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Probing the Ca^{2+}/CaM-induced secondary structural and conformational changes in calcineurin

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ABSTRACT

Calcineurin (CN) is a Ca^{2+}/CaM-dependent Ser/Thr protein phosphatase that plays a critical role in coupling Ca^{2+} signals to a cellular response. Various methods have been applied to explore CN activation. A widely accepted model involves CaM binding to the CaM-binding domain (CN 389–413), inducing displacement of the CN autoinhibitory peptide (CN 467–486) from the active site. However, almost the entire regulatory region (CN 374–521), except the autoinhibitory peptide, is not visible in the electron density map of the reported structures. In the present study, we determined the overall secondary structure of CN in the presence or absence of Ca^{2+}/CaM using FT-IR, and the Ca^{2+}/CaM-induced structural dynamics and conformational changes were monitored by hydrogen–deuterium exchange experiments. The results revealed that the regulatory domain possessed some intrinsic structure. The binding of Ca^{2+} and subsequent binding of CaM generated a sequential folding of CN, transforming it into a more constrained, less flexible conformation.

1. Introduction

Calcineurin (CN), also known as protein phosphatase-2B (PP2B), is the only known protein Ser/Thr phosphatase that is directly regulated by calcium and calmodulin (CaM) [1–3]. CN communicates with a large number of substrates and other proteins after being stimulated by the multifunctional protein CaM, which ensures the coordinated regulation of CN protein phosphatase [4]. Among CN substrates, the nuclear factor of activated T cells (NFAT) family of transcription factors is arguably the best understood [2,5]. Coupling Ca^{2+} signals to cellular responses, CN has diverse biological functions and plays a critical role in many physiological processes, including the immune response, apoptosis, muscle differentiation, bone formation, and neuronal signaling [6–8]. Therefore, dysregulation of CN underlies many diseases, including cardiac hypertrophy [9], glutamate neurotoxicity, brain ischemia [10], Alzheimer's disease [11], and schizophrenia [12].

CN is a heterodimeric protein consisting of a 61-kDa catalytic subunit A (CNA), which contains an active site dinuclear metal center, and a tightly associated 19-kDa Ca^{2+}-binding regulatory subunit B (CNB) (Fig. 1) [13]. This architecture is highly conserved throughout all eukaryotic organisms [4]. The structure of CN indicated that CNA consists of a catalytic domain, CNB binding domain, a regulatory domain including the CaM binding region and an autoinhibitory peptide (AI), and a short C-terminal domain [14,15]. Biochemical results indicate that the auto-inhibitory domain forms a α-helix that binds to the substrate-binding cleft of the enzyme catalytic site [13,16]. A widely accepted mechanism for CN activation involves CaM binding to the CaM-binding domain (CN 389–413), inducing the displacement of the AI (CN 467–486) from the active site. Although many structures of CN have been solved, an electron density map of the entire regulatory domain (CN 374–521) is lacking (Fig. 1). Previous studies with limited proteolysis have suggested that the regulatory domain is disordered and unstructured [17,18]. However, Shen et al. (2008) constructed a separate CN fragment corresponding to the CNA regulatory region (CN 381–521) and demonstrated that this region is predominantly random coil, but with significant amount of β-strand and α-helix structures [19]. In the present study, FT-IR was used to further estimate the secondary structure of the CN regulatory region in full-length CN and investigate the conformational changes and regulation induced by Ca^{2+}/CaM binding.

2. Materials and methods

2.1. Materials

The high-performance phenyl-sepharose chromatography used for purification was purchased from GE Healthcare. The nickel-nitrilotriacetic acid column (Qiagen, Germany) and gel filtration

Abbreviations: CaM, calmodulin; CN, calcineurin; FT-IR spectroscopy, Fourier transform infrared spectroscopy; H–D exchange, hydrogen–deuterium exchange.

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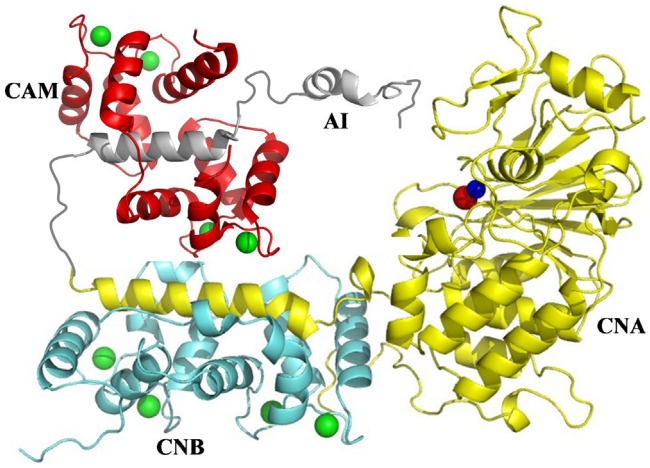

Fig. 1. The structure of Ca^{2+}/CaM–CN complex. Calcineurin A (CNA) is shown in yellow and calcineurin B (CNB) in blue. Iron and zinc in the active site are shown as red and blue spheres, respectively. The four Ca^{2+} in the CNB subunit and calmodulin (CaM) sites are shown as green spheres. A region of CNA whose structure has not been determined is indicated schematically (gray). Upon activation by Ca^{2+}, CaM (red) binds to its target sequence in CNA and induces a structural change that relieves autoinhibitory peptide (AI). The depictions of CN are modified from PDB entry 1AUI (Griffith et al., 1995), and the depiction of CaM is based on its complex with a Dap kinase peptide (PDB entry 1YR5). The figure was generated using Chimera software (Pettersen et al., 2004). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

column Sepharose S-200 column (GE Healthcare) were used for the purification. Deuterium oxide (99.9 at% D) was purchased from J&K Chemical Ltd.

2.2. Protein expression and purification

The CaM gene was purchased from ATCC (Manassas, VA) and cloned into the pET-15b vector between the NcoI and XhoI sites. CaM was expressed and purified using the procedure described by Hayashi et al. [20]. CN was purified from IPTG-induced *Escherichia coli* strain BL21 according to the published standard protocol [19,21]. The purity of CN and CaM was greater than 95% based on the results of SDS-PAGE. Glycerol (20%) was added to the purified protein solution, which was stored at −20 °C. Both protein solutions were dialyzed against a buffer containing 50 mM Tris (pH 7.5), 100 mM NaCl and gradually reduced EDTA (50 mM-0) overnight to remove glycerol and other additives with three buffer changes before being used.

CaM and CN were concentrated to 15 mg/ml and 7 mg/ml, respectively, by centrifugation using an Amicon Ultra-4 (Millipore Corporation, Billerica, USA). The protein concentration was determined spectrophotometrically using the following molar extinction coefficients: CaM, $\varepsilon_{276} = 3006\ M^{-1}\ cm^{-1}$ [22] and CN, $\varepsilon_{279} = 60{,}935\ M^{-1}\ cm^{-1}$ [23]. The samples of CaM/CN complex were mixed in a 1:1 molar ratio of the two proteins. $CaCl_2$ solution (0.5 M) was added to the samples containing Ca^{2+} in a 10-fold stoichiometric excess.

2.3. FT-IR spectroscopy

FT-IR spectra were recorded with an ABB Bomem (Quebec, Canada) MB-3000 Fourier Transform infrared spectrometer equipped with a deuterated triglycine sulfate detector and purged constantly with dry air. Frozen protein samples were thawed at room temperature and loaded into a liquid IR cell (Spacac 20500 series) with CaF_2 windows and a 7.5-μm spacer. For each spectrum, a 128-scan interferogram was collected in single-beam mode with 4-cm^{-1} resolution at room temperature. The reference spectrum was recorded under identical conditions with only the corresponding buffer in the cell. The protein spectra were processed using a previously established protocol [24,25]. A straight baseline between 2000 and 1750 cm^{-1} was used as the standard for judging the success of water subtraction. Second-derivative spectra were obtained using a seven-point baseline-corrected Savitzky–Golay derivative function, and the amide I band area-normalized as described previously [24]. The secondary structure content of the protein was calculated by curve-fitting analysis of the inverted second-derivative amide I band from 1600 to 1700 cm^{-1} [26]. The amide I band was ascribed to the C=O stretching vibration of the peptide bond [27]. The fraction of amino acid residues composing each secondary structural element is proportional to the relative percent area of the associated C=O vibrational bands [25,28].

2.4. Hydrogen–deuterium exchange

Aliquots of protein samples (50 μl) were lyophilized to dryness using a LNG-T98A Lyophilizer. The H–D exchange experiments were performed by reconstituting lyophilized protein samples with 50 μl of D_2O and injecting the reconstituted sample immediately into an IR cell with CaF_2 windows and a 50-μm spacer. The spectral measurements were started 1 min (i.e., lag time = 1 min) after the addition of D_2O using the kinetic scanning mode. The spectra were recorded at 1–11, 15, 20, 30, 40, 50, 60, 90, 120 and 180 min. An 8-scan interferogram was collected at each time interval between 1 and 10 min, whereas a 64- or 128-scan interferogram was recorded at each time interval between 11 and 90 min and longer. For comparison, the amide I band maximum for protein in H_2O was normalized to the amide I′ band maximum for protein for D_2O recorded at 1 min [29].

The H–D exchange process was monitored by following the apparent changes in intensity at the amide II band maxima around 1548 cm^{-1}, which was attributed to an out-of-phase combination of N—H in-plane bending and CN stretching vibrations in the peptide bonds [30]. The absorption bands arising from H_2O, HOD, or D_2O do not adversely interfere with the amide II band [28]. As the protein amide N—H bonds in H_2O change to the N—D bonds in D_2O, the N—H bending vibrational band at 1550 cm^{-1} decreases, but the magnitude of the N—D bending vibrational band at 1450 cm^{-1} increases. The fraction of un-exchanged amide proton, F, was calculated at various time intervals using Eq. (1) [31].

$$F = \frac{A_{II} - A_{II\infty}}{A_{I\omega}} \quad (1)$$

In Eq. (1), A_I and A_{II} are the absorbance maxima of the amide I and II bands, respectively, $A_{II\infty}$ is the amide II absorbance maximum of the fully deuterated protein, and ω is the ratio of A_{II}/A_I, with A_{II} and A_I being the absorbance maxima for the amide II and amide I bands of protein in H_2O, respectively. The exchange kinetic parameters were fitted using Eq. (2) [29].

$$F = A_1 e^{-k1t} + A_2 e^{-k2t} + C \quad (2)$$

In Eq. (2), F is the amide proton fraction at time t, k_1 and k_2 are the intermediate and slow exchange rates, respectively, and A_1, A_2, and C are constants.

3. Results and discussion

CN is the only serine/threonine protein phosphatase under the control of Ca^{2+}/CaM [32], and it plays a critical role in the coupling of Ca^{2+} signals to cellular responses [33,34]. The stimulation of CN by the multifunctional protein, CaM, ensures the coordinated regulation of its protein phosphatase activity with the activities of the many other enzymes, including a large number of protein kinases, under the control of Ca^{2+} and CaM [4,35]. The CN regulatory region