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Calcineurin in a Crowded World

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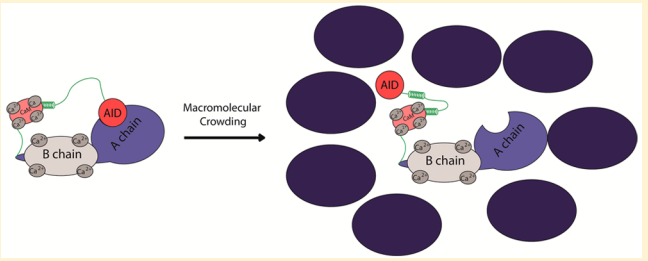
ABSTRACT: Calcineurin is a Ser/Thr phosphatase that is important for key biological processes, including immune system activation. We previously identified a region in the intrinsically disordered regulatory domain of calcineurin that forms a critical amphipathic α-helix (the "distal helix") that is required for complete activation of calcineurin. This distal helix was shown to have a T_m close to that of human body temperature. Because the T_m was determined in dilute buffer, we hypothesized that other factors inherent to a cellular environment might modulate the stability of the distal helix. One such factor that contributes to stability in other proteins is macromolecular crowding. The cell cytoplasm is comprised of up to 400 g/L protein, lipids, nucleic acids, and other compounds. We hypothesize that the presence of such crowders could increase the thermal stability of the distal helix and thus lead to a more robust activation of calcineurin *in vivo*. Using biophysical and biochemical approaches, we show that the distal helix of calcineurin is indeed stabilized when crowded by the synthetic polymers dextran 70 and ficoll 70, and that this stabilization of the distal helix increases the activity of calcineurin.



Calcineurin (CaN) is a calmodulin (CaM)-activated protein Ser/Thr phosphatase that was discovered in the laboratories of Wang and Desai,[1] and Klee and Krinks.[2] Initially thought to be expressed only in neurons (hence the name), CaN is now known to be expressed ubiquitously. CaN is a key regulator of processes such as activation of the immune system, neuronal plasticity, and heart growth.[3−5] CaN mediates immune system activation through its action on members of the transcription factor family nuclear factor of activated T-cells (NFAT), for example, NFAT1 and NFAT2. Once dephosphorylated, these transcription factors translocate to the nucleus where they upregulate the expression of cytokines IL-2, IL-6, and IFN-γ, which are important for further immune system stimulation.[6−9] As a result of infection and initiation of the CaN/NFAT-dependent pathway release of these cytokines, patients can experience symptoms of inflammation, including fever that typically ranges from 38 to 42 °C.[10]

CaN is a heterodimeric protein composed of a catalytic A chain (57−61 kDa) and a regulatory B chain (19 kDa).[5,11] From the N-terminus to the C-terminus, the A chain of CaN contains the catalytic domain, the B chain-binding helix, and regulatory and autoinhibitory domains. The catalytic domain of the A chain contains all the molecular machinery necessary for CaN's catalytic function as a phosphatase.[11] Extending out from the catalytic domain is a helix to which the regulatory B chain binds. The regulatory domain (RD) is an ∼100-residue domain that contains the CaM-binding site.[12] The autoinhibitory domain (AID) of CaN occludes the catalytic site of the catalytic domain in CaN's inactive state. The B chain of CaN is structurally homologous to CaM and is capable of binding up to four Ca^{2+} cations.[2]

In the resting cell, the intracellular Ca^{2+} concentration ranges from 50 to 100 nM and is insufficient for CaN activation.[13] In the inactive state of CaN, the AID occludes the catalytic site, preventing binding of phosphorylated residues. It was suggested by Yang and Klee that the RD is folded onto the interface between the A and B chains, which would prevent CaM binding (Figure 1A).[14] These authors showed that the RD, which contains the CaM-binding site, in full-length CaN is protected from limited digestion by trypsin and chymotrypsin in the absence of Ca^{2+}. This was not the case for Ca^{2+}-bound CaN.

Upon the influx of Ca^{2+} (e.g., triggered by T-cell receptors binding to an antigen), CaN undergoes a multistep conformational change that results in full activation.[14−16] Ca^{2+} binds to the B chain, causing dissociation of the RD from the A chain−B chain interface (Figure 1B). Release of the RD results in the domain becoming disordered, exposing the CaM-binding site.[12,14,17] Concurrently, CaM can bind four Ca^{2+} cations and the newly accessible CaM-binding site. Upon CaM binding, the RD undergoes a conformational change that removes the AID from the catalytic site and results in full activation (Figure 1C).

It was first suggested by Manalan and Klee that the RD containing the CaM-binding site is intrinsically disordered in Ca^{2+}-bound CaN.[17] The RD and AID in full-length CaN in the presence of Ca^{2+} were readily digested in limited proteolysis experiments. Hydrogen−deuterium exchange with mass spectrometry (HD/MS) and circular dichroism (CD) analyses

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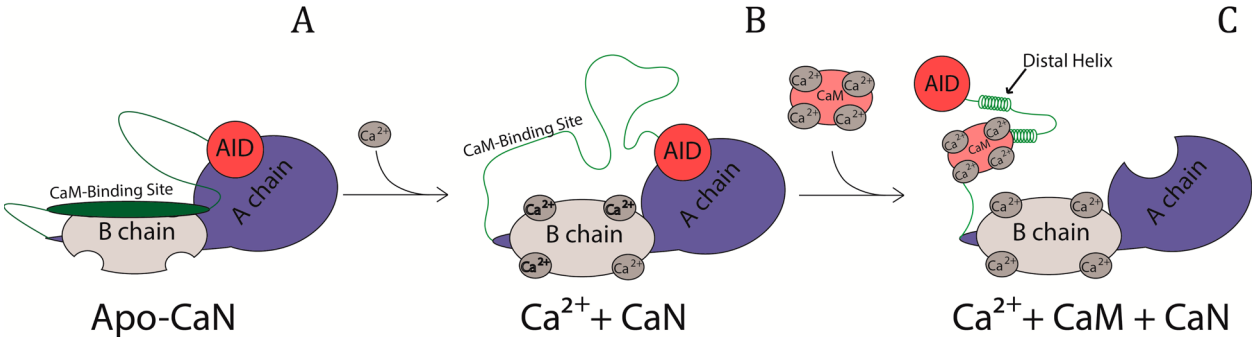

Figure 1. Activation of calcineurin by calmodulin. (A) The CaM-binding site is located on the regulatory domain (RD), which interacts with the A chain−B chain interface in the absence of Ca^{2+}. (B) The RD is released when the B chain of CaN binds Ca^{2+}. (C) When Ca^{2+}/CaM binds to the CaM-binding site of CaN, the distal helix folds onto CaM, and the AID dissociates from the active site, fully activating the phosphatase.

performed in our laboratory showed that a protein construct of the RD alone was intrinsically disordered.[12]

We also observed that CaM binding led the RD to fold.[12] It was known that CaM interacts with an ∼24-residue sequence located in the N-terminal half of the RD (residues 391−414), and that CaM binds to this region with a high affinity (K_D = 1−28 pM).[12,18−20] The HD/MS experiments detected a second RD region (residues 441−459), ∼27 residues downstream of the CaM-binding site, that also folds upon association with CaM.[12,21] CD data revealed this distal structured region to be helical. Thus, this was named the distal helix.[21]

The sequence of the distal helix region suggested that it was an amphipathic α-helix, and it is hypothesized that the hydrophobic face interacts with CaM.[21] To investigate the structure of the distal helix, the isolated RD was expressed and purified. CD measurements of the wild-type RD construct in the presence of CaM revealed that the distal helix had a T_m of 38 °C, close to human body temperature of 37 °C.[21] To further probe distal helix formation, glutamate point mutations (A447E, A451E, and A545E) were introduced into the putative hydrophobic face of the amphipathic α-helix. Glutamate was chosen to replace the native alanines because glutamate both introduces a negative charge and is significantly larger. We hypothesized that this would disrupt interactions with CaM and weaken distal helix formation. Because these mutations were outside of the CaM-binding region, CaM could still bind to the RD with high affinity. RD constructs that contained Ala-to-Glu mutations were found to have significantly weakened, if not abolished, folding of the distal helix.

The functional role of the distal helix was assessed by introducing the same disruptive Ala-to-Glu mutations into the distal helix in full-length CaN and determining the effects on enzymatic activity.[21] Two substrates are typically used to assess the enzymatic activity of CaN, *p*-nitrophenol phosphate (pNPP), a generic small-molecule phosphatase substrate, and RII, which is a phosphorylated peptide derived from an endogenous substrate of CaN, protein kinase-A regulatory subunit type II (PKA RII).[22] Analysis of CaN constructs containing Ala-to-Glu mutations revealed that distal helix formation is required for full and robust activity of CaN against both pNPP and RII substrates.[21] Michaelis−Menten analyses of the enzymatic assay data suggested that disruption of the distal helix allowed the AID to occupy the active site of the catalytic domain of CaN, thereby directly inhibiting pNPP and RII binding.[21]

Interestingly, the T_m of the distal helix is 38 °C, which is close to that of the average human body temperature (37 °C). On the basis of the thermal transition shown in our CD data, the distal helix is predicted to be partially folded at 37 °C.[21] It is curious that the distal helix would not be fully folded at human body temperature when it appears to be critical for robust CaM-dependent activation of CaN. It seems likely that some factor or factors within the cell modulate this, allowing for full activation of this essential phosphatase.

There are many differences in the way proteins are typically studied *in vitro* (i.e., dilute buffers and salts) and how they exist in the context of the cell. One of the most notable differences, on which we focus in this paper, involves macromolecular crowding. The term "macromolecular crowding" was coined by Minton and Wilf in 1981 when they observed that an increase in the concentration of inert proteins ("crowders") changed the conformational equilibrium of GAPDH compared to that observed in dilute buffer.[23] Since then, many laboratories have observed that the inclusion of crowding reagents that are thought to approximately mimic the cell interior can alter the stability and activity of proteins and enzymes.[24−32]

Crowding has the potential to stabilize the distal helix in CaN, thereby giving us a better estimate of its folded state and the activity of the phosphatase *in vivo*. In this study, we utilized the inert crowding reagents dextran 70 and ficoll 70 with CD to determine its effect on the distal helix of CaN. Ficoll 70 and dextran 70 were chosen because they do not contribute an interfering signal to CD unlike protein crowding agents such as bovine serum albumin (BSA) and lysozyme. Therefore, dextran 70 and ficoll 70 offer convenient and easily interpretable effects of macromolecular crowding on the CaN/CaM system. In addition, we measured the enzymatic function of CaN to show that crowding-induced stabilization of the distal helix confers greater activity of CaM-activated CaN.

MATERIALS AND METHODS

Plasmids, Peptides, Protein Expression, and Purification. The bicistronic pETTagHisCN plasmid containing the genes for calcineurin (CaN) subunits αCaN A and B1 was purchased from Addgene (Cambridge, MA) and transformed into *Escherichia coli* BL-21 (DE3) CodonPlus RIL cells (Agilent, Santa Clara, CA) for protein expression. Cells were grown in terrific broth (TB) until an OD_{600} of ∼1.2−1.6 was reached and were induced with 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG). Cell lysates were cleared by centrifugation, and CaN was purified first by Ni-NTA and then calmodulin (CaM)-sepharose chromatography (GE Healthcare, Piscataway, NJ) as described previously.[12,21]

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The C-terminal tail and autoinhibitory domains of CaN were deleted from αCaN A by polymerase chain reaction mutagenesis to yield the CaNΔAID-CT construct. The N-terminal polyhistidine tag and CaM-binding domain of CaNΔAID-CT were left unperturbed, and purification was the same as for wild-type CaN.

Plasmid pETCaM1, which contains the human CALM1 gene and encodes full-length wild-type CaM, was transformed and expressed in BL-21 (DE3) competent *E. coli* (Agilent). Cells were grown in TB until an OD_{600} of ∼1.2−1.6 was reached and were induced with 1 mM IPTG. Cell lysates were cleared by centrifugation, and CaM was purified using a 2-trifluoromethyl-10-aminopropyl phenothiazine-sepharose (TAPP-sepharose) column.[12,21] The TAPP-sepharose column was synthesized at the Center for Molecular Medicine Organic Synthesis Core Facility at the University of Kentucky.

For our RD construct, a gene encoding residues 373−468 of CaN (NCBI NP_000935) was synthesized by Genscript (Piscataway, NJ) and subcloned into a pET303 plasmid (Life Technologies, Grand Island, NY), which adds a C-terminal six-His tag. The resulting RD plasmid was cotransformed with pETCaMI into BL-21 (DE3) (Agilent) *E. coli* for protein expression. Cells were grown in TB until an OD_{600} of ∼1.2−1.6 was reached and were induced with 1 mM IPTG. RD in the cleared and filtered cell lysates was purified by Ni-NTA chromatography in which CaM was removed by washing with 5 M urea, 2 M thiourea, 20 mM Tris (pH 7.5), 200 mM NaCl, and 10 mM imidazole. Ni-NTA-bound RD was then washed with buffers containing 20 mM Tris (pH 7.5), 200 mM NaCl, 10 mM imidazole, and serially diluted urea and thiourea concentrations. The concentrations of each wash in order were 2.5 M urea and 1.0 M thiourea, 1.3 M urea and 0.50 M thiourea, 0.63 M urea and 0.25 M thiourea, 0.31 M urea and 0.13 thiourea, 0.16 M urea and 0.063 M thiourea, and 0.078 M urea and 0.031 M thiourea. The Ni-NTA-bound RD was finally washed with 20 mM Tris (pH 7.5), 200 mM NaCl, and 10 mM imidazole, then eluted with 20 mM Tris (pH 7.5), 200 mM NaCl, 2 mM $CaCl_2$, and 250 mM imidazole, and subjected to CaM-sepharose chromatography for final purification. Once the Ni-NTA elution was applied to the CaM-sepharose column, the column was washed with 20 mM Tris (pH 7.5), 200 mM NaCl, and 2 mM $CaCl_2$. The RD was finally eluted off the CaM-sepharose column with 20 mM Tris (pH 7.5), 200 mM NaCl, and 4 mM EGTA. The purity and concentration of the RD were determined by sodium dodecyl sulfate−polyacrylamide gel electrophoresis and absorbance at 280 nm, respectively. A tryptophan was added to the C-terminus of the RD construct with a glycine linker to increase the accuracy of protein concentration determination by UV−vis spectroscopy.

A peptide encompassing the CaM-binding domain (residues 391−414 from calcineurin A) was synthesized by Atlantic Peptides (Lewisburg, PA) and will be termed pCaN. The sequence of pCaN is WGARKEVIRNKIRAIGKMARVFSVLRGGC with an N-terminal tryptophan included for peptide concentration determination.

Circular Dichroism and Thermal Denaturation. A Jasco-810 spectropolarimeter equipped with a Peltier temperature controller was used for circular dichroism (CD) measurements. All samples were contained in a 1 mm quartz cuvette. Concentrations of all proteins and peptides were 20 μM. Spectra of the RD with or without CaM in the presence of 0, 50, 100, 150, and 200 g/L dextran 70 or ficoll 70 were recorded at 37 °C. Measurements were also taken of RD with CaM in the presence of 0, 5, 10, 20, and 40% 2,2,2-trifluoroethanol (TFE). CD spectra of pCaN with CaM were recorded in the presence and absence of 200 g/L dextran 70, as well as 0, 5, 10, 20, and 40% TFE. All samples were in a buffer consisting of 20 mM HEPES (pH 7.5), 200 mM NaCl, and 4 mM $CaCl_2$. Each spectrum is the average of four accumulations taken at a scanning speed of 20 nm/min with the sample temperature set to 37 °C. Raw data were converted to molar ellipticity.

For thermal denaturation, the ellipticity of RD with or without CaM in the presence and absence of 200 g/L dextran 70 was measured at 222 nm as the temperature was increased from 5 to 95 °C at a rate of 1 °C/min. The concentrations of RD and CaM were 20 μM, and they were in 20 mM HEPES (pH 7.5), 200 mM NaCl, and 2 mM $CaCl_2$. HEPES buffer was selected for the low temperature dependence of its pK_a.[33] The global maximum of the first derivative of the temperature-dependent melting transition was used to estimate the T_m of the distal helix.

CaN pNPP Activity and Kinetic Assays. Michaelis−Menten kinetics of CaN and CaNΔAID-CT using *p*-nitrophenyl phosphate (pNPP) (Sigma-Aldrich, St. Louis, MO) as a substrate were determined at various concentrations of dextran 70 (0−200 g/L) or dextrose (0−200 g/L) using a 96-well plate format with a 100 μL final volume per reaction. Final reaction solutions contained 40 mM Tris (pH 8), 100 mM KCl, 1 mM $CaCl_2$, 30 nM CaN, 90 nM CaM, 5 mM $MnCl_2$, 1 mM DTT, and 0−100 mM pNPP. The CaN enzyme solution and pNPP standards were preincubated at 37 °C before they were mixed. When the components were mixed, dephosphorylation of pNPP was monitored by measuring absorption at 410 nm for 2 h at 37 °C. The linear range of pNPP hydrolysis by CaN was observed to be between 0 and ∼35 min, so initial velocities were determined from the slope of the curve from 0 to 30 min. Initial velocities versus pNPP concentration were plotted in SigmaPlot, and Michaelis−Menten kinetics were determined using the SigmaPlot Enzyme Kinetics module and the assumed Michaelis−Menten equation:

$$V_i = \frac{V_{max}[S]}{K_m + [S]} \quad (1)$$

where V_{max} is the asymptotic velocity at which all binding sites are saturated with substrate, V_i is the initial velocity at a given substrate concentration [S], and K_m is the concentration of substrate that results in 50% V_{max}. Final Michaelis−Menten parameters of each condition (K_m and V_{max}) are representative of six replicates and two protein preparations of CaN.

RESULTS

Inert Polymeric Crowding Agents Increase the Thermal Stability of the RD:CaM Complex. Previously, our group has shown that an α-helix forms in the C-terminal region of the RD construct, distal to the CaM-binding site, and that this helix formation is important for CaM-mediated activation of CaN.[21] Our data demonstrated that this "distal helix" is marginally stable at human body temperature, and destabilization of the distal helix leads to decreased CaN activity. To determine if crowding could stabilize the distal helix, far-UV circular dichroism (CD) was performed on the RD construct in the presence of CaM. Dextran 70 and ficoll 70 are inert polymers commonly used as crowding agents and were chosen because they do not contribute a significant signal

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to CD spectra at wavelengths typically used to characterize protein structure. RD/CaM samples were incubated with varying concentrations of dextran 70 or ficoll 70 at 37 °C, and CD spectra were recorded (Figure 2A,B). CD spectra for the

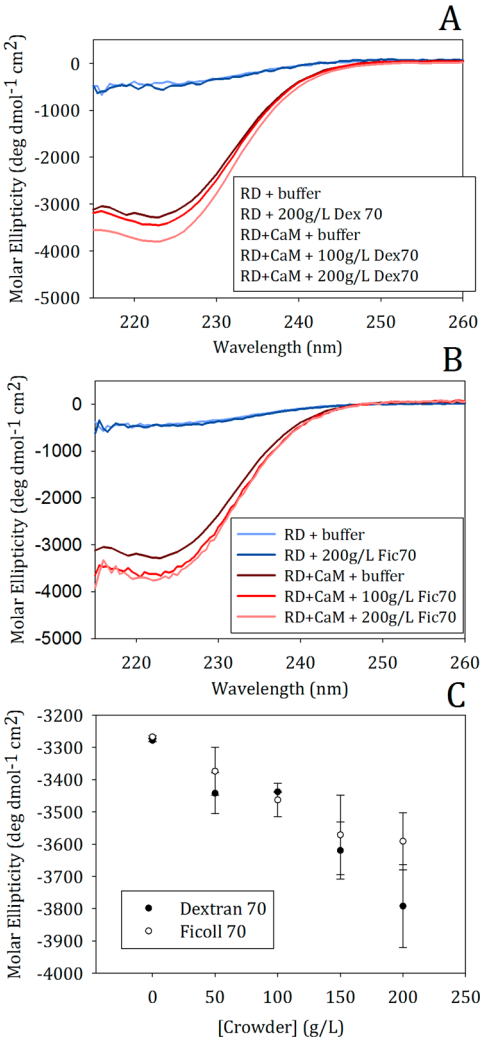

Figure 2. Structural effects of dextran 70 and ficoll 70 on the RD:CaM complex using far-UV CD. RD construct in the presence and absence of CaM at various concentrations of (A) dextran 70 and (B) ficoll 70 (0–200 g/L). (C) Molar ellipticity at 222 nm of RD with CaM as a function of dextran 70 or ficoll 70 concentration showing similar degrees of crowding agent-induced structural content increase.

proteins used in this work were measured down to only 215 nm due to absorption from HEPES buffer. As the concentration of dextran 70 and ficoll 70 was increased, the ellipticity at 222 nm decreased in a nearly linear manner, suggesting an increase in the secondary structural content of the RD:CaM complex (Figure 2C). The addition of either dextran 70 or ficoll 70 to a solution of the RD:CaM complex gave the same increase in ellipticity within error (Figure 2C).

Previously, the RD fragment was characterized and found to be intrinsically disordered.[21] CD measurements of the RD fragment displayed a minimum at approximately 200 nm, which is often interpreted as being indicative of disorder. This conclusion was supported by hydrogen–deuterium exchange/MS (HD/MS) experiments. In contrast to the RD:CaM complex, dextran 70 and ficoll 70 had no effect on the ellipticity of the RD construct in the absence of CaM (Figure 2A,B), which suggests a CaM-dependent structural change. Dextrose and sucrose are the monomeric subunits of dextran 70 and ficoll 70, respectively; 200 g/L dextrose or sucrose did not appear to affect the overall secondary structure of the RD:CaM complex (Figure 3A). Thus, the inert polymers dextran 70 and ficoll 70, but not the lower-molecular weight compounds, are able to increase the helical content of CaM-bound RD.

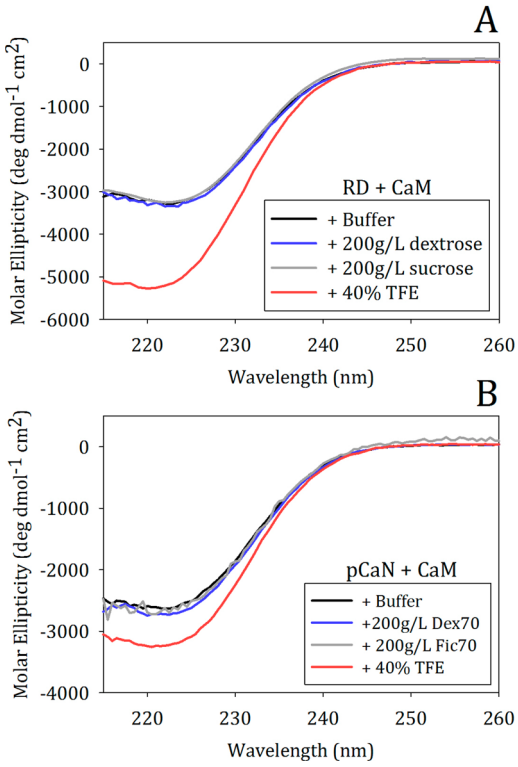

Figure 3. Far-UV CD spectra with small molecular osmolytes. (A) RD construct in the presence of CaM and buffer alone, 200 g/L dextrose, 200 g/L sucrose, and 40% TFE. (B) pCaN:CaM complex in 200 g/L dextran 70, 200 g/L sucrose, and 40% TFE compared to that in buffer alone.

To determine if any of the observed increase in molar ellipticity from crowding is due structural changes apart from the distal helix, we had a 29-residue peptide synthesized corresponding to the CaM-binding site of CaN (peptide termed pCaN). This CaM-binding site is necessary and sufficient for high-affinity CaM binding in the range of 1–28 pM.[18,19] Dextran 70 and ficoll 70 did not appear to have any effect on the ellipticity measured for CaM bound to pCaN (Figure 3B). Because the distal helix is known to be marginally stable[21] and is contained in the RD, but not the pCaN peptide,[12] we surmise that the dextran 70- and ficoll 70-dependent change in ellipticity of the RD:CaM complex is due to stabilization of the distal helix. Further, the distal helical sequence was the only region found to participate in backbone–backbone hydrogen bonding via HD/MS.[12]

The data illustrated in Figure 2C indicate that the ellipticity of the RD:CaM complex exhibits an almost linear dependence on the dextran 70 concentration from 0 to 200 g/L. This would suggest that a higher helical content is possible at higher dextran 70 or ficoll 70 concentrations. Thus, TFE was used to investigate if an even higher helical content could be achieved.

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While the mechanism of action is not fully understood, TFE is capable of stabilizing and inducing α-helical structure in some proteins. TFE was able to decrease the ellipticity of the RD:CaM complex at 222 nm by 58% (Figure 3A), suggesting a large increase in α-helical content is possible. In comparison, 40% TFE was able to induce an only 22% decrease in the ellipticity at 222 nm of the CaM:pCaN complex (Figure 3B). Thus, the remaining ellipticity change is due to TFE's influence on the RD outside the CaM-binding site, most likely the distal helix given that much of the remainder of the RD is enriched with glycine and proline residues.[12]

To determine if the addition of crowding reagents altered the thermal stability of the distal helix, we used CD and measured the ellipticity at 222 nm as a function of temperature. The first derivative was used to determine the T_m of the distal helix in each instance (Figure 4A). All reactions showed reversible folding as the unfolding–folding transition of the distal helix was the same when scanning from 5 to 95 °C or from 95 to 5 °C. The T_m of the distal helix in the RD:CaM complex was determined to be ~38 and ~43 °C in dilute buffer and 200 g/L dextran 70 or ficoll 70, respectively (Figure 4A). The T_m of the distal helix in dilute buffer is in good agreement with the results of Dunlap et al.[21] The RD in the absence of CaM showed no apparent thermal transition with an increase in temperature even when 200 g/L dextran 70 or ficoll 70 was added (Figure 4B). The pCaN:CaM complex showed no apparent thermal transition in dilute buffer from 5 to 95 °C but did show a thermal unfolding transition starting around 80 °C in 8 M urea (Figure 4C). The fact that the pCaN:CaM complex has such a high melting temperature even in the presence of 8 M urea is a result of the high affinity of CaM for the CaM-binding site. Thus, the thermal transition in Figure 4A is entirely due to structural elements outside the CaM-binding site.

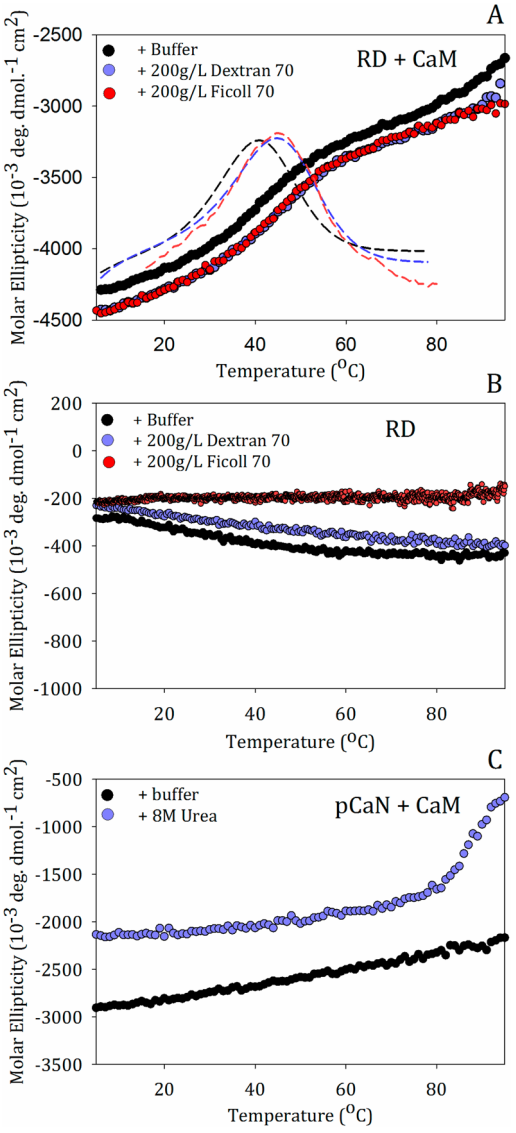

Figure 4. CD temperature scans at 222 nm. (A) RD:CaM complex in the presence and absence of either 200 g/L dextran 70 or 200 g/L ficoll 70 with first derivatives shown as dashed lines of the same color. (B) RD construct alone in the presence and absence of 200 g/L dextran 70 or 200 g/L ficoll 70. (C) pCaN:CaM complex in the presence and absence of 8 M urea.

Crowding Increases the Activity of CaN. Previous research has shown that alterations in the stability of the distal helix can affect the enzymatic activity of CaN.[21] Enzymatic assays of CaN with pNPP as a substrate indicated that destabilization of the distal helix increased the K_m (lower affinity). Experimental results shown in Figure 4A show that crowding with dextran 70 or ficoll 70 can increase the T_m of the distal helix. Therefore, we predict that as the distal helix is stabilized through crowding, the K_m of CaN should decrease, a result of the AID being prevented from binding to the active site. Thus, we determined the enzymatic constants K_m and V_{max} for wild-type CaN in 0, 50, 100, 150, and 200 g/L dextran 70 and ficoll 70 at 37 °C. A generic substrate for phosphatases, pNPP, was used to assess CaN's enzymatic activity. The determined V_{max} and K_m values for the activity of CaN at dextran 70 and ficoll 70 concentrations ranging from 0 to 200 g/L are listed in Table 1. While increasing the dextran 70 and

Table 1. K_m and V_{max} Values for Wild-Type CaN at 37 °C with pNPP as a Substrate

	K_m (mM)	V_{max} (μmol min^{-1} mg^{-1})
dilute buffer	56 ± 8	1.4 ± 0.1
dextran 70		
10 g/L	36 ± 8	1.2 ± 0.11
25 g/L	26 ± 4	1.0 ± 0.10
50 g/L	22 ± 2	1.6 ± 0.05
100 g/L	21 ± 2	1.7 ± 0.05
150 g/L	23 ± 5	1.2 ± 0.09
200 g/L	17 ± 3	1.1 ± 0.07
dextrose		
50 g/L	59 ± 12	1.1 ± 0.11
100 g/L	56 ± 5	0.95 ± 0.04
150 g/L	70 ± 8	0.98 ± 0.06
200 g/L	58 ± 18	1.6 ± 0.2
ficoll 70		
50 g/L	38 ± 5	0.92 ± 0.10
100 g/L	10 ± 2	0.83 ± 0.10
150 g/L	19 ± 7	0.99 ± 0.12
200 g/L	9.2 ± 6	1.4 ± 0.3
sucrose		
50 g/L	69 ± 8	2.1 ± 0.1
100 g/L	59 ± 4	1.5 ± 0.1
150 g/L	76 ± 11	1.5 ± 0.1
200 g/L	63 ± 5	1.3 ± 0.1

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ficoll 70 concentration did not appear to have an effect on V_{max}, the K_m is reduced ~70 and ~91% as the concentration of dextran 70 and ficoll 70, respectively, is increased from 0 to 200 g/L. In contrast, dextrose and sucrose did not induce a significant change in either the K_m or V_{max} at any measured concentrations (Figure 5B and Table 1). Thus, these enzymatic data are consistent with a stabilization of the distal helix with the inert polymers, but not the small molecules dextrose and sucrose.

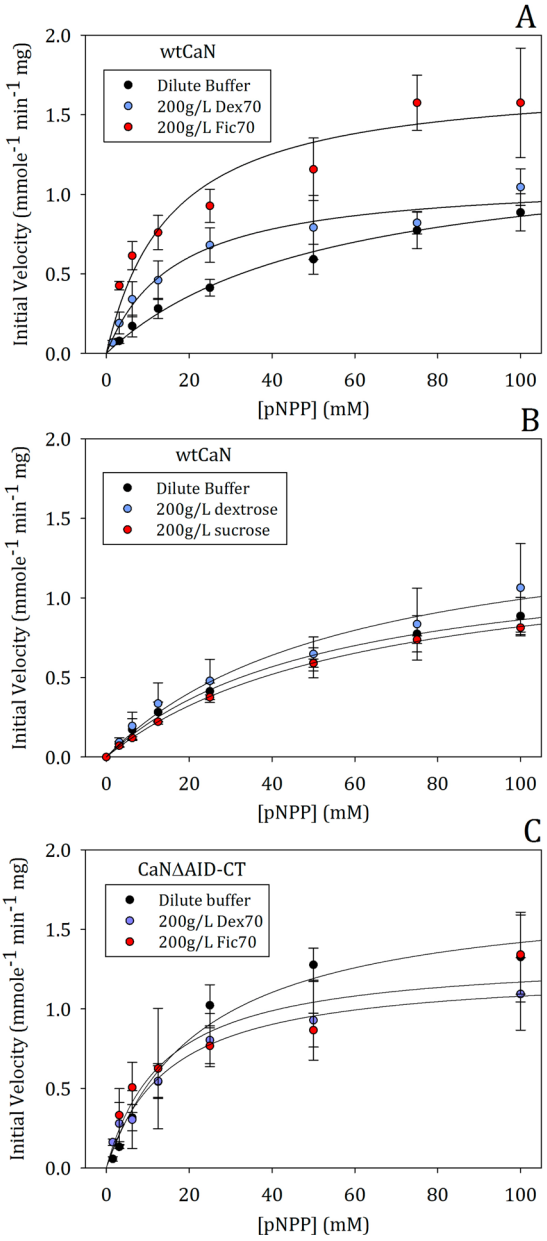

Figure 5. Enzyme kinetic data showing the rate of hydrolysis of pNPP as a function of pNPP concentration at 37 °C. (A) Dextran 70 and ficoll 70 increase the activity of CaN. (B) The small molecules dextrose and sucrose do not alter kinetics. (C) Truncation of CaN and removal of the AID and CT domains and its activity with ficoll 70 or dextran 70 compared to dilute buffer.

CaNΔAIDCT Activity Does Not Change under Crowded Conditions. Because macromolecular crowding can affect global protein conformation and stability, we sought to determine if the increase in CaN activity was due to factors other than stabilization of the distal helix.[32] To accomplish this, we truncated CaN at the first residue of the AID (residue I467). Via truncation of CaN at the AID, CaN is constitutively active, although CaM can still bind and the distal helix can still form. The K_m and V_{max} of CaNΔAIDCT were determined as a function of dextran 70 and ficoll 70 concentration. The K_m of CaNΔAIDCT does not appear to vary significantly from 0 to 200 g/L dextran 70 or ficoll 70 (Figure 5C and Table 2).

Table 2. K_m and V_{max} Values for CaNΔAID-CT at 37 °C with pNPP as a Substrate

	K_m (mM)	V_{max} (μmol min^{-1} mg^{-1})
dilute buffer	23 ± 4	1.7 ± 0.1
dextran 70		
50 g/L	29 ± 7	1.7 ± 0.2
100 g/L	15 ± 3	0.76 ± 0.06
150 g/L	18 ± 2	1.2 ± 0.1
200 g/L	15 ± 3	1.3 ± 0.1
ficoll 70		
50 g/L	16 ± 7	1.7 ± 0.5
100 g/L	9 ± 1	1.03 ± 0.1
150 g/L	16 ± 4	1.3 ± 0.2
200 g/L	12 ± 7	1.2 ± 0.3

Comparing the V_{max} data of wild-type CaN and CaNΔAIDCT for each specific crowding reagent yields no specific trend in the data as the concentration of the crowding reagent increases. The K_m and V_{max} of CaNΔAIDCT in the absence of crowding reagents were similar to those of wild-type CaN under crowded conditions.

DISCUSSION

It is important when characterizing an enzyme or macromolecule to consider its native environment is a crowded milieu of various nucleic acids, proteins, and other macromolecules that range in concentration from 50 to 400 g/L.[27,34,35] While crowding can influence enzyme and protein:protein reactions through slower diffusion rates,[36−38] this study focuses on the effects that macromolecular crowding has on the structure of CaN and the effects that structural changes confer on enzymatic activity. There have been many studies suggesting that macromolecular crowding can increase the stability of folded proteins, and while the mechanism underlying this stability is debated, it nonetheless has important implications for understanding the role of structural stability and enzyme function.[24−26,32,39−43] The Pielak group has published a wealth of information about macromolecular crowding, and recently published data on in-cell nuclear magnetic resonance of the SH3 domain of the *Drosphila* protein drk.[26,39,44−49] Interestingly, they found that this protein does not display an increased stability in the intracellular environment of *E. coli* or in the presence of lysozyme or BSA in *in vitro* stability measurements.[44] There is no doubt that the effects of the cellular environment on proteins are complex and there are many contributing factors to be considered. Here we are considering just the effects of relatively simple inert polymers and realize that the *in vivo* situation is significantly more complicated.

In a previous study, our laboratory found that the activity of CaM-activated CaN is influenced by a helical element, called the distal helix, found at the C-terminal end of the regulatory domain (RD).[12,21] In the presence of Ca^{2+} but absence of CaM,

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the RD of CaN is disordered and the autoinhibitory domain (AID) is bound in the active site (Figure 1). When CaM is saturated with Ca^{2+}, it will bind to the CaM-binding site (residues 390−414), located toward the N-terminal end of the RD, with a high affinity (K_D = 1−28 pM).[18,19] When CaM binds to the RD, the distal helix folds onto CaM and the AID dissociates from the active site.[12,21,50,51] Mutations destabilizing the distal helix were shown to decrease CaN activity toward both pNPP and a phosphorylated peptide from cAMP-dependent protein kinase regulatory subunit type II called the RII peptide.[21]

It is important at this point to understand that holo-CaM (calcium-loaded CaM) is a very stable protein with no observable melting transition from 5 to 95 °C.[52] Holo-CaM binds to pCaN, a 29-residue peptide of the CaM-binding site of CaN, with an estimated K_D of 1−28 pM forming an even more stable complex, also with no observable melting transition (Figure 3C).[18,19,21] In fact, it takes the addition of 8 M urea to a solution of the CaM:pCaN complex for an observable thermal transition to become apparent (Figure 4C). In contrast, the RD:CaM complex was shown to have a thermal transition at 38 °C.[21] This transition was assigned to the unfolding of the distal helix. Inspection of the amino acid sequence of the RD reveals that region could form an amphipathic α-helix with the hydrophobic face potentially interacting with CaM. Via mutation of hydrophobic residues at this interface, folding of the distal helix was abolished.[21] It was also demonstrated that the complex formed when CaM binds to the RD has a stoichiometry that is predominantly 1:1.[20] On the basis of these data and other data, this region was identified as the helical structure that forms when CaM binds.[12,21] Mutations that disrupted this distal helix resulted in an increase in both the V_{max} and the K_m of CaN using pNPP as a substrate.[21] Disruption of the distal helix was also shown to decrease the activity of CaN against the more physiologically relevant substrate, the RII peptide.

The distal helix T_m of 38 °C is puzzling because this would suggest that it is not completely folded at human body temperature, yet folding of the distal helix has been shown to be essential for full activation of CaN.[21] This observation was made using protein in dilute buffer at 37 °C. It is known, however, that macromolecular crowding can stabilize a protein and increase its T_m. Thus, our hypothesis is that via stabilization of the distal helix through macromolecular crowding, the activity of CaN will be increased at body temperature.

To mimic macromolecular crowding, large inert polymers such as dextran 70 and ficoll 70 are frequently utilized and have been shown to increase the structural content of proteins as well as their thermal stability.[32,40] Because the distal helix is marginally stable at human body temperature, our goal was to determine if macromolecular crowding could increase its stability. This investigation was conducted using CD, which can measure the secondary structural content of proteins (α-helices, β-sheets, etc.). The addition of dextran 70 or ficoll 70 increases the helical content of the RD:CaM complex, but not that of the RD in the absence of CaM (Figure 2A,B). Note that the CD signal of RD with CaM at 222 nm (the signal typically used to measure α-helical content) appears to be approximately linearly dependent on the dextran 70 and ficoll 70 concentrations from 0 to 200 g/L (Figure 2C). Further, dextran 70 and ficoll 70 did not seem to affect the secondary structure of the pCaN:CaM complex, which would indicate that regions outside the CaM-binding site are amenable to folding and/or stabilization by macromolecular crowding. Because of the minimal protein concentrations required for CD, we could not measure CD spectra at dextran 70 or ficoll 70 concentrations greater than 200 g/L.

The distal helix is contained within the RD, C-terminal to the CaM-binding region, and is known to be a folded structure that is dependent on CaM binding and is marginally stable at 37 °C.[12,21] Thus, we attribute this gain in structure of the RD:CaM complex using crowding reagents to an increase in the folded fraction of the distal helix. An alternative explanation for the increase in the CD as the concentration of crowding reagents increases is an increase in the affinity of CaM for the RD. We discount this possibility because the dissociation constant of CaM for the RD construct is in the low picomolar range.[18,19] If the protein concentrations of CaM and the RD were 20 μM each, that would leave only around 20 pM RD or CaM not associated. A small increase in the bound fraction on a scale of this magnitude cannot be detected using CD.

In contrast to the polymers, 200 g/L dextrose or sucrose did not have an observable effect on the structure of the RD:CaM complex (Figure 3A). Dextrose and sucrose can theoretically affect the stability of a protein through the osmophobic effect.[53] Here, dextrose and sucrose were chosen as osmolytes because they represent the monomeric analogues of dextran 70 and ficoll 70, respectively. Because dextrose and sucrose did not promote folding of the RD:CaM complex, it is likely the osmophobic effect is not the driving force behind the increase in the level of folding of the distal helix.

Also, direct interactions between the chemical groups of ficoll 70 or dextran 70 and the proteins are possible and are known as "soft" or intermolecular interactions. Soft interactions between proteins have been observed by the Pielak group to destabilize proteins and mitigate the stabilizing effect of excluded volume.[48] Because we do not observe a change in the ellipticity of the RD:CaM complex at 200 g/L in the presence of either dextrose or sucrose, we hypothesize that these "soft" interactions (along with the osmophobic effect) do not have an observable effect on the stability of the distal helix. This would be consistent with a volume exclusion model of crowding, in which extended unfolded states are less favorable than compact folded states.[54,55]

To investigate the propensity of the RD to form α-helical structure and to determine if higher helical content could be obtained, we used TFE; 40% TFE increased the ellipticity (Figure 3A,B) of the RD:CaM complex by 58% and that of the pCaN:CaM complex by 22%. The linear appearance of the data in Figure 2C and the fact that TFE is able to induce more α-helical content than what can be achieved at 200 g/L dextran 70 suggest that the RD:CaM complex could possess a higher structural content at higher crowding agent concentrations. Estimates of macromolecular crowding in the cell range up to 400 g/L and thus the distal helix could be stabilized further *in vivo*.[35]

Our CD data suggest that the thermal stability of the distal helix is increased ∼5 °C when the RD:CaM complex is in the presence of 200 g/L dextran 70 or ficoll 70 (Figure 4A). Because the distal helix is not a part of the pCaN peptide, and because the pCaN:CaM complex does not have an observable thermal transition (Figure 4C), it is our hypothesis that the increase in the thermal stability of the RD:CaM complex in the presence of inert crowding agents is due to the distal helix.

In dilute buffer, the distal helix has an estimated T_m of 38 °C. At 37 °C (average human body temperature), the distal helix

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would be partially unfolded. By adding dextran 70, we can increase the stability of the distal helix at 37 °C and potentially increase CaN activity. A common CaN substrate used to assess CaN activity is the RII peptide that is a 19-residue peptide corresponding to the CaN-binding region of cAMP-dependent protein kinase regulatory subunit type II.[56] This peptide has initial velocities typically calculated from a <10 min linear range of dephosphorylation versus time.[22] Considering this time restraint, dextran 70 and ficoll 70 solutions are too viscous to accurately measure the activity of CaN against the RII peptide. Thus, we used the generic small-molecule substrate pNPP that has a linear range of up to 1 h.

We have shown previously that CaN activity against pNPP is diminished when distal helix-disrupting mutations are introduced into the RD.[21] This decrease in CaN activity is due to an increase in K_m, consistent with competitive inhibition, and was proposed to be due to the AID binding to and occluding the active site upon unfolding of the distal helix. Because pNPP and the AID are known to bind only to the active site of CaN, we proposed that folding of the distal helix promotes CaN activity by preventing the AID from binding.

In our activity data for CaN, we observed that the K_m decreases when dextran 70 or ficoll 70 is added (Table 1), consistent with stabilization of the distal helix. When small molecule dextrose or sucrose was added, the K_m did not appear to change (Table 1). If anything, the K_m may have increased somewhat, although it is difficult to determine that given the experimental error estimates. Such an increase could be due to the sucrose or dextrose competing with pNPP for access to the active site at high sugar concentrations. Unlike the CD data shown in Figure 2C, we did not observe a linear decrease in the K_m of CaN as the dextran 70 or ficoll 70 concentration increased from 0 to 200 g/L (Table 1). One reason for this difference could be that only a partially folded distal helix is required for robust CaN activity. Our CD data indicated that dextrose and sucrose were not able to stabilize the distal helix and thus would not be expected to modulate the K_m of CaN activity against pNPP.

To investigate if crowding was having an effect on the enzymatic activity of CaN aside from its effect on the distal helix, we made a constitutively active form of CaN that is independent of CaM. When CaN is truncated at the AID and C-terminal tail (AID-CT), folding of the distal helix and CaM binding is no longer essential for activity. The V_{max} and K_m of CaNΔAIDCT do not change significantly as the dextran 70 or ficoll 70 concentration increased from 0 to 200 g/L (Table 2). The K_m of CaNΔAIDCT resembles the K_m of CaN in the presence of crowding reagents. Altogether, this suggests that the distal helix is a heat labile structural component needed to disrupt the interaction between AID and the active site cleft of CaN. Upon disruption of interaction of the AID with the catalytic cleft, or deletion of the AID (in the case of CaNΔAIDCT), pNPP is able to freely diffuse into the active site of CaN for dephosphorylation.

In the context of immune system activation, CaN activation leads to eventual release of so-called pyrogens that can lead ultimately to a fever.[6,8,9] Typical fevers range from 38 to 42 °C. Previously, we characterized the distal helix as a heat labile structure whose formation is critical for complete activation of CaN.[21] Here, we characterized the distal helix as having a T_m of ∼38 °C in dilute buffer but increases to ∼43 °C in the presence of dextran 70 and ficoll 70, which mimic macromolecular crowding of the cell. From these data, we hypothesize that the distal helix may impart a feedback mechanism by which CaN activity decreases as a high-grade fever is attained. Thus, the distal helix may be a novel and important modulatory feature of immune system regulation.

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Notes

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ABBREVIATIONS

CaN, calcineurin; CaM, calmodulin; RD, regulatory domain; pCaN, calmodulin-binding peptide from calcineurin; dex 70, dextran 70; fic 70, ficoll 70; AID, autoinhibitory domain; CD, circular dichroism; pNPP, *p*-nitrophenyl phosphate.

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