International Journal of Biological Macromolecules 60 (2013) 122–127

Contents lists available at SciVerse ScienceDirect

International Journal of Biological Macromolecules

journal homepage: www.elsevier.com/locate/ijbiomac

Structural dynamic and thermodynamic analysis of calcineurin B subunit induced by calcium/magnesium binding

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ARTICLE INFO

Article history:
Received 13 April 2013
Received in revised form 10 May 2013
Accepted 13 May 2013
Available online 21 May 2013

Keywords:
Calcineurin B subunit
Thermodynamics
Structural dynamics

ABSTRACT

The structural dynamics and thermodynamics of the interaction of Ca^{2+}/Mg^{2+} with the calcineurin B subunit (CNB) were monitored by Fourier transform infrared spectroscopy (FT-IR) and isothermal titration calorimetry (ITC). The results suggest that CNB activation by Ca^{2+} binding involves significant conformational changes with a marked increase in the α-helix content, whereas Mg^{2+} binds to CNB without inducing changes in secondary structure. The results of hydrogen—deuterium (H—D) exchange and GdnHCl-induced unfolding show that the overall conformation of Ca^{2+}-loaded CNB (CNB-Ca^{2+}) is more stable and has more hydrophobic areas than that of Ca^{2+}-free CNB (apo-CNB) or Mg^{2+}-loaded CNB (CNB-Mg^{2+}). The thermodynamic characterization suggests that there is no competition between Ca^{2+} and Mg^{2+} in their binding to the main CNB Ca^{2+} binding sites. Mg^{2+} is more likely to bind the auxiliary cation-binding sites present on CNB.

1. Introduction

Calcineurin (CN) is the only serine/threonine protein phosphatase under the control of calcium and calmodulin (CaM), which plays a critical role in the coupling of Ca^{2+} signals to cellular responses [1,2]. In CaM-CN-NFAT (NFAT, nuclear factor of activated T cells) pathway, a rise in intracellular Ca^{2+} triggered by certain stimulations leads to the activation of CN's phosphatase activity, which in turn functions in T-cell activation [3]. Structurally, CN is made up of two tightly bound subunits: a 59 kDa catalytic subunit, calcineurin A (CNA) and a 19.3 kDa Ca^{2+}-binding regulatory subunit, calcineurin B (CNB) [4,5]. CNB is a member of the family of EF-hand calcium-binding proteins and contains four helix-loop-helix motifs like CaM [6]. According to the crystal structure of CN-FKBP12-FK506 complexes [7], CNB contains two globular Ca^{2+}-binding domains and forms a long hydrophobic groove into which the top half of the CNB binding domain of CNA. CN has intrinsically bound iron and zinc, but requires exogenous metal for activity [8–12]. Ca^{2+}, Mn^{2+}, Ni^{2+} and Mg^{2+} are typically used for activating CN [13], whereas, in the absence of exogenous metal, CN effectively has no catalytic activity (<1%). It has long been recognized that cells contain relatively high levels of Mg^{2+} (~1–5 mM) [14], and evidence was obtained by mass spectrometry for the presence on CaM of a pair of high-affinity Mg^{2+} binding sites and a pair of low-affinity sites [15]. Ca^{2+} signal and calcium binding proteins (CaBPs) of the EF-hand superfamily are involved in the regulation of almost every process in the life from birth to death [16,17]. As a member of the family of CaBPs, CNB shares 35% sequence homology with CaM [18]. Although it is generally assumed that sequence similarity implies shared structure and function, the calcium binding proteins yet are very diverse in their structures and in the target with which they interact [19].

In the present work, the structural dynamics and conformational changes of CNB were analyzed by Fourier transform infrared (FT-IR) spectroscopy in conjunction with hydrogen—deuterium (H—D) exchange. The secondary structure of CNB in the absence and presence of 4 mM Ca^{2+}/Mg^{2+} was also monitored by circular dichroism (CD) spectroscopy. In addition, the thermodynamic changes of CNB induced by Ca^{2+}/Mg^{2+} binding were determined by isothermal titration calorimetry (ITC).

2. Materials and methods

2.1. Materials

GdnHCl (Analytical grade) was purchased from China National Medicines Corp; NaCl, $CaCl_2$ and $MgSO_4$ were purchased from

Abbreviations: CN, calcineurin; CNB, calcineurin B subunit; CNA, calcineurin A; NFAT, nuclear factor of activated T cells; FKBP12, the FK506-binding protein; CaBPs, calcium binding proteins; IPTG, isopropyl thiogalactopyranoside; CD, circular dichroism; GdnHCl, guanidine hydrochloride; FT-IR spectroscopy, Fourier transform infrared spectroscopy; H—D exchange, hydrogen—deuterium exchange; ITC, isothermal titration calorimetry.

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http://dx.doi.org/10.1016/j.ijbiomac.2013.05.009

Sinopharm Chemical Reagent Co. Ltd.; and deuterium oxide (99.9 atom% D) is purchased from J&K Chemical Ltd.

2.2. Expression of CNB

The human CNB gene was cloned into pET-28a vector between the NcoI and BamHI sites [20]. NcoI and BamHI sites were engineered at the 5′ and 3′ ends of the coding sequence using the primers 5′-CCGCCATATGGGAAATGAGGCGAGT-3′ and 5′-CGCGGGATCCTCACACATCTACCACCA-3′, respectively. The plasmids were transformed into *Escherichia coli* BL21 (DE3) cells and grown in LB medium with 100 μg/mL of kanamycin at 37 °C. The sequence of the complete CNB was verified by DNA sequencing.

2.3. Protein purification and preparation

CNB was purified from IPTG-induced *E. coli* strain BL21 (DE3) according to published procedures [21]. The protein purity was assessed by staining SDS-PAGE gels with Coomassie Blue. The CNB-containing fractions were collected and dialyzed overnight against 50 mM Tris buffer (pH 7.5) containing 100 mM NaCl to remove glycerol and other additives with three buffer changes before being used. The protein concentration was determined spectrometrically using the following molar extinction coefficient: $\varepsilon_{279} = 1578\ M^{-1}\ cm^{-1}$ [22].

2.4. Far-UV circular dichroism spectroscopy

The secondary structures of CNB in the absence and presence of 4 mM Ca^{2+}/Mg^{2+} were monitored by CD spectroscopy. The Far-UV CD spectra of CNB were recorded in a 0.05 mm quartz cuvette as the average of six scans from 250 to 190 nm with 20 nm/min steps using a Jasco J-810 circular dichroism spectrometer purged with N_2 gas. The concentration of the CNB solution was 30 μM in each experiment. GdnHCl-induced unfolding of CNB in the absence and presence of 4 mM Ca^{2+}/Mg^{2+} was monitored at 222 nm in 50 mM Tris–HCl buffer (pH 7.5) containing 100 mM NaCl and concentrations of GdnHCl ranged from 0 to 5.5 M. The protein spectra were obtained by subtracting the spectrum of corresponding buffer solution. The data were expressed as mean residue ellipticity.

2.5. FT-IR spectroscopy

FT-IR spectra were recorded with an ABB Bomem (Quebec, Canada) MB-3000 Fourier transform infrared spectrometer equipped with a dTGS detector and purged constantly with dry air. Protein samples (~1.0 mM) in the absence and presence of 10 mM Ca^{2+}/Mg^{2+} were loaded into a demountable IR liquid cell (P/N 2500 series) with CaF_2 windows and a 7.5-μm spacer. For each spectrum a 128-scan interferogram was collected in single-beam mode and 4 cm^{-1} resolution. 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 [23]. A straight baseline between 2000 and 1750 cm^{-1} was used as the standard for quantifying the completion of water subtraction. Second-derivative spectra were obtained using a seven-point Savitsky–Golay derivative function, baseline-corrected, 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} [25]. The amide I band is ascribed to the C=O stretching vibration of the peptide bond [26]. The fraction of amino acid residues composing each secondary structural element has been empirically validated to be proportional to the relative percent area of the associated C=O vibrational bands [23,27].

2.6. Hydrogen—deuterium exchange

H—D exchange of amide protons was used to analyze the structural dynamics and conformational flexibility of proteins. Aliquots (80 μL) of CNB (1.0 mM) in 50 mM Tris–HCl (pH 7.5) containing 100 mM NaCl in the absence and presence of 4 mM Ca^{2+}/Mg^{2+} were lyophilized using a LNG-T98A lyophilizer. The H—D exchange experiments were performed by reconstituting the lyophilized CNB (80 μL original volume) or buffer with or without 10 mM Ca^{2+}/Mg^{2+} in the same volume of D_2O. The reconstituted sample was injected immediately into an IR cell with a 50-μm spacer. One minute after the addition of D_2O, single-beam spectra were recorded using kinetic scanning mode. The spectra were recorded at 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 30, 40, 50, 60, 90, 120, 150, 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 15 and 90 min and longer. In order to compare the protein FT-IR spectra recorded in H_2O and D_2O, we normalized the amide I band of the protein in H_2O to the amide I band of the protein in D_2O at 1 min. The spectrum collected 24 h after H—D exchange was used as the fully deuterated spectrum [28].

2.7. Calculation of the amide proton exchange rate

The H—D exchange process is monitored by following the apparent intensity changes at the amide II band maxima around 1548 cm^{-1}, which is attributed to an out-of-phase combination of N—H in-plane bending and C—N stretching vibrations in the peptide bonds [29]. The absorption bands arising from H_2O, HOD, or D_2O do not adversely interfere with the amide II band [27]. As the N—H bonds change to N—D bonds, the N—H bending vibrational band at 1550 cm^{-1} decreases, whereas 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) [30].

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

where 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 $A_{II/H_2O}/A_{I/H_2O}$, with A_{II/H_2O} and A_{I/H_2O} being the absorbance maxima for the amide II and amide I bands of CaM in H_2O, respectively. The exchange kinetic parameters were fitted using Eq. (2) [28].

$$F = A_1 e^{-k_1 t} + A_2 e^{-k_2 t} + C \quad (2)$$

where 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.

2.8. Isothermal titration calorimetry

Binding of Ca^{2+}/Mg^{2+} to the main sites of CNB was performed with a MicroCal ITC-200 system (GE Healthcare). All ITC experiments were conducted in 50 mM Tris–HCl (pH 7.5) containing 100 mM NaCl. Protein solution and buffer were thoroughly degassed by stirring under vacuum for 10 min before use. The reaction cell (200 μL) contained 200 μM CNB in 50 mM Tris–HCl (pH 7.5) containing 100 mM NaCl, and the reference cell contained buffer only. The injection syringe (40 μL) was filled with 5 mM Ca^{2+}/Mg^{2+} in 50 mM Tris–HCl (pH 7.5) containing 100 mM NaCl and rotated at 1000 rpm during temperature equilibration and the experiment. The titration consisted of 35 injections: 0.4 μL for the first injection and 1 μL for each of the following injections. The injection speed was 0.5 μL/s with a 60 s interval between injections. A blank titration of Ca^{2+}/Mg^{2+} into the buffer was performed