The Secondary Structure of Calcineurin Regulatory Region and Conformational Change Induced by Calcium/Calmodulin Binding*

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Xianrong Shen,1 Huiming Li,1 Yan Ou,1 Wenbing Tao,1 Aichun Dong,1 Jilie Kong,1 Chaoneng Ji,1 and Shaoning Yu2

From the 1Department of Chemistry and Institutes of Biomedical Science and 2Institute of Genetics, School of Life Science, Fudan University, Shanghai 200433, China, 3Immune Disease Institute, Harvard Medical School, Boston, Massachusetts 02115, and 4Department of Chemistry and Biochemistry, University of Northern Colorado, Greeley, Colorado 80639

The protein serine/threonine phosphatase calcineurin (CN) is activated by calmodulin (CaM) in response to intracellular calcium mobilization. A widely accepted model for CN activation involves displacement of the CN autoinhibitory peptide (CN^{467–486}) from the active site upon binding of CaM. However, CN activation requires calcium binding both to the low affinity sites of CNB and to CaM, and previous studies did not dissect the individual contributions of CNB and CaM to displacement of the autoinhibitory peptide from the active site. In this work we have produced separate CN fragments corresponding to the CNA regulatory region (CNRR^{381–521}, residues 381–521), the CNA catalytic domain truncated at residue 341, and the CNA-CN heterodimer with CNA truncated at residue 380 immediately after the CNB binding helix. We show that the separately expressed regulatory region retains its ability to inhibit CN phosphatase activity of the truncated CN341 and CN380 and that the inhibition can be reversed by calcium/CaM binding. Tryptophan fluorescence quenching measurements further indicate that the isolated regulatory region inhibits CN activity by occluding the catalytic site and that CaM binding exposes the catalytic site. The results provide new support for a model in which calcium binding to CNB enables CaM binding to the CNA regulatory region, and CaM binding then instructs an activating conformational change of the regulatory region that does not depend further on CNB. Moreover, the secondary structural content of the CNRR^{381–521} was tentatively addressed by Fourier transform infrared spectroscopy. The results indicate that the secondary structure of CNRR^{381–521} fragment is predominantly random coil, but with significant amount of β-strand and α-helix structures.

Calcineurin (CN),3 also called protein phosphatase 2B, is a calcium/CaM-dependent Ser/Thr protein phosphatase (1–3) and plays a critical role in the coupling of Ca^{2+} signals to cellular responses (3–10). CN is stimulated by the multifunctional protein, calmodulin (CaM), which ensures the coordinated regulation of CN protein phosphatase activity, together with the activities of many other enzymes, including a large number of protein kinases under Ca^{2+} and CaM control (7). CN has a wide range of physiological substrates (7). The complex regulation observed with CN is expected for an enzyme that is a major player in the regulation of many cellular processes. Various phosphoproteins such as inhibitor 1a, protein kinase A regulatory subunit RII, neurogranin, phosphorylase kinase a, neuromodulin, and small organic substrate p-nitrophenyl phosphate are all dephosphorylated by CN (7). Among CN substrates, the nuclear factor of activated T cells (NFAT) family of transcription factors is arguably the best understood (2, 9). NFAT is a phosphoprotein located in the cytoplasm of the resting cell. In response to physiological signals that elevate intracellular calcium, NFAT is rapidly dephosphorylated by CN, which triggers its import into cell nucleus and subsequent transcriptional activation of NFAT target genes (5, 9).

CN is a heterodimeric enzyme consisting of a 61-kDa subunit (CNA) with catalytic activity and the binding sites for Ca^{2+}/CaM and a 19-kDa subunit (CNB), an EF-hand regulatory protein which binds four Ca^{2+} ions, two with very high affinity and two with more moderate affinity. The crystal structures of the recombinant human CN (11) and of its complex with FKBP12-FK506 (11) and that of the complex with CsA-CyCA (12) of the proteolytic fragment of bovine CN have been determined at 2.1, 3.5, and 2.5 Å, respectively. However, CN residues 1–13 and residues comprising nearly the entire regulatory region (residues 374–468 and 487–521) are not visible in the electron density map of the reported structures (11). Another gap in current knowledge is that the structure of the Ca^{2+}-CaM-CN complex has not been solved.

3 The abbreviations used are: CN, calcineurin; CNA, calcineurin A subunit; CNB, calcineurin B subunit; CN380, calcineurin A subunit from residue 1 to residue 380 and B subunit; CNRR^{381–521}, calcineurin regulatory region from residues 381 to 521 of A subunit; pNPP, p-nitrophenyl phosphate; FT-IR, Fourier transform infrared; NFAT, nuclear factor of activated T cells; GdnHCl, guanidine hydrochloride; CaM, calmodulin.
Despite the lack of detailed structural information, the regulation mechanism of CN has been inferred from biochemical studies (9, 13–17). The enzymatic activity of CN is repressed in its inactive state but becomes fully activated when the regulatory region (residues 381–521) is removed by proteases (15). The regulatory region contains two subregions of known function, a CaM-binding peptide and autoinhibitory peptide (14, 15). The accepted model is that, upon activation by Ca2⁺, CaM binds to its target sequence in CNA and induces a conformational change in CN that leads to the displacement of autoinhibitory peptide from the active site (Fig. 1). More detailed examination has shown that the Ca²⁺ dependence of the phosphatase activity of CN is controlled by the two structurally similar but functionally different Ca²⁺-binding proteins, CaM and CNB subunit. In the presence of less than 10⁻⁷ M Ca²⁺, CNB, with its high affinity Ca²⁺-binding sites occupied, remains bound to CNA subunit, but the enzyme is inactive. Occupancy of the low affinity sites of CNB (Kᵦ in the low micromolar range) at elevated intracellular calcium concentrations causes a small increase in CN catalytic activity and a conformational change, detectable by limited proteolysis, which facilitates the subsequent binding of CaM (5, 16, 17).

There are many questions that need to be addressed regarding the mechanism of CN activation. The regulatory region extending from residues 381 to the C terminus of CNA is not visible in the electron density map, except for a small helical fragment at the active site. What structural features of the regulatory region render it a suitable mediator between Ca²⁺/CaM and CN enzymatic activity? What conformational changes are induced by Ca²⁺/CaM binding? The fact that the CNB-binding site (residues 341–372) and the CaM-binding site (residues 391–414) are distant from the autoinhibitory peptide (residues 467–486) in the linear sequence of CNA also raises a mechanistic question. Given the fact that Ca²⁺ binding to CNB activates both a detectable increase in enzyme activity and an increase in accessibility of the CaM binding segment of the CNA regulatory region, do CNB and CaM work at separate steps of CN activation, or do the two proteins work in concert to favor a single activated conformation of the enzyme? Here, we begin to address these questions using a combination of FT-IR spectroscopy, tryptophan fluorescence quenching, and functional studies of engineered CN fragments.

**EXPERIMENTAL PROCEDURES**

**Plasmid Construction, Protein Expression, and Purification**—pETCNα, a plasmid encoding full-length CN (CNA and CNB), was a gift from Dr. Anjana Rao (Harvard Medical School). CN380 and CNA341 were cloned and amplified by PCR using pETCNα vector as a template. CaM gene was purchased from ATCC (Manassas, VA) and cloned into pET-15b vector between the NcoI and XhoI sites. AI segment was PCR-amplified using pETCNα as a template and cloned into pGEX-6P-1 vector after the glutathione S-transferase coding sequence between the BamHI and XhoI sites. The plasmids were transformed into BL21 (DE3) cells and grown in LB medium with 100 μg/ml of ampicillin at 37 °C. When A₆₀₀ reached 0.6, protein expression was induced by the addition of 0.5 mM isopropylthiogalactopyranoside. The full-length CN, CN380, CNA341, and CNRR₃₈₁–₅₂₁ glutathione S-transferase were grown at 20 °C overnight after induction, and CaM was grown at 37 °C for 5 h. Cells were harvested by centrifugation.

As for the purification of CN, CN380, and CN341, cells were lysed by sonication in lysis buffer (50 mM Tris-HCl, pH 8.0, 100 mM NaCl, 1 mM EDTA, and 1 mM phenylmethylsulfonyl fluoride). After centrifugation, the supernatant was collected and passed through a nickel-nitriiotriacetic acid column (Qiagen, Germany) (18), then washed with 20 times column volume of the lysis buffer without phenylmethylsulfonyl fluoride. The proteins were eluted with 400 mM imidazole and further purified with gel filtration column Sepharose S-200 (GE Healthcare). The purity of the proteins was greater than 95% based on the results of SDS-PAGE.

For CaM, cells were lysed by sonication in lysis buffer (50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 1 mM dithiothreitol, and 1 mM phenylmethylsulfonyl fluoride). After centrifugation, the supernatant was collected and passed through a nickel-nitriiotriacetic acid column (Qiagen, Germany) (18), then washed with 20 times column volume of the lysis buffer without phenylmethylsulfonyl fluoride. The proteins were eluted with 400 mM imidazole and further purified with gel filtration column Sepharose S-200 (GE Healthcare). The purity of the proteins was greater than 95% based on the results of SDS-PAGE.

For glutathione S-transferase-tagged CNRR₃₈₁–₅₂₁ segment, the cell lysate was incubated with glutathione-Sepharose resin at 4 °C overnight. The resin was extensively washed with lysis buffer followed by 10 mM reduced glutathione in the same buffer. CNRR₃₈₁–₅₂₁ segment was cleaved from the fusion protein and purified using PreScission protease (GE Healthcare) as described in the protocol provided by the vendor. All purified proteins were stored at −80 °C. Protein concentrations were determined by Bradford Assay (Bio-Rad).
FT-IR Spectroscopy and Secondary Structure of AI Segment—

FT-IR spectra were measured with a Bomem MB series Fourier transform infrared spectrometer (ABB Bomem, Quebec, Canada) equipped with a deuterated triglycine sulfate detector and purged constantly with dry air. Frozen protein samples (~6 mg/ml) were thawed at room temperature and loaded in an infrared liquid cell with CaF$_2$ windows and a 7.5-μm spacer. For each spectrum, a 256-scan interferogram was collected in a single-beam mode with a 4-cm$^{-1}$ resolution. Reference spectrum was recorded under identical conditions with only the corresponding buffer in the cell. Protein spectra were subtracted using a previously established protocol and criteria (19, 20).

The second derivative spectrum was obtained with a 7-point Savitsky-Golay derivative function. Quantitative analysis was carried out by the curve-fittings of the base-line-corrected, invert second derivative spectrum as described previously (19). It was assumed that the fraction of residues composing each secondary structural element is proportional to the relative percent area of the associated vibrational band (20).

In Vitro Phosphatase Assay—The CN enzyme activity was measured by using pNPP (Sigma) and RII phosphopeptide (Biomol) as substrates (18). The reaction buffer contained 20 mM HEPES, pH 7.4, 2 mM MgCl$_2$, 100 mM NaCl, 1 mg/ml bovine gamma-globulin, and 1 mM dithiothreitol. For assay with pNPP, 20 mM pNPP stock solution was prepared by dissolving pNPP in the assay buffer, 90 μl of which was mixed with 10 μl of CN (reaction buffer in the presence of Ni$^{2+}$) to give the desired final enzyme concentration (2 μM) and Ni$^{2+}$ concentration (2 mM). The reaction mixture was incubated at 30 °C for 20 min. The release of p-nitrophenolate anion was measured at 405 nm by a SpectraMax 340 PC microplate reader (Molecular Devices, Sunnyvale, CA). For assay with RII phosphopeptide, 10 μl of RII phosphopeptide substrates were mixed with 40 μl of CN to give the desired final enzyme concentration (20 mM). The reaction mixture was incubated at 30 °C for 30 min, and Malachite green (100 μl, Calbiochem) was added at the end of incubation. The color was allowed to develop for 30 min, and absorption at 620 nm was monitored.

Fluorescence Acrylamide Quenching—Fluorescence intensity was measured by a Cary Eclipse fluorescence spectrophotometer (Varian, Palo Alto, CA) using a 1-cm quartz cuvette at 25 °C. The excitation and emission wavelengths were 295 and 380 nm, respectively. Protein concentration was ~5 μM. The reaction mixtures were titrated with 4.0 M acrylamide in the reaction buffer (50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 1 mM dithiothreitol, and 1 mM phenylmethylsulfonyl fluoride) at room temperature. Four cuvettes (designated as A, B, C, and D) containing different solutions were used in the measurement. Cuvettes A, B, and C contained 5 μM protein in buffer, and cuvette D contained buffer only. During the experiment no buffer or acrylamide was titrated into cuvette A, and the readings were used for validating instrument stability. Cuvettes B and C were titrated with the same volume of buffer and acrylamide, respectively. Aliquots of acrylamide were titrated into cuvette D. As a consequence of all the effects on the observed fluorescence intensity, the actual fluorescence intensity fraction can be described as $F = (F_C/F_B) - F_D$, where $F_B$, $F_C$, and $F_D$ are the observed signal fractions of protein fluorescence in cuvettes B, C, and D. To increase the precision, slit widths (both $\varepsilon_s$ and $\varepsilon_m$) were adjusted to make the initial values of $F_B$ and $F_C$ close to 950. For every point the fluorescence intensity was read at least 10 times to ensure that the result was not located at an extreme deviation region. Quenching data were plotted using the Stern-Volmer equation (21), $F_0/F = (1 + K_{SV} (Q))(1 + V(Q))$, where $F_0/F$ is the fractional decrease in fluorescence due to the quencher ($Q$), and $K_{SV}$ and $V$ are the collisional and static quenching constants, respectively.

RESULTS

FT-IR Spectra and Secondary Structure of the CN Regulatory Region—Fig. 2 shows the primary FT-IR spectrum of the isolated CN regulatory region, CNRR$_{381-521}$ segment, in solution. The second derivative analysis provides a mathematical solution to the overlapping components under the amide I band contour (1700–1600 cm$^{-1}$) and reveals basic band components assignable to various secondary structural elements (Fig. 3). The curve-fitting of the inverted secondary derivative spectrum provides additional quantitative information about the secondary structure of CNRR$_{381-521}$ segment, which consists of 10% α-helices, 34% β-strands, 20% β-turns, and 36% random coils (Table 1).

The spectral evidence does not imply, however, that the isolated regulatory region has a single static conformation, but it does suggest that the region possesses some intrinsic structure
even in the absence of the CN catalytic domain. The finding motivated us to explore whether or not the isolated regulatory region can undergo conformation changes in a functionally relevant fashion.

Calmodulin and the Function of Isolated CN Regulatory Region—To understand how the CN regulatory region responds to CaM and CN active site, we prepared three recombinant forms of CN; they are the wild-type heterodimer of CNA and CNB, a recombinant fragment of CNA truncated at residue 380, which contains the catalytic domain and the CNB binding helix expressed in complex with CNB, and a recombinant fragment of CNA truncated at residue 341, which contains only of the catalytic domain. The three proteins were designated as the full-length CN, CN380, and CN341, respectively. The full-length CN and the truncated forms CN380 and CN341 were all catalytically active in pNPP and RII phosphopeptide assay (data not shown). The phosphatase activity of the full-length CN was stimulated, as expected, by CaM binding in the presence of Ca2+/CaM (Fig. 4).

Interestingly, the disjoint segments, CN380, and the regulatory region CNRR381–521 recapitulated the CaM-dependent regulation of the full-length enzyme. CNRR381–521 was able to inhibit the CN phosphatase activity of CN380 (Fig. 5A; due to the experimental error, RII phosphopeptide inhibition data were not shown here), which is consistent with the findings of other researchers and with the presence of the autoinhibitory peptide within the regulatory region (15). The inhibition could be reversed by the addition of Ca2+/CaM (Fig. 5B).

It has been reported in previous work (17) that the CN regulatory region folds against CNB and the CNB binding helix when CN is inactive at low Ca2+/CaM concentrations. Thus, a plausible interpretation of the results is that CNB (in the absence of Ca2+) and the CNB binding helix may serve as a template to stabilize the inhibitory conformation of the regulatory region, the activated CNB (in the presence of Ca2+), and the CNB binding helix may stabilize the activated conformation or both. To test these possibilities, we repeated the experiment with CN341, which lacks CNB and the CNB binding helix. The phosphatase activity of CN341 was still inhibited by CN regulatory region CNRR381–521 (Fig. 6A, RII phosphopeptide inhibition data were not shown), and the inhibition was reversed by Ca2+/CaM (Fig. 6B). These findings rule out a direct requirement for CNB and the CNB binding helix in the conformational change
that relieves autoinhibition and suggest that the primary locus of a CaM-dependent reorganization is the CN regulatory region, CNRR381–521.

Solvent Accessibility of Tryptophan Residues—Tryptophan residues, with their intrinsic fluorescence, can report on protein conformational changes and protein-protein interactions. There are 4 tryptophan residues in CNA catalytic domain; Trp-134 is buried in the interior of the catalytic domain, Trp-232 is located at the catalytic site, and Trp-342 and Trp-352 are located in the hinge region that links the catalytic domain and CNB binding helix. There is no Trp residue in the CNA regulatory region, in CNB, or in CaM (Fig. 1).

To probe conformational changes in CNA and interactions of CNA with separate regulatory domain fragment, the accessibility of tryptophan residues was quantitatively assessed using fluorescence collisional quenching by acrylamide (Figs. 7–9). A control comparison between full-length native CN and CN in a partially unfolded state (in 1.0 M GdnHCl, and 1 mM EDTA) showed that tryptophan residues are substantially shielded from collisional quenching in the native protein. Tryptophans in the full-length protein are more accessible in the presence of 1 mM Ca<sup>2+</sup>, 0.5 mM CaM than in the presence of 1 mM EDTA, as indicated by a steeper slope in Stern-Volmer plots in Fig. 7. This result is consistent with expected exposure of the catalytic cleft tryptophan Trp-232 upon displacement of the autoinhibitory peptide, although we have not directly demonstrated that the tryptophan residue being exposed to increased quenching is Trp-232.

The experiments with CN380 yielded parallel results. In the presence of EDTA, CNRR<sup>381–521</sup> caused a decrease in tryptophan accessibility, and the decrease was reversed by the addition of 1 mM Ca<sup>2+</sup>, 0.5 mM CaM (Fig. 8). In fact, tryptophan accessibility in the presence of CNRR<sup>381–521</sup> and Ca<sup>2+</sup>/CaM exceeded that in the EDTA control conditions, possibly due to an effect of Ca<sup>2+</sup>/CaM on Trp-342 and Trp-352 in the CNA hinge region or to the effect of Ca<sup>2+</sup> binding to CNB. Rather than disect the fluorescence signals by replacing individual tryptophan residues, which could compromise the function of CN, we simplified the experimental situation by further truncation of CNA.
Use of a CN fragment consisting of the catalytic domain only, CN341, permitted examination of the accessibility of Trp-232 because this CN fragment contains no other tryptophan except the buried Trp-134, which is unlikely to participate in collisional quenching. The fragment also lacks CNB and the CNB binding helix, the other likely source of Ca\(^{2+}\)-induced conformational change. Fig. 9 shows that inclusion of CNRR381–521 in the assay in the presence of 1 mM EDTA decreases accessibility of Trp-232. Ca\(^{2+}\)/CaM reverses this change in accessibility, albeit partially. We conclude that CNRR381–521 interacts with CN341 and occludes the active site of CN341 and that the addition of CaM results in displacement of CNRR381–521 from the active site.

**DISCUSSION**

The CN regulatory region is the key to precise control of CN activity in cells, where enzyme activity responds to changes in Ca\(^{2+}\) concentration in a narrow window of physiological Ca\(^{2+}\) signaling. The wide separation in a linear sequence of the CaM binding (residues 391–414) and autoinhibitory (residues 467–486) sites of CN indicates that Ca\(^{2+}\)/CaM cannot activate CN merely by binding the autoinhibitory peptide and competitively displacing it from the catalytic site. It suggests that an extensive conformational change in the regulatory region accompanies the activation. The documented dual control of CN activity by Ca\(^{2+}\) binding to CNB and to CaM (16) further raised the possibility that this is a concerted transition involving both CNB and CaM. In current study we produced separate CN fragments corresponding to the CNA regulatory region (CNRR381–521), the CNA catalytic domain truncated at residue 341, and the CNA-CNβ heterodimer with CNA truncated at residue 380 immediately after the CNB binding helix and attempted to identify the secondary structure and regulation mechanism of CN regulatory region. The full-length CN and the truncated forms CN381 and CN341 were all catalytically active in the pNPP and RII phosphopeptide assay. The phosphatase activity of full-length CN was stimulated as expected by CaM binding in the presence of Ca\(^{2+}\). Interestingly, the CN380 and regulatory region CNRR381–521 recapitulated the CaM-dependent regulation of the full-length enzyme. CNRR381–521 was able to inhibit CN phosphatase activity of CN380 (Fig. 5A), which is consistent with the findings of other groups and with the presence of the autoinhibitory peptide within the regulatory region (15). The inhibition can be reversed by addition of CaM, and the reversal was complete with only a modest excess of CaM.

FT-IR spectroscopic analysis of recombinant CNRR381–521 reveals that the secondary structure of CNRR381–521 consists of ~36% random coil, 10% α-helix, 34% β-sheets, and 20% β-turns. Although the accuracy of the estimated values may suffer somewhat due to the extensive overlap of various secondary structural components, however, there are defined signals of α-helix and β-sheet structures. We considered that the CNRR381–521 segment contains not only a random coil but also other secondary structural components. It is worthy of noting that the evidence does not imply that the isolated regulatory region has a single static conformation, but it does suggest that the region possesses some intrinsic structure even in the absence of the CN catalytic domain.

Tryptophan residues can report on protein conformational changes and protein-protein interactions. The results of full-length CN tryptophan fluorescence quenching show that in the presence of CaM, tryptophan residues are more open to solvent, indicating that CaM binding causes CNRR381–521 to leave CN active site and exposes Trp-232 to solvent. Comparing the fluorescence quenching results of full-length CN with CN380 (Fig. 10), one can see that in the native state tryptophan residues in full-length CN are more open than that of CN380, which is unexpected. One may conclude that the hinge region of CN380 is more closed without the CNRR381–521 segment than that of the full-length CN, so that Trp-342 and Trp-352 become less exposed to solvent. Contrarily, in the full-length CN, Trp-342 and Trp-352 are more open to solvent with regulatory region binding to CNB. The results of CN, CN380, and CN341 trypto-
phan fluorescence quenching indicate that the isolated regulatory region inhibits CN activity by occluding the catalytic site, and the CaM binding exposes the catalytic site. These observations are consistent with the results reported by other biological and biochemical experiments (15).

The principal finding of this work is that Ca\(^{2+}/\text{CaM}\) by itself imposes reorganization on the isolated regulatory region, which is sufficient to relieve autoinhibition. In particular, this effect is seen in experiments with the isolated catalytic domain (residues 1–341) and the isolated regulatory region (residues 381–521). Thus, the participation of CNB and CNB binding helix is not essential for this activation step, although it is possible that their presence in intact CN provides additional stabilization to the resting and activated conformations of the regulatory region. We interpret these observations as direct experimental support for the model (17) that Ca\(^{2+}/\text{CaM}\) binding to the low affinity sites of CNB first releases the CaM-binding site and that this constitutes the primary regulatory role of CNB. CaM then separately initiates and stabilizes the activating conformational change of the regulatory region.

In summary, the secondary structure composition of CNRR\(^{381–521}\) fragment is predominantly random coils (~36%) but with a significant amount of \(\alpha\)-sheet and \(\alpha\beta\)-helix structures. It serves not only as a regulatory element but also impacts the structure of calcineurin. When CN was truncated at the residue 380, the two separated fragments, CN380 and regulatory region CNRR\(^{381–521}\), recapitulated the CaM-dependent regulation of the full-length enzyme. The regulatory region CNRR\(^{381–521}\) was able to inhibit the phosphatase activity of CN380 and was reversed by Ca\(^{2+}/\text{CaM}\) binding. Tryptophan fluorescence quenching measurements further indicate that the isolated regulatory region inhibits CN activity by occluding the catalytic site and that CaM binding exposes the catalytic site. The results provide new support for a model in which calcium binding to CNB enables CaM binding to the CNA regulatory region, and CaM binding then instructs an activating conformational change of the regulatory region that does not depend further on CNB.

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