Calcineurin is an essential calcium-activated serine/threonine phosphatase. The six NMR-observable methionine methyl groups in the catalytic domain of human calcineurin Aα (CNA) were assigned and used as reporters of the presence of potential cis-trans isomers in solution. Proline S4 is found in the cis conformation in most calcineurin X-ray structures, and proline 309, which is part of a highly conserved motif in phosphoprotein phosphatases, was modeled with a cis peptide bond in one of the two molecules present in the asymmetric unit of CNA. We mutated each of the two prolines to alanine to force the trans conformation. Solution NMR shows that the P84A CNA mutant exists in two forms, compatible with cis-trans isomers, while the P309A mutant is predominantly in the trans conformation.

Database
PDB depositions mentioned PDB 5C1V and 2JOG.

Introduction
Calcineurin (CN) or phosphoprotein phosphatase-3 (PPP3) is a Ca$^{2+}$/calmodulin-regulated serine/threonine phosphatase involved in various key signal transduction pathways. Dephosphorylation of substrates by CN, including the cytosolic Nuclear Factors of Activated T cells 1 to 4 (NFATc1-c4) transcription factors, is achieved by CN activation after increasing the intracellular concentration of calcium [1]. Once dephosphorylated, NFATc translocate to the nucleus where, binding to DNA and in cooperation with other

**Keywords**
$^{13}$C-methyl methionine NMR; calcineurin; cis-trans isomerization; NMR; phosphatase

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transcription factors, modulate many major biological processes such as development, immune response, memory, or cardiac functioning [2,3].

Calcineurin is the target of immunosuppressant drugs cyclosporine A (CsA) and FK506 [4] which, in complex with immunophilins cyclophilin A (CypA) and FKBP-12, respectively, inhibit T-cell activation and consequently the immune response. Interestingly, CypA and FKBP-12 are both cis-trans proline isomerases and FKBP-12 itself is an endogenous regulator of CN activity [5].

Calcineurin is a heterodimer formed by a large catalytic subunit (CNA, 59 kDa for the human α isoform) tightly bound to a small Ca$^{2+}$-binding regulatory subunit (CNB, 19 kDa). Activation of CN by Ca$^{2+}$ is also depending on the interaction of the Ca$^{2+}$-binding protein calmodulin with CN.

The structure of the CNA subunit alone or in complexes with peptides from substrates and regulatory proteins of CN [6,7] has been previously determined by X-ray diffraction. The human CNA α subunit is organized as an N-terminal catalytic domain (347 residues) and a regulatory C-terminal region comprising the CNB and calmodulin-binding regions and the autoinhibitory domain (AID), which can block access of substrates to the active site. The active site in the catalytic domain of CNA contains two metal ions ligated by histidine and aspartic acid side chains contributed by the immediate extensions at one end of both sheets of a central β-sandwich that are bridged at the other end by a loop that contains a cis peptide bond at P84. The β-sandwich and the cis bond containing loop are well-conserved among PPPs.

In spite of the overall similarity, there are relevant differences among the CNA structures determined in various contexts. For example, P309 within a highly conserved Ser-Ala-Pro-Asn-Tyr (SAPNY) sequence in PPPs, is most often found forming a trans peptide bond but is connected by a cis bond in one of the two molecules from the asymmetric unit of a single crystal (PDB 5C1V) [8], although the low resolution available (3.4 Å) might question the model proposed. In contrast, the structure of the complex between CNA and a 14-residue peptide with the PxxIxxT motif was shown with a trans peptide bond to P84 (PDB 2JOG) [9] while it is cis in all other reported structures.

These observations suggest a considerable dynamic plasticity and prompt for the need of additional structural studies in solution to obtain a more accurate view of the CN conformational landscape and of the interactions with other proteins and with drugs.

Pioneering NMR work by the Wagner group [9,10] has shown that these studies are feasible, although they were hampered by incomplete deuterium back exchange, severe signal overlap and the presence of paramagnetic atoms in the active center broadening the lines for a large number of signals [10]. Some of the assigned residues showed more than one peak, indicating the presence of structural heterogeneity, but the inherent complexity of the spectra prevented a more detailed study.

Methionine residues are often located in protein–protein interaction sites, sometimes forming clusters. Methionine selectively $^{13}$C labeled at the ε-methyl carbon can be readily incorporated biosynthetically and used as a sensitive NMR probe of their environment. CNA has methionine residues located in the proximity of functionally and structurally important sites that can be exploited strategically as reporter probes: the binding sites for modulatory proteins and substrates, including the NFATc peptides, the region experiencing large rearrangements in a crystal form in which P309 was assumed to adopt the cis conformation, and P84 which is in the cis form in most structures.

The complete assignment of the methyl methionine spectra in CNA provides a tool to follow the changes in the environment of key regions upon mutations designed to force the P309 and P84 peptide bonds in the trans form.

These results provide a new, more complex, view of the structure and dynamics of CNA in solution and suggest a relevant role of proline isomerization in CN regulation.

**Results**

The 2–347 truncated CNA construct designed by Aramburu et al. [11] that included the complete catalytic domain of human CNA $\alpha$ contains seven methionine residues. Its sequence differs from the wild-type by three substitutions (Y341S, L343A, and M347D) introduced to increase protein stability, this is the construct used in this study, as well as in previous crystallographic [8] and NMR studies [9,10]. For simplicity, we will refer hereafter to the truncated and optimized form simply as CNA or CNA-WT to distinguish from the mutations applied in this study. Five of the seven methionine residues form two clusters: M51 and M99, with their sulfur atoms at less than 4.5 Å and M191, M290 and M329 with distances of 7–8 Å between M329 and the other two methionines. M179 and M227 show a S-S distance of 13 Å between them and, thus, are not considered a cluster (Fig. 1A).
Methyl methionine $^1$H-$^{13}$C HSQC NMR spectra

An expansion of the $^1$H-$^{13}$C HSQC spectrum of CNA-WT at 230 μM concentration is shown in Fig. 1B, with the assignments explained below. The main methionine signals are labeled A-F. Peaks A-D, have similar integrated intensities, corresponding to individual methionine residues (Fig. 1C), while E and F display lower intensities. Thus, six of the seven methionine residues are resolved and one of the methionine residues is not observed. Assignment of the methionine signals was achieved by site-specific mutations (Fig. 2).

Methionines located in α-helices were individually replaced by leucine, while M191, M290, and M329, which are in β-strands, were replaced by isoleucine. All CNA methionine mutants showed similar NFATc activity to CNA WT (Fig. 2G), confirming the conservative character of the mutations and validating the assignment protocol.

Mutation of individual methionine residues to isoleucine in the cluster formed by M191, M290, and M329 caused, in addition to the disappearance of the signal from the mutated residue, chemical shift perturbations or intensity changes in the peaks of neighbor methionines. However, by comparing the effects of the three mutations unambiguous residue assignments could be achieved. Fig. 2 panels A-C show the effect of the M191I, M290I, and M329I mutations in the HSQC spectra of CNA. The M191I mutation caused the minimum perturbation: the peak E disappeared and peak D was shifted while the remaining main signals were nearly unchanged. Therefore, peak E was assigned to M191. Mutation of M290 to isoleucine, M290I, resulted in the complete disappearance of peak D and a very strong broadening of peak E, which is visible only at a very low contour threshold, but only a small shift in peak C and M290 was assigned to peak D. Finally, the mutation M329I caused the disappearance of peaks C, D, and E. Together with the previous assignments, peak C was assigned to M329. The disappearance of multiple methionine signals when a single methionine is mutated reinforces the concept of methionine clusters. The more widespread changes caused by M329I mutation are consistent with the central position of M329 in the cluster. The observed M329 He chemical shift agrees with a previous assignment [9,10].

Peak F was assigned to M99 as its replacement by leucine resulted in the disappearance of this peak (Fig. 2D). The M99L mutant also showed small chemical shift perturbations in peak A. Methionine 51 is very close to M99 and therefore signal A was tentatively assigned to M51, although the M51L mutant was expressed in very low yields and no spectra could be measured. Mutation M227L caused the selective disappearance of peak B (Fig. 2E) while the M179L mutation preserved all the methionine signals, suggesting that M179 is the unobservable methionine residue (Fig. 2F). The available crystal structures of CNA show that the methionine residues closer to the paramagnetic Fe$^{3+}$ ion in the catalytic center are M179 (at
15 Å) and M227 (at 16 Å). M99 is separated more than 18 Å from the iron center and the other four methyl groups have distances longer than 20 Å.

The observed chemical shifts were compared with the predictions made using quantum chemistry calculations. Using a set of structures with resolution higher than 1.7 Å, we have shown that density functional methods enable in favorable cases complete assignments of methionine methyl groups (manuscript in preparation). Although the resolution of the available calcineurin X-ray structures is lower, a reasonable agreement between the calculated and observed chemical shifts was observed for the 5C1V chain A structure that corresponds to the same construct studied by NMR. The 5C1V chain A structure has proline 309 in trans. The calculated and experimental chemical shifts are compared in Table 1. The predicted chemical shifts for the invisible methionine 179 are 19.0 ppm and 0.88 ppm for 13C and 1H respectively, which fall in the vicinity of the M99 signal. Methionine 290 was not correctly predicted. Interestingly, methionine 290 shows completely different environments in the two structures present in the asymmetric unit in 5C1V.

**Cis peptide bonds in conserved phosphatase motifs**

The human CNA subunit (residues 2–347, Uniprot Q08209) contains 21 proline residues, most of them conserved in other protein phosphatases of the PPP family. In the crystal structure of CNA reported by Guasch et al. [8], the unit cell contained two CNA molecules with large structural differences resulting, at least in part, from the modeling of the peptide bond between A308 and P309, in the well-conserved Ser-Ala-Pro-Asn-Tyr (SAPNY) motif, in a cis conformation. Proline 84, forming a cis peptide bond with the preceding residue in all the X-ray structures of CN, is also highly conserved (Fig. 3A). In CNA, these conserved prolines are connected to the active site through the central β-sandwich (Fig. 3B) that contains three reporter methionine residues: M191, M290, and M329.

**Table 1.** Observed and calculated chemical shifts for CNA methionine methyl groups

| Residue | 13C δ exp | 13C δ cal | Error | 1H δ exp | 1H δ cal | Error |
|---------|-----------|-----------|-------|----------|----------|-------|
| 51      | 16.5      | 16.6      | 0.1   | 2.12     | 1.42     | –0.70 |
| 99      | 20.2      | 18.4      | –1.8  | 1.01     | 0.76     | –0.24 |
| 191     | 17.2      | 18.5      | 1.3   | 0.98     | 1.56     | 0.59  |
| 227     | 17.0      | 17.3      | 0.3   | 2.03     | 2.09     | 0.05  |
| 290     | 17.9      | 22.9      | 5.0   | 1.43     | 2.31     | 0.88  |
| 329     | 18.1      | 16.9      | –1.2  | 2.03     | 1.33     | –0.70 |
| 179     | –         | 19.0      | –     | –        | 0.88     | –     |

*Hartree-Fock approximation using the 6-311G (d,p) basis set and the 5C1V PDB structure with trans P309.
We decided to investigate the role of cis peptide bonds in these positions. Our strategy was to force the formation of a trans bond by individually mutating these proline residues to alanine. The structural effects were monitored through reporter methionines, and the functional consequences were investigated by testing the phosphatase and NFATc activities in solution (Fig. 3C,D).

**Potential cis-trans isomerization sites: Proline 309**

The possible presence of the cis form in the 308–309 peptide bond suggests that, although packing forces in the crystal could have forced it, the cis form of this peptide bond might be energetically accessible and we decided to explore if the cis P309 form was present in solution. The chemical shift of M329 was hardly affected by the P309A mutation (Fig. 4A–D). M329 has a completely different environment in the cis and trans forms coexisting in the crystal and provides an excellent probe to test this hypothesis (Fig. 4E,F). The other potential reporter is M290, which is part of a well-defined β-strand in the trans form but is in a disordered region in the cis isomer. Although M290 shows a small perturbation in the P309A mutant (Fig. 4B), the observed shifts are far from the random coil values (1H: 2.3 ppm, 13C: 15.0 ppm) expected for a disordered chain. Thus, the population of the cis P309 form, if present, is not detectable by NMR. The most significant change is observed in the signal from M99 (Fig. 3C), which is the methionine residue closest to the mutation site (at around 12 Å). The other perturbed residues M51 (Fig. 4D) and M290, are located 15–17 Å away and the observed effects suggest an extensive network of interactions affecting distant regions in the entire protein. Interestingly, M51 and M290 signals become narrower, suggesting the P309A mutation affects the dynamics of distant sites. Thus, although the presented experiments do not prove the existence of an observable cis P309 conformation, they...
point toward the existence of mechanisms of fine-tuning of CNA internal dynamics.

We next investigated the impact of the P309A mutation on the phosphatase activity of CNA toward the small phosphosubstrate p-nitrophenyl phosphate (pNPP) and their effect to induce CN-mediated NFATc activity measured using a luciferase reporter gene driven by an NFATc–dependent promoter. As negative controls, we used ZnCl₂ as phosphatase inhibitor and FK506 as inhibitor of the CN-mediated NFATc activity. The phosphatase activity of the P309A CNA mutant toward pNPP was reduced by 35% and NFATc activity by 30% with respect to the WT form (Fig. 3C,D).

Potential CNA cis-trans isomerization sites: Proline 84

The peptide bond between A83 and P84 is found in the cis conformation in all CNA structures deposited in the PDB with the exception of PDB 2JOG [9], an energy minimized model incorporating NMR restraints to a bound peptide, where the A83-P84 bond is found in trans conformation. The experimental restraints did not involve P84 or its immediate vicinity, thus the trans conformation may have resulted from the energy minimization protocol. However, a comparison of the alpha carbon coordinates of the 2JOG and the 1AUI structure from which it was derived, shows a RMSD of 0.90 Å indicating that both the cis and trans peptide bonds to P84 can be accommodated without major distortions in the CNA structure (Fig. 3C,D).

The 83-84 peptide bond can be locally compensated and does not cause drastic changes in the overall protein structure. However, the environment of methionine 191 is strongly affected. In order to force this peptide bond into the trans form, we mutated P84 to alanine. A comparison of the HSQC spectra of wild-type and P84A CNA is presented in Fig. 5A. The P84A mutation has a major effect in the M191 signal where now two well-resolved signals are observed: a major one shifted in both dimensions, with respect to that of CNA WT and a minor one that is found at the same chemical shift of the WT M191 signal. The relative integrals are 1:0.4. The sum of the two integrals is comparable to that of M329 or M290, the other methionine residues in the same cluster. M191 is the closest methionine to P84 and therefore we assigned the new major signal to the chemical environment sensed by M191 when Ala84 is in the trans form. Interestingly, the fact that the minor peak retains the original position as in the wild-type cis form raises the intriguing hypothesis that a cis conformation may be accessible also to the P84A mutant. The chemical shift of the other methionine residues is unaffected by the P84A mutation, although signals from M51, M290, and M329 become narrower and M99 shows increased intensity. Thus, the P84A mutation preserved the structure of CNA WT. The possibility that the new observed signal could come from the M179 methyl group that is not observed in the WT form is unlikely on the basis of its predicted chemical shifts.

The P84A CNA mutant retained ca. 80% of the phosphatase activity toward pNPP and 80% of the 3C,D).

Fig. 4. Cis and trans forms in P309 and P309A CNA. (A) 1H-13C HSQC NMR spectrum of CNA P309A mutant (gold) superimposed to CNA-WT (black). (B-D) Expansions of the regions of (B) M290, (C) M99, and (D) M51. (E) Zoom in the crystal structure 5C1V showing CNA molecules with P309 in the cis (left, blue) and trans (right, pink) forms. P309 (gold), M290, M329, M191, M99, and M51 are highlighted. The position of P84 (purple) is also indicated. (F) Topology diagram showing the differences observed between the P309 cis and trans forms, where the position of M329 in the trans form is highlighted in blue. Adapted from Guasch [8] under license CC BY 4.0.
NFATc activity compared to the wild-type (Fig. 3C, D); the minor form of the P84A mutant has a population of 30%. Thus, one should conclude that the two forms are both active, unless the mutation causes a strong activation of the minor form. P84 is located more than 25 Å away from the active site and the similarity of the chemical shifts of the other methionine signals compared to the WT indicates that the overall structure of CNA is preserved upon P84A mutation. Thus, the observed effect is clearly allosteric. We hypothesized that perturbing the loop that bridges the two sheets in the β-sandwich that extends into the active site may be causing the observed changes. To test this hypothesis, we mutated A83, in the same loop, to tyrosine. This results in a nearly complete loss of phosphatase activity (Fig. 3C). In contrast, the decrease in NFATc activity of the A83Y mutant was not statistically significant, confirming this mutant is at least partially well-folded. These experiments show a functional connection between the loop containing A83-P84 residues and the phosphatase active site.

Discussion

The central role of CN in many signaling pathways and key biological processes has prompted a large number of structural studies of crystals of the complete protein or its catalytic subunit (CNA) alone or in complex with substrates or inhibitors. A partial assignment of the NMR spectra of human CNA by the Wagner group [9,10] has helped to map the CN-binding sites of peptide ligands. The presence of paramagnetic metal ions complicates the structural studies in solution using NMR. Here, we have shown that 13C-methyl labeled methionine, readily incorporated into CNA, provides a useful probe to study the catalytic domain of CNA in solution. Six of the seven methionine residues present in CNA give resolved signals that could be assigned by site-specific mutagenesis. Methionine chemical shifts are sensitive reporters of their environment. The vicinity of aromatic rings or other methionine residues affects proton chemical shifts. Carbon chemical shifts show a strong dependency on the side-chain torsion angles ($\chi_1-\chi_3$). The singular chemical shift values of M99 are consistent with the proximity of three aromatic rings according to density functional theory calculations.

Methionine residues are located in the proximity of potentially important proline residues in CNA. A sequence alignment of a number of phosphatases shows two highly conserved proline residues, at positions 84 and 309 in CNA. Interestingly, P84 is forming a cis peptide bond in all the reported structures, with only one exception, while P309 was found in the cis peptide conformation only in a recent crystallographic study at relatively low resolution [8].

Calcineurin is the target of immunosuppressant drugs cyclosporine A (CsA) and FK506 [4] that recruit prolyl isomerases CypA and FKBP-12 respectively, forming ternary complexes. The physiological role of the direct interaction between immunophilins and CN, in the absence of immunosuppressant drugs, has been controversial. Recently, Caraveo et al. [5] demonstrated that FKBP-12 is an endogenous regulator of CN activity thus, the possibility that proline isomerization plays a role remains an intriguing possibility.

In an attempt to force the corresponding peptide bond to be trans, we individually mutated P84 and P309 to alanine. We used methionine NMR to study the structural effects of the mutation and measurements of
the phosphatase and NFATc activities to determine the functional consequences.

From the structural point of view, the P309A mutation caused only small changes although the perturbation extended across the entire protein. Methionine peaks show complex shapes suggesting that methionine residues sample multiple conformations in slow exchange. In general, NMR peaks were sharper in the P309A mutant, suggesting a faster dynamics.

Overall, the conserved chemical shifts, especially that of M329 that is in completely different environments in the cis and trans forms observed by Guasch et al., [8] confirm that P309 is predominantly in the trans conformation in solution, although a small percentage of cis form cannot be ruled out. Thus, the P309 cis peptide bond observed in the crystals may be the result of conformational selection driven by the crystalization conditions. The P309A mutant retained 65% of the phosphatase and 70% of the NFATc activities of the wild-type. Our structural and functional data suggest that the region around P309 is highly important, in agreement with high conservation of the sequence around P309 in other phosphoprotein phosphatases (Fig. 3).

Although the P84A mutant was expected to convert the cis A83-P84 bond into a trans A83-A84 peptide, the chemical shifts from most methionine methyl residues remained unaltered, suggesting that the overall structure was not affected. The only significant chemical shifts changes were observed for M191, which is the residue closest to the mutation site. Interestingly, the NMR signal from this residue appears duplicated with one of the peaks retaining a chemical shift very similar to the one found in wild-type, where position 84 is occupied by proline in a cis peptide bond. This raises the intriguing hypothesis that the two forms observed by NMR could correspond to the trans and cis conformations of the 83-84 bond. Nonproline cis peptide bonds, although rare, have been previously detected representing 0.03% of the peptide bonds in a nonredundant set of 571 proteins [12]. However, the mutation of cis Pro 202 to alanine in carbonic anhydrase II retained the cis conformation [13] and this was also observed in cis Pro 93 of Ribonuclease A [14]. Interestingly, duplicate signals were also observed in the NMR spectra reported by Takeuchi et al. [9], in some sites including alanine 83, suggesting that the trans form may also be populated in CNA in solution, although it has never been observed in the CNA crystals. We want to emphasize that the observation of a second environment for Met 191 in the P84A mutant does not represent an unequivocal demonstration of the presence of a conserved nonproline cis peptide bond in CNA. It could be that the minor species has a different conformation from the major one, also with a trans peptide that, fortuitously, causes the chemical shifts of Met 191 to take the same values than in wild-type (cis) CNA Met. Thus, the possible existence of a cis peptide bond in P84A remains speculative.

The P84A CNA mutant has phosphatase activity and NFATc activities quite similar to the control wild-type sample, suggesting that the widespread cis conformation is not an absolute requirement for CNA activity. This raises the question of the functional reason for the overwhelming observation of a cis proline in this position. Prolyl isomerases, like CypA or FKBP, present very high affinities for CN when bound to immunosuppressant drugs CsA and FK506, respectively. Thus, the possible role of cis-trans isomerization in CN activity is an intriguing possibility that has been previously suggested [5,8,15]. Interestingly, P84 is located in a loop closing one of the edges of a β-sandwich, which on the opposite edge displays residues directly involved in metal binding in the binding site. The cis and trans forms at position 84 can adopt very similar geometries, as confirmed with the nearly identical chemical shifts of most methionine residues. However, they may sustain different dynamics affecting the functional properties of CN. Interestingly, the A83Y mutation, retaining proline in position 84, has very little effect on NFATc activity, therefore indicating that the overall structure is well preserved, but has nearly lost completely its phosphatase activity, in spite of the fact that position 83 is located more than 25 Å away from the active site. This result supports the notion that the loop containing A83-P84 has a regulatory function on the phosphatase activity. We speculate that proline isomerases, like FKBP-12, may be participating also in this regulation.

Materials and methods

Preparation of calcineurin subunit A

Expression was achieved via a pGEX-6P-1-CNAα vector encoding Glutathione S-transferase protein linked to the human calcineurin catalytic subunit α isoform (CNA) (NCBI NP_000935.1, residues 2–347 [11]. Rosetta™ 2 cell strains were used for expression of 13C e-Methyl methionine labeled CNA and mutant proteins, applying an adapted protocol for production of selenomethionine-labeled proteins using autoinduction medium containing 10 mg/mL of 13C e-Methyl methionine, at 37 °C for 6 h followed by a 20 h incubation at 25 °C. Harvested cells were suspended in lysis buffer containing Phosphate Buffered Saline, 2 mM MgCl2, 5 mM dithiothreitol (DTT), 1 mM ethylene glycol tetraacetic acid
(EGTA), 40 mg·mL⁻¹ lysozyme, 2 mg·mL⁻¹ DNase and protease inhibitors (Complete) and then ruptured using a cellular disruptor at 20 kPa. Cell debris was removed by centrifugation. Following further centrifugation at 25 000 g for 1 h, the supernatant was treated with glutathione-sepharose-4B beads (GE Healthcare, Chicago, IL, USA). The resin was first washed with a buffer containing 150 mm NaCl, 50 mm Tris pH 7, 1 mm DTT, 1 mm EDTA and protease inhibitor (Complete) five times, and then with the same buffer, without protease inhibitors, five times. Bound CNA was released by digestion with PreScission™ protease (GE Healthcare) overnight at 4°C and the flow through purified using a Superdex 75 size-exclusion column equilibrated with 100 mm NaCl and 50 mm Tris pH 8.0. CNA eluted as a single peak corresponding to 40 kDa.

Methionine and Proline mutants were prepared using the following forward primers 5’ to 3’: M51L (gcgctcctcct caaaagatgctgctaatag), M99L (ccgactaaccagaagctcaacacatc aagaattgtctcat), M179L (caaacatcacaagaagctcatactacgctctg tctg), M227L (caaaagatgctgctaatag), M191I (ccgctcctcct caaaagatgctgctaatag), M290I (gacgctcctcct caaaagatgctgctaatag), M329I (tattgaagtatgagaacaatgttataa atacagcaattacatgcttc), P84A (gttctgaaattcagcagctaattcagcagctggcagtcgctgctctgctctctgctctctgctctgctc) and the corresponding reverse primers.

Calcineurin phosphatase activity
Calcineurin Az phosphatase activity toward the p-nitropheno-lyphosphate (pNPP) substrate was measured. Briefly, 0.5 μM human CNAz wt (amino acids 2–347) and corresponding mutants were incubated with 5 mm pNPP in 100 μL final volume in 1x colorimetric assay buffer (50 mm Tris-HCl pH 7.5, 0.5 mm MnCl₂, 0.5 mm DTT, 6 mm MgCl₂, 2 mm CaCl₂, 0.1 mg·mL⁻¹ BSA) for 30 min at 30°C in a 96-well microtiter plate. Reaction was stopped by adding 10 μL 5 N NaOH. Fluorescence produced after pNPP dephosphorylation is read at 405 nm wavelength on a Victor™ X5 2030 Multilabel Reader (PerkinElmer Life Sciences, Waltham, MA). Fifty micrometer ZnCl₂ was used as a negative control of CNA phosphatase activity. Background values obtained from a sample containing pNPP but not CNA was subtracted to each sample. Data were obtained from triplicates of three independent experiments.

NFATc activity assay
HEK 293T cells were seeded at 30% confluence in 24-well plates. After 24 h, each well was transfectioned with 100 ng of 3xNFAT-luc reporter plasmid, 100 ng of pBJ5-mCNB, 1 ng of pRLNull as an internal transfection control and decreasing concentrations of pFLAG-CNA, 2–389 (400, 200, and 100 ng). The total amount of plasmid DNA was kept constant in all conditions using FLAG empty vector. All FLAG-CNA constructs bear the Y341F mutation that confers resistance to cyclosporin A (CsA). Twenty-four hours after transfection, cells were stimulated with 1 μm ionomycin, 10 ng·mL⁻¹ phorbol-12-myristate 13-acetate sodium salt (PMA), and 10 mm CaCl₂; then, 1 μm CsA was used to inhibit endogenous CN phosphatase activity. FK506 at 1 μm was used as a positive control of FLAG-NFATc activity inhibition. After 6 h of stimulation, cells were analyzed for luciferase gene expression using the Dual-Luciferase Reporter Assay (Promega, Fitchburg, Wisconsin) following the manufacturer’s protocol on a Victor X5 2030 multiplate luminometer. As a negative control of NFATc activity, the ΔNIR mutant with an inactivating deletion of the CNA PxixIT-binding sequence 328VMNIR332 was included. NFATc activation was normalized to the activity of renilla luciferase and the results are given relative to the activity observed in the transfection using 400 ng of wild-type CNA 2-389 Y341F. Data were obtained from triplicates of two independent experiments.

Nuclear magnetic resonance
Sensitivity-enhanced HQSC experiments were performed on a Bruker 600 MHz Avance III spectrometer (Billericia, MA) equipped with a TCI Cryoprobe. All spectra were collected using 10 mM Sodium Phosphate, 150 mM NaCl, pH 6.8, 1 mM DTT, 1 mM EDTA and 90%/10% H₂O/D₂O at 298K. Spectra were analyzed with CCPN Analysis Suite v2 [16].

Methionine chemical shift calculations
All the calculations were performed with the Gaussian 09 package using the 6-311G (d,p) basis set and the Hartree-Fock approximation. Calculations of methionine chemical shifts in proteins were performed using the geometry deposited in the PDB for the relevant methionine residue surrounded by the residues that contain some of its atoms located at a distance of 6 Å or less from the methionine methyl group, fixed also at the position reported in the X-ray structure. Conversion of isotropic magnetic shielding to chemical shifts was performed using the magnetic shielding constant of 2,2-Dimethyl-2-silapentane-5-sulfonate sodium salt (DSS) as a reference. Chemical shift predictions were further corrected using the linear correlation observed between experimental and calculated CS in a set of structurally well-defined methionine residues in high-resolution crystal structures. A detailed computational protocol and comparison of alternative calculation methods will be reported elsewhere.

Reagents
¹³C ε-Methyl Methionine was purchased from Cambridge Isotope Laboratories (Tewksbury, MA, CAS#: 49705-26-2), CsA was purchased from Sandoz (Holzkirschen, Germany);
Ionomycin sodium salt, PMA, and pNPP were obtained from Sigma (St. Louis, MI).

Data availability
Raw data and spectra are available from the corresponding author.

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Competing interests
The authors declare no competing interests.

Author contributions
JMCT measured and assigned and analyzed NMR spectra. AG expressed and purified CNA variants for NMR studies and analyzed data. AA-I and AB prepared CNA mutants. AB performed functional studies and analyzed data. SC and JCP calculated theoretical chemical shifts of methionine residues and contributed to assignment. MP-R, IF, AG, and MP designed the study and analyzed data. MP and JMCT wrote the paper with contributions from all the authors.

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