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Structural basis of calcineurin activation by calmodulin

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ABSTRACT

Calcineurin is the only known calmodulin (CaM) activated protein phosphatase, which is involved in the regulation of numerous cellular and developmental processes and in calcium-dependent signal transduction. Although commonly assumed that CaM displaces the autoinhibitory domain (AID) blocking substrate access to its active site, the structural basis underlying activation remains elusive. We have created a fused ternary complex (CBA) by covalently linking three polypeptides: CaM, calcineurin regulatory B subunit (CnB) and calcineurin catalytic A subunit (CnA). CBA catalytic activity is comparable to that of fully activated native calcineurin in the presence of CaM. The crystal structure showed virtually no structural change in the active site and no evidence of CaM despite being covalently linked. The asymmetric unit contains four molecules; two parallel CBA pairs are packed in an antiparallel mode and the large cavities in crystal packing near the calcineurin active site would easily accommodate multiple positions of AID-bound CaM. Intriguingly, the conformation of the ordered segment of AID is not altered by CaM; thus, it is the disordered part of AID, which resumes a regular α-helical conformation upon binding to CaM, which is displaced by CaM for activation. We propose that the structural basis of calcineurin activation by CaM is through displacement of the disordered fragment of AID which otherwise impedes active site access.

1. Introduction

Calcineurin is the only known calcium (Ca^{2+})/calmodulin (CaM)-dependent serine/threonine protein phosphatase [1,2], which plays critical roles in many cellular and developmental processes as well as in Ca^{2+}-dependent signal transduction [3–5]. Extensive studies have been conducted on the CN-NFAT (nuclear factor of activated T cells) signaling pathway and Ca^{2+}-mediated signaling pathways, including the effects of these pathways on a variety of pathogenic conditions such as schizophrenia [6], Alzheimer's disease [7–9], Down's syndrome [10], cardiac hypertrophy [11–13], diabetes [14], various cancers [15–17], abnormalities in osteoclastic bone resorption [18], and glaucoma [19]. As a heterodimeric protein, calcineurin is composed of a catalytic subunit A (CnA, ~61 kDa) and a regulatory subunit B (CnB, ~19 kDa) [5,20]. CnA is further subdivided into four functional domains: a catalytic domain, a CnB binding domain (BBH), a CaM binding domain (CBD) and an autoinhibitory domain (AID) [21,22]. CBD and AID are located in the C-terminal region of CnA and are readily cleaved by proteases [21–23]. Originally, the CBD was designated as residues 389–413 while the AID was thought to encompass residues 457–482 (native calcineurin numbering is used throughout). However, a more recent study demonstrated that CBD comprises a larger domain (at least residues 389–456) and also exhibits an autoinhibitory function along with AID [24]. Based on that work, the AID has been refined (residues 389–511), and is now considered to include both the CBD in addition to sequences corresponding to the originally designated AID. In this work, we shall use the updated AID definition unless otherwise stated.

The active site in CnA, where substrate binds and dephosphorylation takes place, is masked by AID. Although much of AID is disordered and not observed in calcineurin structures, it is generally thought that the highly flexible AID hovers near the entrance of the active site and impairs protein substrate access. Furthermore, it is thought that, upon CaM binding to AID, there is a conformational change and/or displacement of AID from its position guarding the entrance to the active center, effectively removing the steric obstacle that compromises protein substrate access and leading to full phosphatase activation. While this CaM-dependent activation model explains the biochemical and biophysical results, little corroborating structural evidence has been published to date. Indeed, all calcineurin structures currently available were determined in the absence of CaM. In these structures, including apo calcineurin and complexes with immunosuppressant FK506 and cyclosporin, a major part of AID is highly disordered and missing in the structure, with only a short, ordered AID helix (Ser469–Arg486)

Abbreviations: CaM, calmodulin; AID, autoinhibitory domain; CnB, calcineurin regulatory B subunit; can, calcineurin catalytic A subunit; Ca^{2+}, calcium; BBH, CnB binding domain; CBD, CaM binding domain; SAXS, small angle X-ray scattering.

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observed in the apo structure near the active site [25–28]. Previously, we have shown that a disordered 25-residue fragment in the CBD subdomain of AID becomes ordered and assumes an α-helical structure upon binding to CaM [29,30]. The X-ray study of CaM in complex with this peptide has revealed the structural determinants underlying the specific binding of AID to CaM. However, some 18 years after the first calcineurin structure was determined [28] the structural basis of its CaM-mediated activation still remains to be elucidated.

In the current work, we set to obtain a complex structure of calcineurin with CaM. We made numerous efforts to co-crystallize calcineurin with CaM using various calcineurin constructs which, unfortunately, have all failed, either in crystallization or lack of CaM in the final structure. Previously, we were successful in determining the structure of CaM with a peptide corresponding to sequences within the AID by covalently fusing the two partners via a glycine linker [29], later confirming that this structure is identical to the actual, unfused, complex structure [30]. Based on this experience, we generated a fused construct (termed CBA) by linking together the three polypeptides corresponding to CaM, CnB and CnA, respectively, in hopes of breaking the deadlock of its structural characterization. In previous studies, we have observed that fused calcineurin subunits behave better during expression and purification, and have been successful in reconstituting the active calcineurin enzyme from these individual subunits [31]. Herein, we report the crystal structure of CBA and propose a new model for CaM-dependent activation.

2. Experimental procedures

2.1. Cloning, expression and protein purification

Cloning of CBA [CaM–CnB–CnA1−482, containing CaM UniProt: P62161; CnB UniProt: P63100, and residues 1–482 of CnA (RatCNα isoform δ type) UniProt: P63329] with a C-terminal 6xHis tag was carried out using standard procedures. The final construct is CaM–Gly9–CnB–Gly6–CnA–His6. The protein was expressed in BL21 (DE3) *E. coli* cells (Novagen) and was purified as described previously [32] with some modifications. Briefly, cells were lysed by sonication in 300 mM NaCl, 20 mM Tris–HCl (pH 8.0), 5.0 mM imidazole, and 0.2 mM PMSF (buffer A), and clarified by centrifugation at 75,000 ×*g* for 45 min. The supernatant was applied to a Ni-NTA column (Novagen), and washed with buffer A. Specifically-bound protein was eluted with 200 mM NaCl, 200 mM imidazole and 20 mM Tris–HCl (pH 8.0) (buffer B), and the resulting material was collected. CBA was precipitated from this material with 45% saturated $(NH_4)_2SO_4$, followed by centrifugation at 14,600 ×*g* for 30 min. The resultant pellet was dissolved in 20 mL of buffer A and subsequently applied to a second Ni-NTA column. Following a second elution with buffer B, CBA was dialyzed into a third buffer containing 50 mM NaCl, 20 mM Tris–HCl (pH 7.4), 1 mM $CaCl_2$, 20 mM β-mercaptoethanol and 0.2 mM PMSF. A final gel filtration step using a Superdex 200 column (Amersham Biosciences) was performed prior to its use for crystallization. The CBA sample was analyzed by 12% SDS-PAGE and visualized using Coomassie Blue. Expression and purification of CnA was performed as described previously [31].

For SAXS analyses, CaM fused with a peptide corresponding to an AID fragment (residues 389–456) of CnA (CaM–AID) was expressed and purified. The pET21a-CaM–AID were based on previous descriptions [29] with some modifications to the purification protocol. In brief, the cell pellet was resuspended in lysis buffer consisting of 300 mM NaCl, 20 mM Tris–HCl, pH 8.0, 1.0 mM EDTA, 0.1% Triton X-100 and one tablet protease inhibitor cocktail. The cells were lysed by sonication and the lysate clarified by centrifugation at 75,000 ×*g* for 40 min. The supernatant was heated in an 85 °C water bath for 25 min and subsequently centrifuged at 75,000 ×*g* for 30 min. The resultant supernatant was then collected and 5 mM $CaCl_2$ added. Thereafter, the sample was loaded onto a phenyl sepharose column which had been pre-equilibrated with buffer consisting of 50 mM Tris–HCl pH 7.5, 20 mM β-mercaptoethanol, 0.5 mM $CaCl_2$ and a protease inhibitor cocktail. The column was washed with the same buffer and protein eluted with 50 mM Tris–HCl, pH 7.5, 1.0 mM EGTA containing protease inhibitor cocktail. Before the SAXS experiment, a final gel filtration was performed on a Superdex 200 column (Amersham Biosciences). The purity of CaM–AID was analyzed by 12% SDS-PAGE.

2.2. Phosphatase activity assay

A phosphatase activity assay was performed using *p*-nitrophenyl phosphate (*p*NPP) in 50 mM Tris–HCl, pH 7.4, 0.5 mM $MnCl_2$, 0.5 mM dithiothreitol (DTT), 1 mM $CaCl_2$, 0.2 mg/ml BSA, and 20 mM *p*NPP in accordance with previously published protocols [31,33]. Reactions were performed in 0.2 ml volumes at 30 °C for 15 min, and terminated by adding 1.8 ml 0.5 M Na_2CO_3 and 20 mM EDTA. The absorbance was recorded at 410 nm. A molar extinction coefficient of 17,500 M^{-1} cm^{-1} was used to convert absorbance values into molar concentrations, and relative specific activity was converted to nmol/(min·mg).

2.3. Crystallization and data collection

CBA was crystallized by the hanging drop vapor diffusion method at 20 °C by mixing equal volumes of protein (~6 mg ml^{-1}) and well solutions containing 8% (w/v) PEG 3350, 12% 1-propanol, 0.1 M citric acid (pH 5.6) and 1 mM DTT. Crystals grew over a 3-week period and were flash-frozen in liquid nitrogen in a cryo-protectant containing 25% ethylene glycol, 12% PEG 3350, 12% 1-propanol and 0.1 M citric acid. Diffraction data was collected at the F1 station at the Cornell High Energy Synchrotron Source (CHESS). The crystal diffracted to 3.0 Å and the dataset was processed and scaled using HKL2000 [34] and XDS [35].

2.4. Structure determination and refinement

The crystal belongs to the space group $P2_1$, with four molecules in asymmetric unit. The CBA structure was solved by molecular replacement using the human calcineurin complex structure devoid of cyclosporin A and cyclophilin as a search model (PDB 1MF8). Iterative cycles of refinement and manual building were carried out using CNS [36,37], REFMAC5 [38] and Coot [39]. Structure diagrams were prepared using PyMol [40].

2.5. Detection of CaM in CBA crystal

Western blot analyses were performed in order to verify the presence of CaM in the CBA crystals. CBA crystals were first transferred to a new drop containing crystallization well solution devoid of CBA protein. The crystals were then washed more than three times with the well solution. Subsequently, these crystals were dissolved in Millipore water, resolved using 12% SDS-PAGE, and transferred onto a nitrocellulose membrane in 39 mM glycine, 48 mM Tris, and 0.1% methanol, using a semidry apparatus (BioRad). The membrane was blocked with 5% skim milk (GE Healthcare) in T-TBS (20 mM Tris–HCl (pH 7.5), 150 mM NaCl, 0.05% Tween 20) for one hour and then incubated with monoclonal mouse anti-CaM (1:1000, Thermo Scientific) in T-TBS/2% skim milk overnight at 4 °C. After incubation with an appropriate HRP-conjugated secondary Ab (1:10000, BioRad), an immunoreactive band was detected using an ECL system (GE Healthcare). The washed and dissolved crystal sample was also subject to MALDI-TOF mass spectrometry analysis.

2.6. Analytical ultracentrifugation

Sedimentation velocity experiments were performed using a Beckman Optima XL-1 instrument equipped with an An-60-Ti rotor and schlieren interference optics system. CBA was studied at a various