn^{2+} at the active site and is regulated by many exogenous metals including vanadyl ions [24,25]. Calcineurin requires exogenous metal ions for structural stability and full activity. There are different Mn^{2+} and Ni^{2+} binding sites on calcineurin A and the enzyme has different affinities for Mn^{2+} and Ni^{2+} [26]. Generally, the Ni^{2+}-stimulated calcineurin activity toward nonproteins is greater than Mn^{2+}-stimulated calcineurin activity [7]. This is consistent with our experimental results. Matsui et al. [27] showed that a monoclonal antibody against calcineurin can inhibit Ni^{2+}-stimulation but not Mn^{2+}-stimulation of the enzyme and they suggested that Ni^{2+} and Mn^{2+} induce different active conformations of calcineurin. CD and fluorescence spectra as shown by the closeness of data coordinates on our graphs demonstrate that the Ni^{2+} stimulation in higher than the Mn^{2+} stimulation.

The most important characteristic of a protein that determines its feasibility for functional analysis is its solubility. High solubility is also strongly correlated with the success of structural studies using either NMR or X-ray crystallography. In this paper, we found another manner for solving the solubility of calcineurin, a single chain type of calcineurin was constructed by inserting six glycines between the A and B subunits. It allowed for the purification to near-homogeneity of sufficient quantities of recombinant calcineurin for further biochemical characterization and structural analysis. Previously people obtained soluble recombinant calcineurin by coexpressing both A and B subunits in *E. coli*, but the sequence length of the A subunit was threefold that of the B subunit, so did not assure the agreement of the translation rate of the two subunits in the coexpressing process. Thus, the extra B subunits expressed in *E. coli* will aggregate and the soluble portion of the yield may decrease. Mondragon et al. [10] harvested only about 1.8 mg of soluble holoenzyme from 1 L of the culture in this way. The single chain calcineurin, however, was absolute in the ratio of one to one due to the space–time synchronization of transcription, translation, and the translation rate of the two subunits. Because of this, the yield was about 20 mg of soluble protein from 1 L of the culture.

Acknowledgments

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