International Journal of Biological Macromolecules 79 (2015) 235–239

Contents lists available at ScienceDirect

International Journal of Biological Macromolecules

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

Binding of calmodulin changes the calcineurin regulatory region to a less dynamic conformation

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ARTICLE INFO

Article history:
Received 2 April 2015
Received in revised form 28 April 2015
Accepted 29 April 2015
Available online 5 May 2015

Keywords:
Calcineurin
Calcineurin regulatory region
Calmodulin
FT-IR spectroscopy
ITC

ABSTRACT

Calcineurin (CN) is a Ca^{2+}/calmodulin (CaM) activated serine/threonine phosphatase, and its regulatory region (CNRR) plays a critical role in the coupling of Ca^{2+} signals to cellular responses. Ca^{2+}/CaM binds to the CNRR, resulting in a conformational change that removes an autoinhibitory domain (CN467–486) from the active site of the phosphatase and activates the enzyme. However, almost the entire regulatory region (CN374–521) is not visible in the electron density maps of reported structures. In this study, we produced separate CN fragments corresponding to the CNRR ($CNRR_{381–521}$, CN residues 381–521) and determined the binding affinity of $CNRR_{381–521}$ for Ca^{2+}/CaM using isothermal titration calorimetry (ITC). The structural change in $CNRR_{381–521}$ induced by Ca^{2+}/CaM binding was also investigated by Fourier transform infrared spectroscopy (FT-IR). The results indicate that Ca^{2+}/CaM binding to $CNRR_{381–521}$ is an exothermic reaction with a dissociation constant of 2.0×10^{-6} M. Binding of calmodulin changes the calcineurin regulatory region to a less dynamic conformation.

1. Introduction

As an essential serine/threonine protein phosphatase, calcineurin (CN) plays a critical role in the coupling of Ca^{2+} signals to cellular responses [1–4], implicating it in a number of pathological states, including cardiac hypertrophy [5], skeletal muscle growth [6], Alzheimer's disease [7], and immunological rejection [8,9]. CN has a wide range of physiological substrates after being stimulated by the multifunctional calmodulin (CaM), which ensures the coordinated regulation of CN protein phosphatase [10]. One of the best characterized CN substrates is the nuclear factor of activated T cells (NFAT) family of transcription factors [11,12], which is a phosphoprotein located in the cytoplasm of quiescent cells [13]. In response to physiological signals that elevate intracellular calcium, NFAT is rapidly dephosphorylated by CN, which triggers its entrance into the cell nucleus and subsequent transcriptional activation of NFAT target genes [14,15].

CN is a heterodimer composed of a catalytic subunit (CNA) and a calcium-binding regulatory subunit (CNB) [16]. CNA consists of a catalytic domain (residues 1–323), a B-subunit binding domain (residues 324–369), a CaM-binding domain (residues 391–414), and an autoinhibitory (AI) domain (residues 467–521) [17]. The regulatory region of CNA (CNRR) can be subdivided into two subregions of known function: a CaM-binding peptide and an AI peptide [18]. Although many CN structures have been solved, CN residues comprising nearly the entire regulatory region (residues 374–521), except for a small helical fragment at the active site (CN467–486), are not visible in electron density maps (Fig. 1) [19]. Recently, Dunlap et al. crystallized the CaM–CNRR (residues 391–414) complex and determined the structure of the complex [1].

Previous work in our laboratory revealed that the secondary structure composition of the $CNRR_{381–521}$ fragment is predominantly random coil (~36%), but with a significant amount of α-helix and β-sheet structures [18]. When CN was truncated at residue 380, the two fragments (CN380 and regulatory region $CNRR_{381–521}$) recapitulated the CaM-dependent regulation of the full-length enzyme. $CNRR_{381–521}$ was able to inhibit the phosphatase activity of CN380, which was reversed by Ca^{2+}/CaM binding [18]. In the present study, we determined the binding affinity and

Abbreviations: CN, calcineurin; CaM, calmodulin; FT-IR spectroscopy, Fourier transform infrared spectroscopy; ITC, isothermal titration calorimetry; EGTA, glycol-bis-(2-aminoethylether)-N,N,N′,N′-tetraacetic acid.

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http://dx.doi.org/10.1016/j.ijbiomac.2015.04.069
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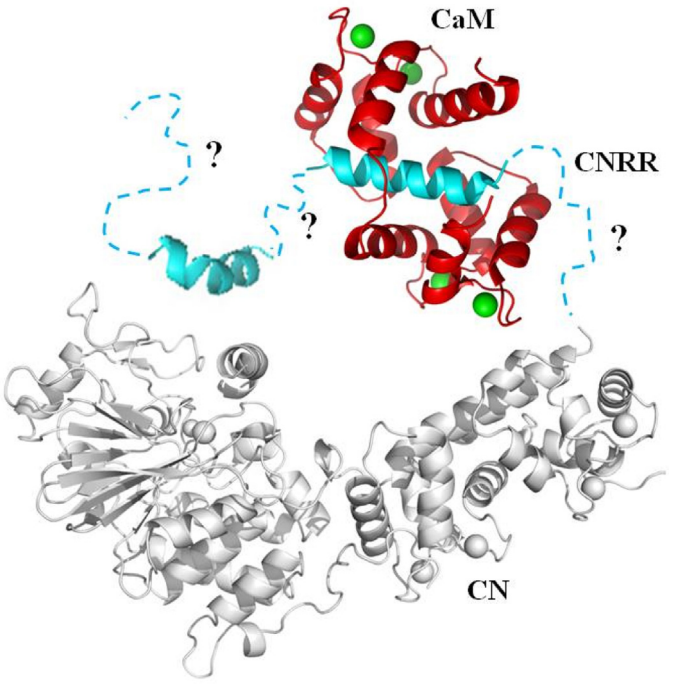

Fig. 1. Structure of the $CNRR_{381-521}$–Ca^{2+}/CaM complex. CNA1–380 and CNB are shown in gray, $CNRR_{381-521}$ is shown in cyan, and CaM is shown in red. Ca^{2+} ions are shown as spheres. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

thermodynamic parameter of the $CNRR_{381-521}$–Ca^{2+}/CaM interaction using ITC, and the structural changes in the $CNRR_{381-521}$ segment induced by Ca^{2+}/CaM binding were investigated by FT-IR spectroscopy.

2. Materials and methods

2.1. Materials

The high-performance phenyl-Sepharose chromatography and gel filtration Sepharose Superdex 75 column used for purification was purchased from GE Healthcare. A nickel-nitrilotriacetic acid column (Qiagen, Germany) was also used for purification.

2.2. Protein expression and purification

Plasmid pET-15b containing human *CaM* was transformed into *Escherichia coli* strain BL21(DE3). The recombinant CaM protein was expressed and purified using the procedure described by Hayashi et al. [20]. The $CNRR_{381-521}$ segment was purified from IPTG-induced *E. coli* strain BL21 according to the published standard protocol [18]. Protein purity was assessed by staining SDS–PAGE gels with Coomassie Blue. All purified proteins were stored at −80 °C before use. The additives in protein solutions were removed by dialysis against a buffer containing 50 mM Tris (pH 7.5), 100 mM NaCl, and gradually reduced EGTA (50 mM-0) overnight. All other experiments were conducted in buffer containing 50 mM Tris and 100 mM NaCl at pH 7.5 and 25 °C.

CaM and $CNRR_{381-521}$ were concentrated to 16 mg/ml and 8 mg/ml, respectively, using an Amicon Ultra-4 centrifugal filter unite (MWCO 3 kDA, Millipore). The CaM concentration was determined spectrophotometrically using the molar extinction coefficient $\varepsilon_{279} = 1578\ M^{-1}\ cm^{-1}$ [21]. The $CNRR_{381-521}$ segment concentration was determined by Bradford assay (Bio-Rad). The $CNRR_{381-521}$/CaM complex was prepared by mixing the two proteins in a 1:1 molar ratio. $CaCl_2$ solution (100 mM) was added to the samples until Ca^{2+} was in 10-fold stoichiometric excess to CaM.

2.3. FT-IR spectroscopy

Infrared spectra were measured 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. Protein solutions were loaded in a liquid IR cell with CaF_2 windows and 6-μm path length. For each spectrum, a 128-scan interferogram was collected in single beam mode with a $4\ cm^{-1}$ resolution. Reference spectra were recorded under identical conditions with only the buffer in which the protein was dissolved. Data processing was carried out using a previously established protocol [22,23]. Second-derivative spectra were obtained using Savitzky–Golay derivative function software for a seven data point window [22]. The relative secondary structure content was determined from the infrared second-derivative amide I spectra by manually computing the areas under the bands assigned to a particular substructure [24,25].

2.4. Isothermal titration calorimetry

All ITC experiments were conducted in 50 mM Tris buffer (pH 7.5) on a MicroCal ITC-200 system (GE Healthcare). The protein concentration was 0.5 mM. A total of 40 μl of Ca^{2+}/CaM solution was titrated into the $CNRR_{381-521}$ solution with a fixed stirring speed of 1000 rpm. Titration consisted of 20 injections, 0.5 μl for the first injection and 2 μl each for all following injections. To achieve complete equilibration, the time between each injection was 120 s. The first injection of 0.5 μl was ignored in the final data analysis. The contents of the sample cell were stirred throughout the experiment at 200 rpm to ensure thorough mixing. Raw data were obtained as a plot of heat (cal) against injection number and featured a series of peaks for each injection. These raw data peaks were transformed using the instrument's software to obtain a plot of enthalpy change per mole of injectant (ΔH, $J\ mol^{-1}$) against the molar ratio. Fitting the data according to the independent binding model resulted in the stoichiometry of binding (n), the equilibrium binding constant (K), and enthalpy of complex formation (ΔH). The standard changes in free energy (ΔG) and entropy (ΔS) were calculated using the following equations [26]:

$$\Delta G = -RT \ln K \quad (1)$$

$$\Delta G = \Delta H - T\,\Delta S \quad (2)$$

2.5. Protein stability

GuHCl-induced $CNRR_{381-521}$/CaM unfolding was carried out with fluorescence spectroscopy. In a typical denaturation experiment, 24 samples at a fixed $CNRR_{381-521}$/CaM concentration (20 μM) in 50 mM Tris were mixed with varying amounts of a stock GuHCl solution to final GuHCl concentrations ranging from 0 to 5 M. The fluorescence intensity was recorded with an HORIBA fluromax-4 spectrofluorometer at 25 °C. The excitation and emission wavelengths were 279 nm and 330 nm, respectively.

3. Results and discussion

CN plays a critical role in the coupling of Ca^{2+} signals to the cellular response. At low calcium concentrations, the AI domain is thought to block the enzyme active site within the catalytic region, keeping CN inactive [27,28]. The increase in intracellular calcium concentration leads to the activation of CaM, which interacts with the CaM-binding domain of the CNA subunit. The