N-terminal Myristoylation Regulates Calcium-induced Conformational Changes in Neuronal Calcium Sensor-1*

Neuronal calcium sensor-1 (NCS-1), a Ca\textsuperscript{2+}-binding protein, plays an important role in the modulation of neurotransmitter release and phosphatidylinositol signaling pathway. It is known that the physiological activity of NCS-1 is governed by its myristoylation. Here, we present the role of myristoylation of NSC-1 in governing Ca\textsuperscript{2+} binding and Ca\textsuperscript{2+}-induced conformational changes in NCS-1 as compared with the role in the non-myristoylated protein. The 45Ca binding and isothermal titration calorimetric data show that myristoylation increases the degree of cooperativity; thus, the myristoylated NCS-1 binds Ca\textsuperscript{2+} more strongly (with three Ca\textsuperscript{2+} binding sites) than the non-myristoylated one (with two Ca\textsuperscript{2+} binding sites). Both forms of protein show different conformational features in far-UV CD when titrated with Ca\textsuperscript{2+}. Large conformational changes were seen in the near-UV CD with more changes in the case of non-myristoylated protein than the myristoylated one. Although the changes in the far-UV CD upon Ca\textsuperscript{2+} binding were not seen in E129Q mutant (disabling EF-hand 3), the near-UV CD changes in conformation also were not influenced by this mutation. The difference in the binding affinity of myristoylated and non-myristoylated proteins to Ca\textsuperscript{2+} also was reflected by Trp fluorescence. Collisional quenching by iodide showed more inaccessibility of the fluorophore in the myristoylated protein. Mg\textsuperscript{2+}-induced changes in near-UV CD are different from Ca\textsuperscript{2+}-induced changes, indicating ion selectivity. 8-Anilino-1-naphthalene sulfonic acid binding data showed solvation of the myristoyl group in the presence of Ca\textsuperscript{2+}, which could be attributed to the myristoyl-dependent conformational changes in NCS-1. These results suggest that myristoylation influences the protein conformation and Ca\textsuperscript{2+} binding, which might be crucial for its physiological functions.

Neuronal Ca\textsuperscript{2+} sensor-1 (NCS-1)\textsuperscript{1} belongs to a growing family of EF-hand Ca\textsuperscript{2+}-binding proteins, which is conserved from yeast to human (1, 2). The EF-hand is formed by a loop of 12 amino acids flanked by helices E and F (named after helices in parvalbumin), which are positioned like the forefinger and thumb of the right hand (3). This three-dimensional arrangement of the EF-hand motifs in various Ca\textsuperscript{2+}-binding proteins is conserved. The proteins of calcium sensor family have in common a fatty acid-modified N terminus, one pseudo or primitive non-canonical EF-hand as the first Ca\textsuperscript{2+} binding site, and three canonical EF-hands.

NCS-1, a 22-kDa protein (theoretical pI 4.7), is a mammalian homologue of frequenin in Drosophila and Xenopus. Frequenin has been shown to enhance synaptic efficacy at the neuromuscular junctions (4, 5). NCS-1 is expressed in neurons and neuromuscular junctions. It also is expressed in other secretory cell types similar to chromaffin cells and in the kidney, suggesting a more generalized role in exocytosis (6–9) that is dependent critically on the spatial and temporal regulation of the intracellular Ca\textsuperscript{2+} concentration (for example see Ref. 10).

The NCS family includes several proteins such as recoverin, neurocalcin, hippocalcin, frequenin, p22, GCAP1, GCAP2, VILIP, and the budding yeast homologue, Fp1. All of them are myristoylated and possess four EF-hand units. The first EF-hand (EF-1) does not bind to Ca\textsuperscript{2+} because of the presence of Gly in place of invariant Asp at the +x position (11). Of all of the aforementioned NCS proteins, recoverin is the most studied protein. It is a Ca\textsuperscript{2+} sensor in retinal rod and cone cells wherein, besides the EF-1, EF-4 is also non-functional and Ca\textsuperscript{2+} binds only to EF-2 and EF-3 sites, leading to the exposure of the buried N-terminal myristoyl group known as Ca\textsuperscript{2+}-myristoyl switch (12). Such a movement of the myristoyl group is supposed to expose a hydrophobic pocket that might interact with target proteins and membranes (12, 13). The role of myristoylation is found to be particularly interesting because the molecular mechanisms underlying the physiological role of NCS-1 and its interaction with target proteins such as phosphatidylinositol 4-OH kinase \( \beta \) are poorly understood, even though myristoylation is required for the interaction of NCS-1 and phosphatidylinositol 4-OH kinase \( \beta \) and up-regulation of its activity (14).

The physiological role of myristoylation varies among the various members of the Ca\textsuperscript{2+} sensor family. For example, the association of p22 with microtubules is dependent both on Ca\textsuperscript{2+} and myristoylation (15), whereas in GCAP1, GCAP2, and VILIP, it is just the Ca\textsuperscript{2+} binding that regulates their function (16, 17). Hippocalcin, neurocalcin, and NCS-1, all of Ca\textsuperscript{2+} sensor family, use their myristoyl group differently. NCS-1 is targeted to membranes via its myristoyl tail (18). In recoverin,
the N-terminal myristoylation stabilizes the T conformation (Ca\textsuperscript{2+}-free conformation) of the protein, suggesting a role of the myristoyl group as an intrinsic allosteric modulator (19). Based on NMR and fluorescence data, it has been shown that myristoylation does not affect the structure and the Ca\textsuperscript{2+} binding characteristics of yeast Frq1 (20). Myristoylated NCS-1 was proposed to be able to bind isolated membranes (6). It plays a more vital role in the regulation of neurotransmitter release and in synaptic plasticity, a highly Ca\textsuperscript{2+}-sensitive process (4). NCS-1 not only directly regulates the activity and trafficking of Ca\textsuperscript{2+} channels (21, 22) but also has a direct effect on vesicle availability (23). NCS-1 is implicated in regulating voltage-gated channels (21, 22). In many of these processes, the role of myristoylation and the presence of the Ca\textsuperscript{2+}-myristoyl switch in NCS-1 is not defined clearly. Therefore, it is desirable to understand the Ca\textsuperscript{2+} binding properties of NCS-1 and differences between the myristoylated and non-myristoylated forms of the protein. In particular, the data for affinity of mammalian-myristoylated NCS-1 (the biologically active form in a variety of assays) are not available and conflicting results exist for non-myristoylated NCS-1 (24) and yeast Frq1 (20).

In view of these discrepancies, the exact role of myristoylation on the structure and function of NCS-1 is not yet clear. This motivated us to improve an understanding of the structure-function relationships of NCS-1 and, in particular, the role of myristoylation in this protein. In this paper, we report the influence of myristoylation on Ca\textsuperscript{2+} binding and Ca\textsuperscript{2+}-induced conformational changes in NCS-1 based on various experimental studies. We observe that myristoylation regulates and restricts the Ca\textsuperscript{2+} binding affinity and conformational changes by increasing the cooperativity for ion binding. This is of physiological significance, because Ca\textsuperscript{2+} binding renders these proteins active for their functional roles by introducing conformational changes. Our results suggest the presence of a Ca\textsuperscript{2+}-myristoyl switch and that myristoylation influences the Ca\textsuperscript{2+}-modulated conformational changes in NCS-1, which might have functional implications. Myristoylated NCS-1 has three Ca\textsuperscript{2+} binding sites with a positive cooperativity among them and a higher apparent affinity compared with non-myristoylated NCS-1, which is found to have just two metal ion binding sites. In addition, the Ca\textsuperscript{2+}-myristoyl switch is functional in the E120Q mutant, although other properties such as Ca\textsuperscript{2+}-dependent surface hydrophobicity were altered by this mutation.

**EXPERIMENTAL PROCEDURES**

**Overexpression of Myristoylated and Non-myristoylated NCS-1 and Their Mutants—** Overexpression of myristoylated and non-myristoylated NCS-1 was performed following the protocol published previously (25, 26). The rat NCS-1 cDNA was subcloned into pTREC2 (kindly provided by Dr. S. Zozula, Stanford University) and either transformed alone or co-transformed with pH11001 (kindly provided by Dr. Jeffery Gordon, Washington University, St. Louis, MO) to express N-myristoyltransferase into competent BL21(DE3) cells (Novagen). pTREC2 drives the expression of NCS-1 from the temperature-sensitive phase promoter, whereas the expression of N-myristoyltransferase in pH11001 is dependent on the lacZ promoter. Non-myristoylated NCS-1 was produced by growing cells containing NCS-1 in pTREC2 at 30 °C to an A\textsubscript{600} of 0.7–1.0 and rapidly shifting the temperature to 42 °C. Myristoylated NCS-1 was produced by growing cells co-transformed with NCS-1 in pTREC2 and pH11001 at 30 °C initially to an A\textsubscript{600} of 0.2, and 4 mg/liter myristic acid and 12.5 mg/liter sodium myristate were added. N-Myristoyltransferase was expressed by the addition of 1 mM isopropyl-β-D-thiogalactopyranoside (Roche Applied Science) when A\textsubscript{600} reached 0.7–1.0. After 30 min, the cells were heat-induced for overexpressing the myristoylated NCS-1 as described above. Cells were harvested by centrifugation and resuspended in 5 ml of buffer (100 mM KCl, 1 mM dithiothreitol (DTT), 1 mM MgCl\textsubscript{2}, 50 mM HEPES-K, pH 7.5) per 500 ml of culture medium.

Alternatively, to improve the yield of protein as well as the level of myristoylation, the gene was subcloned in pET21a vector. To produce the myristoylated NCS-1, the vector was co-transformed with pH11001 carrying the gene for myristoyltransferase. Protein expression was performed as described above. The data were repeated with proteins purified and prepared in different batches.

**E120Q Mutants—** The third EF-hand (EF-3) was disabled by creating a site-directed mutation of Gln at position 120 (→Gln) by replacing it with Glu (E120Q) using the QuickChange mutagenesis kit (Stratagen). The mutated gene was subcloned in pET21a vector. To produce the myristoylated mutant, the vector was co-transformed with pH11001 to express N-myristoyltransferase. Protein overexpression was performed as described above (26).

**Protein Purification by Hydrophobic Interaction Chromatography—** Hydrophobic interaction chromatography was performed using prepacked columns and resins obtained from Tosohaas (Montgomeryville, PA) as described previously (26). All of the purifications were done on a PharmaCia fast protein liquid chromatography column with Hewlett-Packard Chromatation software for data acquisition. Large-scale purifications were done on a Toyopearl Phenyl-650C column (0.16 × 20 cm). The sample lysates were loaded in 50 mM HEPES, pH 7.5, containing 100 mM KCl, 1 mM DTT, 1 mM MgCl\textsubscript{2}, and 1 mM CaCl\textsubscript{2}. A step elution was effected using 50 mM HEPES, pH 7.5, containing 1 mM DTT, 1 mM MgCl\textsubscript{2}, and 2 mM EGTA, and a single peak was collected. Alternatively, we have purified proteins on in-house-built columns of phenyl-Sephrose and eluted the protein with 3 mM EGTA after exhaustive washing. The protein purity was checked on SDS-PAGE, and in case of any impurity in the preparation, a final purification was performed on a Superose 12 column attached to a fast protein liquid chromatography.

**Biochemical Analysis—** The myristoylation of the protein was confirmed by the incorporation of [\textsuperscript{3}H]-labeled myristic acid and fluorography (data not shown). Homogeneity of the protein fractions was confirmed by SDS-PAGE, calcium shift assay, and further by Western blotting using mammalian anti-NCS-1 antibodies. Myristoylation was quantitated and analyzed by electrospray ionization mass spectroscopy as described earlier (26). Protein concentration was determined by its absorption at 280 nm using the extinction coefficient of 22,000 M\textsuperscript{-1} cm\textsuperscript{-1} for NCS-1 (24). Proteins were decalcified immediately before use in the titration experiments. Protein solutions were dialyzed or solvent exchanged against EDTA followed by calcium-free buffer, which was prepared by passing through a Chelex 100 (Bio-Rad) column and stored in plastic bottles. After complete dialysis, protein solution was passed over a column of Chelex 100 that had been washed exhaustively with MilliQ water followed by the appropriate buffer.

**\textsuperscript{45}Ca\textsuperscript{2+} Binding by Ultrafiltration—** \textsuperscript{45}Ca\textsuperscript{2+} binding to myristoylated and non-myristoylated NCS-1 was evaluated and compared by the protocol described earlier (27). Protein concentration was 100 μM in both cases. Total calcium concentration was varied from 0 to 500 μM. Samples (500 μl) dissolved in 50 mM Tris buffer, pH 7, containing 100 mM KCl and 1 mM DTT containing \textsuperscript{45}Ca\textsuperscript{2+} were loaded on Centricon (Amicon) column and centrifuged for 30 s. Equal aliquots (10 μl) from the retentate and filtrate were used for radioactivity counting for the calculation of free Ca\textsuperscript{2+} concentration in a liquid scintillation counter. Protein and Ca\textsuperscript{2+} concentrations in the column (retentate) were compensated by adding an equal amount of protein. Known concentrations of cold calcium were added in the ultrafiltrate and centrifuged. This was repeated several times, and data were analyzed as shown in Equation 1,

\[
Ca^{2+} (\text{free}) = R_f/R_t \times Ca^{2+} (\text{total})
\]  

where \(R_f\) is the radioactivity in the filtrate, \(R_t\) is radioactivity in protein sample, and \(Ca^{2+} (\text{total})\) and \(Ca^{2+} (\text{free})\) are the total and free Ca\textsuperscript{2+} concentration, respectively. The experiments were repeated at least three times with the proteins prepared in different batches. Data were analyzed as described by Senin et al. (27) and fitted using the program Origin, version 6.0.

**\textsuperscript{45}Ca\textsuperscript{2+} Titration by Isothermal Titration Calorimetry (ITC)—** ITC measurements with non-myristoylated and myristoylated NCS-1 were carried out using a Microcal isothermal titration calorimeter. Samples were centrifuged and degassed prior to titration and examined for precipitation, if any, after the titration. In the case of non-myristoylated NCS-1, a typical titration consisted of injecting 1.2-μl aliquots of 10 mM CaCl\textsubscript{2} solution into the protein solution of 0.145 mM (1.43 μl). A total of 47 injections were carried out. In the case of myristoylated NCS-1, the titration was carried out by injecting 2.0-μl aliquots of 10 mM CaCl\textsubscript{2} solution into the protein solution of 0.100 mM (1.43 μl). A total of 40 injections were carried out. In a separate run, the aliquots of the ligand solution were injected into the buffer solution (without the protein) to subtract the heat of dilution. All of the experiments were repeated at

\[
\text{Eq. 1}
\]
least twice. The ITC data were analyzed using Origin (supplied with Omega Micro Calorimeter).

Circular Dichroism Spectroscopy—Circular dichroism spectra were recorded on a Jasco J-715 spectropolarimeter at room temperature. Far- and near-UV CD of proteins were performed in 50 mM HEPES, 100 mM KCI, pH 7. To study the effect of cations, proteins were titrated with Ca\(^{2+}\) or Mg\(^{2+}\) and CD spectra were recorded. Ellipticities were expressed in terms of millidegrees.

Fluorescence Measurements—All of the steady-state fluorescence measurements were done on a Hitachi F-4010 spectrofluorimeter. Emission spectra were recorded in the correct spectrum mode and also corrected for the solvent baseline. For quenching experiments, the concentrated stock solutions (5 \(\text{M}\)) of quencher (potassium iodide and acrylamide) were prepared in water and the protein solution was titrated. The emission spectra were recorded, and the data were analyzed by the Stern-Volmer equation (28) as shown in Equation 2,

\[
F/F_0 = 1 + K_{sv}[Q]
\]

(Eq. 2)

or by the modified Stern-Volmer equation as shown in Equation 3,

\[
F/F_0 = 1/f_{EM}K_{sv}[Q]+1/f_{em}
\]

(Eq. 3)

where \(F\) and \(F_0\) are the fluorescence intensities in the absence and presence of quencher, \([Q]\) is the molar concentration of quencher, \(f_{EM}\) is the Stern-Volmer quenching constant, and \(f_{em}\) gives the fractional accessibility of the NCS-1 fluorophore to quencher.

8-Anilino-1-naphthalene Sulfonic Acid (ANS) Binding—ANS binding experiments were performed by titrating equal amounts of myristoylated and non-myristoylated proteins with ANS (100 \(\mu\text{M}\)). Fluorescence spectra were recorded by excitation at 385 nm in the correct spectrum mode. Ca\(^{2+}\) or Mg\(^{2+}\) was added successively, and the fluorescence was recorded. The spectra were corrected for ANS fluorescence in buffer without protein.

Lipid Vesicle Binding—The binding of myristoylated and non-myristoylated NCS-1 to large unilamellar vesicles of \(~100\ \text{nm}\) in size of palmitoyl oleyl phosphatidylcholine (POPC) and palmitoyl oleyl phosphatidylyserine (POPS) in the absence and the presence of Ca\(^{2+}\) (100 \(\mu\text{M}\)) was performed as described previously by Ehrlich et al. (29) using intrinsic fluorescence or Laurdan-labeled vesicles. The binding was assayed by the change in intrinsic fluorescence upon the binding of the protein to membranes after correction for dilution and the scattering contribution from the lipid. The binding data were normalized using this change.

**RESULTS**

Overexpression, Purification, and Biochemical Characterization of Myristoylated and Non-myristoylated NCS-1 and Its Mutants—Recombinant rat NCS-1 and its myristoylated form were overexpressed in *Escherichia coli* under the control of a heat-inducible A-promoter. Myristoylated NCS-1 was produced by co-expression of N-myristoyltransferase driven by a separate lacZ promoter. The protein was purified at various scales with a single-step purification procedure of hydrophobic interaction chromatography (26). All of the protein preparations were analyzed by SDS-PAGE, Ca\(^{2+}\)-mobility shift assays, and Western blotting using affinity-purified mammalian anti-NCS-1 antibodies (26). The incorporation of myristoylation was analyzed by electrospray ionization mass spectroscopy. Electrospray ionization mass spectroscopy yielded an average mass of 21,747.79 for the myristoylated NCS-1 and an average mass of 21,533.44 for the non-myristoylated protein, which are in good agreement with their predicted molecular weights of 21,533.44 for the non-myristoylated protein, which are in good agreement with their predicted molecular weights of 21,747.47 and 21,537.11, respectively.

To improve the degree of myristoylation of NCS-1, we adopted another approach of overexpression of NCS-1 in the pET-21a vector (Novagen) co-expressed with N-myristoyltransferase. This expression system was more efficient since a higher yield was obtained for both myristoylated and non-myristoylated NCS-1. With this protocol, the myristoylation level was increased to \(\geq 90\%\). Purification was achieved by a single-step chromatography on a phenyl-Sepharose resin. The loss-of-function E120Q mutation was created by site-directed mutagenesis since replacing Glu (at \(\sim z\) position in the EF-3) by Gln, which inactivates this site. The mutant proteins also were purified by the same method with the exception that another step of purification by gel filtration on fast protein liquid chromatography was introduced.

The aim was to identify the role of myristoylation on Ca\(^{2+}\) binding properties and protein conformation. For this purpose, we studied Ca\(^{2+}\) and Mg\(^{2+}\) binding and the Ca\(^{2+}\) - and Mg\(^{2+}\)-induced changes in conformation of myristoylated NCS-1 and compared the data with non-myristoylated protein under identical conditions. In addition, the role of N-terminal myristoylation in E120Q mutants (with EF-3 inactive) in influencing the Ca\(^{2+}\)-myristoyl switch and Ca\(^{2+}\)-induced conformational changes also was compared.

**Myristoylation Increased Cooperativity and Affinity for Ca\(^{2+}\)**

**45Ca binding and ITC Titration**—We evaluated the Ca\(^{2+}\) binding affinity under identical conditions using the protocol described by Senin et al. (27). All of the parameters such as temperature, protein concentration, volume, and CaCl\(_2\) concentration were controlled carefully by performing the experiments simultaneously. The direct binding assay data showed that the amount of bound Ca\(^{2+}\) was greater for myristoylated NCS-1 than for non-myristoylated protein (Fig. 1a). The binding data were evaluated and analyzed as described by Senin et al. (27). The binding sites (\(n\)) for myristoylated and non-myristoylated NCS-1 were 3 (\(n = 2.85 \pm 0.15\)) and 2 (\(n = 1.8 \pm 0.1\)), respectively (Fig. 1a). Myristoylated NCS-1 showed non-linearity in the Scatchard plot, suggesting a higher degree of cooperativity (Fig. 1b, arrow).

**Ca\(^{2+}\) Titration of Non-myristoylated NCS-1 by ITC**—The

![Image](http://www.jbc.org/Downloaded from July 24, 2018)
above results of 45Ca binding were confirmed by ITC measurements. The outcome of calorimetric reaction upon the addition of aliquots of Ca2+ ligand to non-myristoylated NCS-1 is shown in Fig. 2a, and the heat-exchanged ΔQ versus the metal:protein ratio is shown in Fig. 2b. The calorimetric isotherms were fitted to equations of a single class of sites model proposed by Wiseman et al. (30). Only this model could give the best fit with the lowest χ² value. The thermodynamic parameters thus obtained are listed in Table I. The binding isotherms indicate exothermic nature, implying the release of energy upon Ca2+ binding to the protein. Most importantly, the thermodynamic parameter fit results in the values of n = 1.94 (the number of binding sites) and the binding constant K = 5.47 × 10⁵ M⁻¹. These results reveal the presence of two identical Ca2+ binding sites with equal affinity. The horizontal nature of binding isothersms and sudden fall to saturation around a metal:protein ratio of 2.0 indicate the parallel filling of the two equal sites without any cooperativity.

**Ca2+ Titration of Myristoylated NCS-1 by ITC.**—The calorimetric reaction of the myristoylated NCS-1 upon the addition of aliquots of the Ca2+ ligand to myristoylated NCS-1 is shown in Fig. 2c, and the heat-exchanged ΔQ versus the metal:protein ratio is shown in Fig. 2d. The calorimetric isotherms were fitted to equations of a model of three sequential binding sites proposed by Wiseman et al. (30). Only this model could give the best fit with the lowest χ² value. The thermodynamic parameters thus derived are given in Table II. The fitting reveals with clarity two sites of exothermic nature and one site of endothermic nature with the three binding constants: K₁ = 1.67 × 10⁴ M⁻¹; K₂ = 1.89 × 10⁵ M⁻¹; and K₃ = 2.65 × 10⁴ M⁻¹. Further, the sigmoidal nature of binding isotherms indicates cooperative binding. The second macroscopic binding constant K₄ is 10³ higher than the first macroscopic binding constant K₁. The Hill coefficient between first and second Ca2+ binding is 1.95, indicating very high positive cooperativity in Ca2+ binding.

**Ca2+ Induced Differential Changes in Far-UV CD Spectra of Both NCS-1.**—We performed Ca2+ titration with wild type myristoylated and non-myristoylated NCS-1 and their E120Q mutants and measured the changes in far-UV CD. In the Ca2+-saturated form, the negative value of ellipticity of myristoylated and non-myristoylated NCS-1 increased. Ion binding to the wild type NCS-1 showed two different patterns. In myristoylated NCS-1, the Ca2+-induced changes were more prominent around 222 nm (Fig. 3a), which are due largely to a nπ* transition (31, 32). In non-myristoylated NCS-1, the changes are more pronounced at 210 nm (Fig. 3b), which are because of exciton splitting of the ππ* absorption band (33). At saturation, there was a 35 and 15% change in the ellipticity at 222 nm upon binding Ca2+ in myristoylated and non-myristoylated NCS-1, respectively (Fig. 3, a and b). These results suggest that myristoylation affects the apparent affinity as well as the pattern of Ca2+ binding. We have also studied the effect of Mg2+ on far-UV CD spectra. Mg2+ bound to both myristoylated and non-myristoylated NCS-1 and increased the ellipticity (12% in non-myristoylated and 30% in myristoylated NCS-1 at 222 nm). The change in ellipticity induced by Mg2+ was smaller than the change induced by Ca2+ (Fig. 3, c and d). In case of the E120Q mutants, Ca2+ did not induce any change in the far-UV CD (data not shown), indicating that a functional site 3 is required for exhibiting these conformational changes.

**Conformational Changes in Near-UV CD upon Ca2+ Binding.**—The near-UV CD spectra of both forms of NCS-1 are shown in Fig. 4. NCS-1 is rich in aromatic amino acid residues (2 Trp, 7 Tyr, and 15 Phe), and the bands for the corresponding amino acid residues were resolved in near-UV CD. The tertiary structure of NCS-1 showed a positive band of Trp at 294 nm followed by a negative broad band at the 280-nm region for both Trp and Tyr. The vibronic Phe bands were well resolved with the minima at 262 and 268 nm. The addition of Ca2+ caused drastic changes in the CD spectra of myristoylated and non-myristoylated NCS-1 (Fig. 4, a and b). The ellipticity of the 294-nm band decreased, whereas the overall ellipticity of Tyr and Phe bands (280, 268, and 262 nm) increased concomitantly when titrated with Ca2+ (Fig. 4). The increase in the ellipticity at 269 nm was 2.4- and 3.6-fold for myristoylated and non-myristoylated proteins, respectively (Table III). The E120Q mutants of non-myristoylated and myristoylated proteins also exhibited a change in near-UV CD, almost similar to that seen in wild type proteins (Fig. 4, c and d).

We then studied the effect of Mg2+ on near-UV CD spectra of both forms of NCS-1. In contrast to Ca2+, the addition of Mg2+ increased the ellipticity of the Trp band (an ~1.4-fold increase at 292 nm), whereas the ellipticities of the Tyr and Phe bands (minima at 280, 268, and 262 nm) decreased (Fig. 4, e and f). Mg2+-induced conformational changes were identical for both
myristoylated and non-myristoylated proteins. These results show that Mg$^{2+}$/H$^{11001}$-induced changes in near-UV CD are different from the Ca$^{2+}$/H$^{11001}$-induced changes and that myristoylation does not influence Mg$^{2+}$/H$^{11001}$ binding.

Inaccessible and Dynamic Fluorophore in Ca$^{2+}$/H$^{11001}$-bound Myristoylated Protein Monitored by Trp Fluorescence and Potassium Iodide Quenching—Fluorescence spectroscopy was used to ascertain more subtle structural differences between myristoylated and non-myristoylated proteins and the influence of Ca$^{2+}$. There are two Trp residues in the protein, Trp$^{103}$ in the E helix of EF-1 and Trp$^{105}$ in the E helix of EF-3. Both myristoylated and non-myristoylated NCS-1 showed the emission maximum at $\sim336$ nm with 28% less emission intensity for myristoylated protein than for non-myristoylated NCS-1 (Fig. 5a).

This is attributed to the restrictions on Trp imposed by the myristoyl group. When myristoylated NCS-1 was titrated with increasing concentrations of Ca$^{2+}$/H$^{11001}$, the fluorescence intensity decreased by $\sim11\%$ (Fig. 5, b and d). The non-myristoylated protein exhibited a similar behavior with the exception that the decrease in emission intensity (8%) (Fig. 5, c and d) was less than that seen in the case of the myristoylated protein (11%). Ca$^{2+}$/protein molar ratios at saturation were 1.23 and 1.07 for non-myristoylated and myristoylated proteins, respectively, indicating a higher affinity for Ca$^{2+}$ for the myristoylated NCS-1.

| TABLE I |
| Thermodynamic parameters obtained from Ca$^{2+}$/H$^{11001}$ titration of non-myristoylated NCS-1 (one set of sites model) |
| Macroscopic binding constants | Gibbs free energy ($\Delta G$)kJ/mol | Enthalpy ($\Delta H$)kJ/mol | Entropy (T$\Delta S$)kJ/mol | Total number of binding sites $n$ |
| $K_1$ | -32.718 | -0.924 ± 0.073 | 31.794 | 1.94 ± 0.0057 |

| TABLE II |
| Thermodynamic parameters obtained from Ca$^{2+}$/H$^{11001}$ titration of myristoylated NCS-1 (three sequential sites model) |
| Macroscopic binding constants | Gibbs free energy ($\Delta G$)kJ/mol | Enthalpy ($\Delta H$)kJ/mol | Entropy (T$\Delta S$)kJ/mol |
| $E_4$ ($K_1$) | -24.078 | 3.457 ± 0.45 | 27.535 |
| $E_7$ ($K_2$) | -41.464 | -2.289 ± 0.23 | 39.175 |
| $E_6$ ($K_3$) | -36.625 | -12.54 ± 1.27 | 24.085 |

Fig. 3. The effect of Ca$^{2+}$/H$^{11001}$ on far-UV CD of myristoylated protein (a) and non-myristoylated NCS-1 (b) is shown. The additions of calcium were 0, 1, 6, 16, 36, and 66 $\mu$m. Protein concentration in each case was 1.33 mg/ml. The effect of Mg$^{2+}$/H$^{11001}$ on far-UV CD of myristoylated (c) and non-myristoylated NCS-1 (d) is shown. Mg$^{2+}$/H$^{11001}$ added were 0, 1, 6, 16, 36, and 66 $\mu$m. Protein concentration was 1.33 mg/ml. All of the spectra were recorded in 50 mM Tris buffer, pH 7, containing 100 mM KCl and 1 mM DTT. Path length was 1 cm.

Fig. 4. The effect of Ca$^{2+}$/H$^{11001}$ on near-UV CD of myristoylated protein (a) is shown. Ca$^{2+}$/H$^{11001}$ added were 0, 2, 7, 12, 22, 32, and 52 $\mu$m. b, non-myristoylated NCS-1. Ca$^{2+}$/H$^{11001}$ added were 0, 2, 12, 22, 32, and 52 $\mu$m. Protein concentration in each case was 0.86 mg/ml. The effect of Ca$^{2+}$/H$^{11001}$ on near-UV CD myristoylated mutant (c) and non-myristoylated (d) mutant is shown. Concentrations of myristoylated and non-myristoylated mutants were 37 and 48 $\mu$m, respectively. The effect of Mg$^{2+}$/H$^{11001}$ on near-UV CD of myristoylated (e) and non-myristoylated (f) NCS-1 is shown. Mg$^{2+}$/H$^{11001}$ added were 0, 5, 10, 20, and 70 $\mu$m. Protein concentration was 1.3 mg/ml. All of the spectra were recorded in 50 mM Tris buffer, pH 7, containing 100 mM KCl and 1 mM DTT in a 1-cm path length cuvette with seven iterations.
In case of the E120Q mutants, we did not see any change in Trp fluorescence upon Ca\(^{2+}\)/H11001 titration. Mg\(^{2+}\)/H11001 induced only a marginal decrease (2–3\%) in myristoylated NCS-1 and an increase (3–4\%) in non-myristoylated NCS-1 (data not shown). Mg\(^{2+}\)/H11001 did not induce any changes if added to the Ca\(^{2+}\)/H11001-saturated myristoylated NCS-1. However, Ca\(^{2+}\)/H11001 induced large changes in Mg\(^{2+}\)-saturated non-myristoylated and myristoylated NCS-1 (data not shown).

The accessibility and dynamics of the fluorophore in myristoylated and non-myristoylated proteins were compared by iodide (ionic quencher) and by acrylamide (neutral quencher) quenching. As seen in the Stern-Volmer plot (Fig. 6a), the plot for myristoylated NCS-1 was curved downward toward the x axis, indicating that the quenching was dynamic in the case of the myristoylated protein. The graph for non-myristoylated, although followed closely, was less curved (Fig. 6a). However, the presence of Ca\(^{2+}\) altered the accessibility of fluorophore in myristoylated NCS-1, and the Ksv values for myristoylated and non-myristoylated NCS-1 were 3.68 and 2.76 m\(^{-1}\), respectively, suggesting that myristoylation causes solvent inaccessibility (Fig. 6b). The neutral quencher acrylamide, which generally does not penetrate inside the protein, was not able to distinguish solvent accessibility of Trp of myristoylated protein from non-myristoylated NCS-1 (data not shown), most probably because of the lower penetration of acrylamide.

**Influence of Ca\(^{2+}\) on ANS Fluorescence**—We have used the ANS fluorescence to monitor the Ca\(^{2+}\)-induced exposure or solvation of the myristoyl group. ANS bound with myristoylated and non-myristoylated NCS-1 showed an emission maximum at 474 nm, indicating a high degree of surface hydrophobicity; however, the intensity was 27\% less in the case of Ca\(^{2+}\)-free myristoylated NCS-1 (Fig. 7a). The addition of Ca\(^{2+}\) to non-myristoylated NCS-1 drastically decreased the fluorescence intensity (58\%), indicating inaccessibility of ANS to binding sites rather than lower solvent exposure due to Ca\(^{2+}\) binding (Fig. 7, b and f). The effect of Ca\(^{2+}\) on ANS-bound myristoylated protein was in contrast to that seen in the case of the non-myristoylated protein. For myristoylated NCS-1, the ANS fluorescence increased (16\%) upon the addition of Ca\(^{2+}\) (Fig. 7, d and f) because of increased surface hydrophobicity and accessibility of binding sites for ANS. This increased ANS fluorescence of Ca\(^{2+}\)-bound myristoylated NCS-1 could be attributed to the solvation of the myristoyl group, which becomes exposed for ANS binding.

We monitored the effect of Ca\(^{2+}\) on ANS fluorescence of E120Q mutants. In case of non-myristoylated forms of the
E120Q mutant, there was an increase (1.5-fold) in ANS fluorescence when Ca\(^{2+}\) was added (Fig. 7c). However, in case of the myristoylated E120Q mutant, the addition of Ca\(^{2+}\) caused a 2-fold increase in ANS fluorescence with a 4–6-nm blue shift in the maximum (Fig. 7e). This increased fluorescence can be attributed to the accessibility of the myristoyl group in the presence of Ca\(^{2+}\). These results suggest that disabling EF-3 does not affect the movement or solvation of the myristoyl group, although subtle changes occurred in surface hydrophobicity and packing.

Effect of Mg\(^{2+}\) on ANS Fluorescence—To monitor whether Mg\(^{2+}\) can modulate the exposure/solvation of the myristoyl group, the effect of Mg\(^{2+}\) on ANS-protein fluorescence was studied. Upon titration with Mg\(^{2+}\), the fluorescence intensity decreased concomitantly in myristoylated and non-myristoylated NCS-1 (Fig. 8, a and b) with a greater decrease (41%) in the non-myristoylated protein. In myristoylated NCS-1, the change in ANS fluorescence was comparatively less (7%). Mg\(^{2+}\) decreased the ANS fluorescence of E120Q mutants but to a lesser extent than the wild type proteins (Fig. 8, c and d). These
results indicate that, although Mg$^{2+}$ was able to induce changes in the protein conformation, it was not able to change the environment of the myristoyl group as Ca$^{2+}$ binding did. Therefore, the solvation or accessibility of myristoyl group is specific to Ca$^{2+}$ and is not Mg$^{2+}$-dependent. In Mg$^{2+}$-saturated NCS-1, the addition of Ca$^{2+}$ increased the ANS fluorescence, confirming the above observations of the solvation of the myristoyl group by Ca$^{2+}$.

Myristoylated NCS-1 Binds to Lipid Vesicles in a Ca$^{2+}$-dependent Manner—To determine whether myristoylated and non-myristoylated NCS-1 proteins bind lipid and membranes and to determine their dependence on the binding of Ca$^{2+}$, we performed binding experiments with various lipids. Upon binding protein, a large decrease (43 ± 8%) in intrinsic fluorescence was observed. The non-myristoylated protein showed only a weak interaction with POPC vesicles regardless of the Ca$^{2+}$ concentration (Fig. 9a). It bound to the POPC vesicles with a $K_D$ of 74 ± 28 μM (in the absence of Ca$^{2+}$) and 75 ± 9 μM (with 100 μM Ca$^{2+}$), indicating negligible dependence on Ca$^{2+}$. Hence, this concentration of Ca$^{2+}$ was chosen to supersaturate all of the binding sites, and it has been analyzed that a more detailed titration is required to reveal the Ca$^{2+}$ binding properties of NCS-1 to various lipids. The myristoylated NCS-1 showed nonspecific weak binding to POPC in the presence and the absence of Ca$^{2+}$ (Fig. 9b). A similar pattern also was seen for POPS in the absence of Ca$^{2+}$ (data not shown). However, in the presence of excess calcium (100 μM Ca$^{2+}$), the myristoylated form specifically bound to POPS with an affinity of 17 ± 6 μM. Further, myristoylated NCS-1 showed a weak specificity for phosphatidylinositol (PI) 4-phosphate when compared with other PI lipids (data not shown), whereas non-myristoylated NCS-1 did not. These results show a Ca$^{2+}$-dependent binding of only myristoylated NCS-1 to POPS lipid vesicles.

**FIG. 8.** Change in the ANS fluorescence upon the addition of Mg$^{2+}$. a, non-myristoylated NCS-1. b, myristoylated NCS-1. Protein concentration in both cases was 1.15 mg/ml. ANS (100 μM) was added in protein solution in 50 mM Tris buffer, 100 mM KCl, and 1 mM DTT, pH 7, and excited at 285 nm. Mg$^{2+}$ added were 0, 10, 20, 45, and 110 μM. The effect of Mg$^{2+}$ on ANS spectra complexed with non-myristoylated (c) and myristoylated (d) mutants is shown. Protein concentration was 6 μM. Ca$^{2+}$ added were 2, 4, 6, 16, 36 μM.

**FIG. 9.** Normalized change in the fluorescence energy upon POPS and POPC vesicle binding to non-myristoylated NCS-1 (a) and myristoylated NCS-1 (b) in the presence of calcium is shown. The curves of POPC (a) and POPS (b) are the fitted curves.

**DISCUSSION**

The effect of N-terminal myristoylation in modulating the structural and conformational dynamics of NCS-1 has been analyzed and presented. N-myristoylation is a covalent modification that occurs cotranslationally in many proteins in eukaryotes (34). It has been known that the Ca$^{2+}$-myristoyl switch plays a significant role in the Ca$^{2+}$ sensor family of Ca$^{2+}$-binding proteins where, upon Ca$^{2+}$ binding to EF-hands, the generally buried N-terminal myristoyl chain protrudes out, facilitating membrane and target binding (12, 35). We have used several approaches, such as $^{45}$Ca binding, Ca$^{2+}$ titration by ITC, Trp fluorescence and fluorescence quenching, CD, ANS, and lipid vesicle binding, for understanding the role of myristoylation in regulating protein structure and its impact on Ca$^{2+}$ binding. Further, we have corroborated the above data using a decrease-of-function mutant, E120Q, by disabling the EF-3 unit in NCS-1. Ca$^{2+}$ binding to myristoylated NCS-1 increases the helical content; we have seen that Ca$^{2+}$ binding to myristoylated protein induces comparatively large conformational changes (Table III). A 2-fold change in the ellipticity at 268 nm in near-UV CD shows that Ca$^{2+}$ binding induces a large change in the conformation of both proteins. Ca$^{2+}$-induced changes in near-UV CD (tertiary structure) are different from Mg$^{2+}$-induced changes, suggesting selective ionic sensor role for NCS-1. The use of E120Q mutants suggests that, although this mutation almost abolished the changes in far-UV CD, it did not affect the changes in the tertiary structure (near-UV CD), suggesting that most of the conformational transitions take place outside of EF-3. This might be attributed...
to the fact that most of the aromatic residues are not in the vicinity of EF-3.

$^{45}$Ca binding and ITC experiments with non-myristoylated and myristoylated NCS-1 revealed the number of Ca$^{2+}$ binding sites as two and three, respectively. In the case of myristoylated NCS-1, with three Ca$^{2+}$ binding sites, the binding is highly cooperative. The curvature seen in the Scatchard plot (Fig. 1b) also reflects a greater degree of cooperativity in the case of the myristoylated protein. It is interesting to note that, upon the binding of Ca$^{2+}$ ($K_1$) to the first site in myristoylated NCS-1, the apparent binding affinity of Ca$^{2+}$ ($K_2$) to the second site increases almost 1000 times, which is 50 times larger than the apparent binding affinity for the two equal sites in non-myristoylated NCS-1 ($K_2$ for myristoylated protein is $1.89 \times 10^7$ M$^{-1}$, whereas $K$ in the case of the non-myristoylated protein is $5.47 \times 10^5$ M$^{-1}$). These apparent binding affinities and binding sites measured by ITC were supported by our $^{45}$Ca binding experiments, also showing the difference in the Ca$^{2+}$ binding affinity of myristoylated and non-myristoylated NCS-1. Our results corroborate the data of Cox et al. (24), which report only two sites but an allosteric effect (1660-fold) for non-myristoylated NCS-1. In comparison, Ames et al. (20) have reported three sites for yeast frq1 under saturation in the absence of Mg$^{2+}$ ($K_{EFP}$ = 10 μM; $K_{EPF}/K_{FEP} = 0.4$ μM). These variations might be the result of the differences in the structure of yeast frq1 and mammalian NCS-1.

The data showing that myristoylation affects Ca$^{2+}$ binding and dynamics were supported by Trp fluorescence and potassium iodide quenching. The two Trp residues of NCS-1 at positions 30 and 103 form part of the E-helices of EF-1 and EF-3, indicating a dynamic role played by myristoylation in the presence of Ca$^{2+}$, which becomes accessible to ANS binding in the presence of Ca$^{2+}$. Quenching was different when Ca$^{2+}$ binding decreased the ANS fluorescence of non-myristoylated NCS-1 and increased the fluorescence of myristoylated NCS-1. In comparison, Ames et al. (20) have reported three sites for yeast frq1 under saturation in the absence of Mg$^{2+}$ ($K_{EFP}$ = 10 μM; $K_{EPF}/K_{FEP} = 0.4$ μM). These variations might be the result of the differences in the structure of yeast frq1 and mammalian NCS-1.

We have used ANS fluorescence to study myristoylation-dependent conformational changes in NCS-1. This probe has been used earlier to monitor the presence of the switch by measuring the increase in the accessibility of the Ca$^{2+}$-dependent solvation of recoverin (36). Ca$^{2+}$ binding decreased the ANS fluorescence of non-myristoylated NCS-1 and increased the fluorescence of the myristoylated protein. This increased ANS fluorescence upon binding Ca$^{2+}$ seen in the case of myristoylated NCS-1 reflects the possible exposure of the myristoyl group, which becomes accessible to ANS binding in the presence of Ca$^{2+}$. Mg$^{2+}$ was not able to increase the ANS fluorescence of myristoylated NCS-1 as Ca$^{2+}$ did, indicating the myristoyl-sensitive conformational changes by Ca$^{2+}$ and not by Mg$^{2+}$. In both non-myristoylated and myristoylated E120Q mutants, the ANS fluorescence was increased in the presence of Ca$^{2+}$, with a greater increase in the myristoylated mutant. This indicates that myristoylation affects the packing and hydrophobic properties in relation to Ca$^{2+}$ binding. The effect of Mg$^{2+}$ on both mutants has not been very different from the non-myristoylated NCS-1 mutant, indicating that Mg$^{2+}$ binding properties were not altered by the E120Q mutation.

Ca$^{2+}$-dependent binding of myristoylated NCS-1 to POPC also indicates the possible extrusion of myristoyl chain from the protein core, although the binding to other lipids was not enhanced significantly in the presence of Ca$^{2+}$. Because of the complex nature of the interaction of NCS-1 with membranes, this does not allow us to explain the preference for particular

lipid vesicles. The membrane interaction of NCS-1 is not likely to be mediated by electrostatic interactions, because there is no net charge on the N terminus. However, we can suggest that myristoylation facilitates the binding of NCS-1 to specific membranes and thus allows interactions with target proteins. The interaction of NCS-1 with phosphatidylinositol 4-OH kinase β and its modulation is dependent on myristoylation (37). Myristoylation of NCS-1 is important for the direct interaction with phosphatidylinositol 4-OH kinase β and up-regulation of its activity, corroborating the importance of myristoylation for regulating the physiological activity of NCS-1. Interestingly, the myristoylation mutant of yeast Frq1 is also less efficient in rescuing the phenotype of the null mutant, suggesting that myristoylation is important for the biological activity of yeast Frq1 as well (10).

In conclusion, our data show that the myristoylation influences the Ca$^{2+}$ binding. We believe that related Ca$^{2+}$-binding proteins would show a similar pattern in their myristoylated form with respect to Ca$^{2+}$ binding. In fact, Ladant (38) has made similar observations of the restricted conformational changes in myristoylated neurolacin whose crystal structure is similar to the human non-myristoylated NCS-1 structure (39). It will be important to determine how the myristoylation-restricted calcium-induced changes in conformation and affinity relate to the various physiological roles of these calcium sensors/calcium-binding proteins. Determining the solution structure of myristoylated NCS-1 will help dissect out common principles that govern the structure-function relationships of these proteins. This is of utmost importance, because the release of the neurotransmitter and exocytosis in general is regulated strongly by the intracellular calcium concentration.

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N-terminal Myristoylation Regulates Calcium-induced Conformational Changes in Neuronal Calcium Sensor-1

Andreas Jeromin, Dasari Muralidhar, Malavika Nair Parameswaran, John Roder, Thomas Fairwell, Suzanne Scarlata, Louisa Dowal, Sourajit M. Mustafi, Kandala V. R. Chary and Yogendra Sharma

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