Equilibrium Unfolding of Neuronal Calcium Sensor-1

N-TERMINAL MYRISTOYLATION INFLUENCES UNFOLDING AND REDUCES PROTEIN STIFFENING IN THE PRESENCE OF CALCIUM*

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Neuronal calcium sensor-1 (NCS-1), a Ca\(^{2+}\)-binding protein of the calcium sensor family, modulates various functions in intracellular signaling pathways. The N-terminal glycine in this protein is myristoylated, which is presumably necessary for its physiological functions. In order to understand the structural role of myristoylation and calcium on conformational stability, we have investigated the equilibrium unfolding and refolding of myristoylated and non-myristoylated NCS-1. The unfolding of these two forms of NCS-1 in the presence of calcium is best characterized by a five-state equilibrium model, and multiple intermediates accumulate during unfolding. Calcium exerts an extrinsic stabilizing effect on both forms of the protein. In the absence of calcium, the stability of both forms is dramatically decreased, and the unfolding follows a four-state equilibrium model. The equilibrium transitions are fully reversible in the presence of calcium. Myristoylation affects the pattern of equilibrium transitions substantially but not the number of intermediates, suggesting a structural role. Our data suggest that myristoylation reduces the stiffening of the protein during initial unfolding in the presence of calcium. The effects of myristoylation are more pronounced when calcium is present, suggesting a relationship between them. Inactivating the third EF-hand motif (E120Q mutant) drastically affects the equilibrium unfolding transitions, and calcium has no effect on these transitions of the mutants. The unfolding transitions of both forms of the mutants are similar to the transitions followed by the apo forms of myristoylated and non-myristoylated NCS-1. These results suggest that the role of myristoylation in unfolding/refolding of the protein is largely dependent on the presence of calcium.

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Neuronal calcium sensor-1 (NCS-1), also known as frequenin in Drosophila and Xenopus, is a Ca\(^{2+}\)-binding protein of the calcium sensor family (1). It has three functional EF-hand motifs and one non-functional motif (EF1), and it is myristoylated at the N terminus (2). There are several proteins with N-terminal modifications that have two or three functional EF-hand motifs, such as recoverin, hippocalcin, and so forth (3). In some of these proteins, the generally buried myristoyl group is ejected out upon binding Ca\(^{2+}\). This switch, called the Ca\(^{2+}\)-myristoyl switch (4, 5), is known to function in many of these proteins.

Protein myristoylation is a post-translational covalent attachment of myristate, a 14-carbon chain of saturated fatty acid, to the N-terminal Gly (6). In proteins, this lipid modification is performed by an enzyme, myristoyl CoA-protein N-myristoyltransferase. The various physiological functions of the proteins of this subfamily largely depend on myristoylation that participates in a variety of signal transduction cascades. Various functions of NCS-1, such as neurotransmitter release and modulation of phosphatidylinositol 4-kinase, depend on myristoylation (7). In some proteins, such as guanylate cyclase-activating proteins, myristoylation is required for the fine-tuning of their activity (8). However, the actual structural and functional role of N-terminal myristoylation is not known. These calcium sensor proteins function via two interrelated switches, a calcium switch and a myristoyl switch (5). Although the structural role of calcium in these proteins has been studied in modulating conformational transitions from one form to another, the role of myristoylation has not yet been defined clearly.

NCS-1 is a multifunctional protein (9) and is expressed not only in neuronal tissues but also in other non-neuronal tissues. It is a high affinity Ca\(^{2+}\)-binding protein with three functional sites (10). It participates in neurotransmitter release, is a modulator of phosphatidylinositol 4-kinase β (11) and potassium channels (12), and modulates short-term plasticity (13). Although the three-dimensional structures of non-myristoylated frequenin from Drosophila and human NCS-1 were solved recently (14, 15), the structure of myristoylated NCS-1 has not yet been solved. The presence of a functional calcium-myristoyl switch in NCS-1 or its functional role is not yet known. The conformation and structure of the myristoylated form of NCS-1 are not precisely characterized. It is also not clear whether N-terminal myristoylation influences the conformational stability or structure of the protein. The role of Ca\(^{2+}\) on the stability of NCS-1 is also not understood. To understand the role of myristoylation and Ca\(^{2+}\) and the interplay between them, it would be pertinent to study the unfolding behavior of NCS-1 under various conditions. Apart from our interest in the NCS-1 structure and mechanism, the protein folding studies are of general interest as an example of a complex unfolding process as well as of the interaction of calcium and the myristoyl group.

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† The abbreviations used are: NCS-1, neuronal calcium sensor-1; ANS, 8-anilino-1-naphthalene sulfonic acid; GdmCl, guanidinium chloride.
With this objective, we have studied the equilibrium unfolding of myristoylated NCS-1 and compared it to non-myristoylated protein. We have studied the role of calcium on the conformational stability of both forms of the protein and folding pathways. We have shown that holo NCS-1 undergoes multistate (five-state) unfolding transitions, whereas in the absence of Ca$^{2+}$, there is a four-state transition. In the presence of calcium, the stability of protein increases, suggesting an extrinsic stabilization effect. There are substantial differences in the pattern of folding of myristoylated and non-myristoylated NCS-1, particularly in the presence of calcium. Myristoylation appears to decrease protein stiffening, thereby increasing the flexibility. The equilibrium unfolding transitions of apo forms of myristoylated and non-myristoylated NCS-1 are similar to both forms of a mutant (in calcium-bound form) in which EF3 was disabled by replacing E120Q.

**MATERIALS AND METHODS**

Guanyldinium chloride was purchased from Serva, phenyl-Sepharose was purchased from Amersham Biosciences, and standard calcium chloride solution was obtained from Sigma.

Preparation of Myristoylated and Non-myristoylated NCS-1 and E120Q Mutants—Myristoylated and non-myristoylated NCS-1 were prepared by overexpressing rat cDNA cloned in pET21a vector. For preparation of the myristoylated protein, it was co-transformed with pBB131 (kindly provided by Dr. Jeffery Gordon, Washington University, St. Louis, MO) to express N-myristoyltransferase as described previously (9, 16). The third EF-hand (EF3) was disabled by site-directed mutation in amino acid 120 replacing Glu with Gln (E120Q) using the QuikChange mutagenesis kit (Stratagene). The mutated gene was subcloned in pET21a vector. Proteins were purified by hydrophobic interaction chromatography using a phenyl-Sepharose column. The proteins were bound to the column in binding buffer (50 mM Tris, 1 mM CaCl$_2$, and 1 mM MgCl$_2$) and eluted with elution buffer (50 mM Tris, 3 mM EGTA, and 1 mM MgCl$_2$). Proteins were concentrated and made Ca$^{2+}$-free by exchange with Chelex-treated buffer in ultrafiltration concentrator (Amicon).

**CD Measurements**—All CD spectra were recorded on a Jasco J-715 spectropolarimeter, using the appropriate path length for near- and far-UV CD. All spectra were recorded in 50 mM Tris buffer (pH 7.2), containing 100 mM KCl, 1 mM dithiothreitol, and the appropriate concentration of GdmCl, in the presence of calcium or EDTA. Spectra were corrected for the buffer baseline.

**Equilibrium Unfolding Experiments**—Unfolding experiments were performed by denaturation in GdmCl. An 8 M stock solution of GdmCl was prepared, and the concentration of this solution was determined by a digital refractometer. Protein solutions containing 0–7 M GdmCl in the presence of 5 mM calcium chloride solution was obtained from Sigma.

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was prepared in methanol. ANS was added to final concentration of 100 μM in protein solutions prepared for unfolding experiments in GdmCl. The samples were incubated for 30 min, and fluorescence emission spectra were recorded with the excitation wavelength set at 365 nm. ANS spectra were corrected for ANS fluorescence taken in buffer. The spectra were analyzed by intensity and wavelength.

Controlled Trypsin Digestion—Partial trypsin digestion was carried out at room temperature with protein:enzyme ratio maintained at 100:1. Proteolysis was stopped at the desired time by the addition of SDS-PAGE sample buffer and phenylmethylsulfonyl fluoride. The samples were analyzed by SDS-PAGE on a 15% polyacrylamide gel.

RESULTS

Purification of Myristoylated and Non-myristoylated NCS-1—NCS-1 was overexpressed as described under “Materials and Methods.” Myristoylation was catalyzed by N-myristoyl transferase co-expressed with NCS-1 in bacterial cells. The expressed proteins were purified by single-step hydrophobic interaction chromatography using phenyl-Septharose. The purity and proper folding of the recombinant protein were checked by SDS-PAGE and far-UV CD. In case of any impurity, a final step of purification on Superose 12 was employed. NCS-1 was myristoylated, which was confirmed by electrospray ionization mass spectrometry analysis; the mass of the myristoylated protein is 210 Da more than that of the non-myristoylated protein.

Unfolding of NCS-1 Monitored by Trp Fluorescence—To study the transitions of unfolding and the influence of myristoylation and calcium, we performed equilibrium unfolding of myristoylated and non-myristoylated NCS-1 monitored by Trp fluorescence. NCS-1 has two Trp residues at positions Trp30 and Trp103, probably each located in a separate domain. The emission maximum of NCS-1 is about 336 nm, suggesting that these are not deeply buried inside the protein core. Fig. 1 shows the fluorescence emission spectra of myristoylated and non-myristoylated NCS-1 at various concentrations of GdmCl in the presence and absence of calcium. As seen in Fig. 1, a–d, there are substantial differences in the spectra of myristoylated and non-myristoylated NCS-1. The fluorescence intensity increased to a greater degree in the holo myristoylated protein than in the holo non-myristoylated NCS-1, before decreasing at higher concentrations of GdmCl. In the absence of calcium, there is no increase in the fluorescence intensity. This is clearly seen when the fluorescence intensity of myristoylated and non-myristoylated NCS-1 at emission maxima in the absence and presence of excess calcium is plotted against the GdmCl concentration (Fig. 2). At subdenaturing concentrations of GdmCl (up to 1.5 M), there was an increase in the emission intensity followed by a decrease at a higher denaturant concentration (Fig. 2). There was a sharp increase in the intensity maximum followed by decrease at a higher concentration of GdmCl in the case of non-myristoylated protein in its holo form (Fig. 2a). The increase in the intensity was greater and remained at a higher value than the original intensity in the case of myristoylated NCS-1 with up to 1.5 M of GdmCl. The difference between these two proteins was also seen in the absence of calcium (Fig. 2b). These results suggest that myristoylation influences the unfolding of NCS-1, indicating that myristoylation plays an important structural role.

Unfolding Transitions of EF3 Mutants E120Q and Apo Wild Type NCS-1 Are Similar—EF3 is known to be the strongest calcium-binding site; hence, it was disabled by mutation to a non-functional site (E120Q). The effect of this point mutation was studied by equilibrium unfolding. The fluorescence and CD spectral properties of mutants were similar to those of wild type proteins, indicating that E120Q mutants have similar global secondary structures comparable to wild type proteins (10). Fig. 3a shows the changes in wavelength maxima versus GdmCl concentration of myristoylated and non-myristoylated NCS-1. From the graph, it appears to be a multiphasic transition. Although plots for both proteins in the presence of calcium follow similar patterns, there are minor differences in the presence of 1–2 and 4–5 M GdmCl. In the absence of calcium, there was a sharp shift in the wavelength maxima, and the spectra of both proteins overlapped (Fig. 3a). In this case, the mutant proteins were unfolded at lower concentrations of GdmCl (3.5 M). These results suggest that disabling EF3 decreases the protein stability and that calcium or myristoylation does not influence the unfolding of mutant proteins.

Fig. 3b shows the unfolding of myristoylated and non-myristoylated E120Q mutant in the presence of calcium. There was apparently no effect of calcium or myristoylation on the equilibrium unfolding transitions of both forms of the mutant, suggesting that mutating this residue alone severely reduces the stability of the protein and that calcium no longer exerts its extrinsic stabilizing effect. As seen from this figure, the unfolding pattern of mutants is similar to the unfolding transition of wild type proteins in the absence of calcium (Fig. 3a). In the absence of calcium, the unfolding transitions of myristoylated
and non-myristoylated NCS-1 and the E120Q mutant followed a similar pattern, suggesting that calcium and the third EF-hand in the E120Q mutant play important roles in modulating the unfolding pathways.

**Unfolding of NCS-1 in Ca²⁺-bound Form Follows the Five-state Equilibrium Model**—The equilibrium unfolding transitions of myristoylated and non-myristoylated NCS-1 were studied by measuring the intensity of emission fluorescence at 340 nm. We quantitated the equilibrium transitions by fitting them to the equations as described under “Materials and Methods.” The fit was not satisfactory when we performed the unfolding experiments with 30 data points between 0 and 6 M GdmCl (residual value was >30%). Increasing the number of data points improved the fit. It was suggested previously that if the number of data points was lower, there is a possibility of missing some intermediates (17). We therefore performed the unfolding experiment with a large data set of 88 points between 0 and 7 M GdmCl for apo and holo myristoylated and non-myristoylated NCS-1. This way, we could take care of any anomaly in the fitting, which could occur in the unfolding of a complex protein such as NCS-1.

In the case of myristoylated and non-myristoylated proteins in their holo forms, the unfolding data best fitted a five-state transition model (Fig. 4, a and b). For the apo form, the data were best fitted to the four-state transition model (Fig. 4, c and d), suggesting that calcium influences the transitions significantly. However, we did not see an effect of myristoylation on the number of intermediates during unfolding transitions in the absence of calcium.

The thermodynamic parameters for unfolding process are shown in Table I. We did notice the significant differences in free energy change ($\Delta G^0$) of unfolding of myristoylated and non-myristoylated NCS-1; the $\Delta G^0_2$ and $\Delta G^0_3$ were comparatively higher in case of holo myristoylated (3.35 and 6.65 kcal mol⁻¹) than non-myristoylated (1.3 and 4.2 kcal mol⁻¹) NCS-1, suggesting higher conformational stability of these intermediates in the case of myristoylated protein (Table I). $\Delta G^0_1$ is smaller for holo myristoylated protein, suggesting the lower stability of its first intermediate as compared with non-myristoylated NCS-1. The differences were also obvious when the unfolding of apo and holo proteins was compared (Table I). The global $\Delta G^0$ for N to D transition was greater in case of holo myristoylated NCS-1 (19.1 kcal mol⁻¹) than non-myristoylated (14.5 kcal mol⁻¹) protein, suggesting that myristoylation increases protein stability and complements the extrinsic stabilizing effect of calcium. When myristoylated and non-myristoylated NCS-1 were compared, we found that myristoylation increased the free energy of unfolding, suggesting that it plays an important role in protein stability.

To determine the fractional accumulation of the different species upon GdmCl-induced unfolding, the equilibrium distribution of the four or five species, $N$, $I_1$, $I_2$, $I_3$, and $U$, as a function of GdmCl concentration could be reconstituted using the fitted values of $\Delta G^0_1$, $\Delta G^0_2$, $\Delta G^0_3$, $m_1$, $m_2$, and $m_3$ (Fig. 5). As seen from this figure, there was no apparent difference between the apo form of myristoylated and non-myristoylated NCS-1. However, noticeable differences were seen in the $f_1$ and $f_2$ fractions, which were obviously greater in the case of the holo form of myristoylated NCS-1; $f_3$ was less in case of myristoylated NCS-1. These results suggest that myristoylation influences the unfolding transitions when calcium is present, whereas myristoylation has a minimal effect on unfolding in the absence of calcium.
Sharp Increase in Negative Ellipticity of Holo Non-myristoylated NCS-1 at Subdenaturing GdmCl Concentrations Monitored by Far-UV CD—

The unfolding transitions were also followed by monitoring the changes in the far-UV CD spectra of myristoylated and non-myristoylated wild type and their E120Q mutants. In the presence of Ca$^{2+}$, the negative ellipticity of myristoylated and non-myristoylated NCS-1 increased at a subdenaturing concentration of GdmCl (up to 1 M), with a greater increase in the case of the non-myristoylated protein (25% at 222 nm) as compared with myristoylated NCS-1 (4% at

Fig. 4. Equilibrium unfolding of myristoylated and non-myristoylated NCS-1. Unfolding was measured by fluorescence emission intensity at 340 nm with an excitation wavelength of 295 nm. Symbols represent experimental values, and the line through the symbols represents the best fit according to the equation described under “Materials and Methods.” a, holo myristoylated NCS-1; b, holo non-myristoylated NCS-1; c, apo myristoylated NCS-1; d, apo non-myristoylated NCS-1. Fitting residues are shown for each set of data.
The increase in optical activity is seen at the ultra-subdenaturing concentrations (in lower micromolar limits) of GdmCl (Fig. 7, inset). This increase in the negative value of ellipticity at subdenaturing concentrations of GdmCl was not seen in the absence of calcium (data not shown). The increase in negative value of far-UV CD is also seen in case of E120Q non-myristoylated NCS-1 mutants, albeit comparatively very low (3–4%) (Fig. 7).

At higher concentrations of GdmCl, the ellipticity decreased, and finally, the protein was converted to random coil at 6 M GdmCl. In the absence of calcium, the protein is completely unfolded at a much lower concentration (3.5 M).

More Hydrophobic Patches Exposed during Unfolding of Holo Non-myristoylated NCS-1—ANS fluorescence is frequently used for characterization of the hydrophobicity of native proteins and identification of non-native conformations in globular proteins (21, 22). ANS is also used to investigate whether unfolding intermediates are formed. We monitored the change in ANS fluorescence during unfolding (Fig. 8). The fluorescence of the dye in the presence of protein increased with increasing concentration of GdmCl (Fig. 8a) and then declined at higher concentrations with no change in the emission maximum (480 nm). At a concentration of up to 1 M GdmCl, ANS fluorescence increased in both myristoylated and non-myristoylated proteins, suggesting that intermediates with more hydrophobic patches are formed during early unfolding. The increase in fluorescence emission was significantly greater in the non-myristoylated protein than in myristoylated NCS-1 (Fig. 8a), suggesting a greater exposure of hydrophobic residues in the case of non-myristoylated NCS-1 than in myristoylated protein. Because this variation in ANS fluorescence is solely due to myristoylation, it suggests that myristoylation plays a structural role in the presence of calcium. In the absence of calcium, there is only a transient increase in ANS fluorescence (Fig. 8b), suggesting that calcium plays a major role in the unfolding of these proteins. In both proteins, at 4–6 M GdmCl, the fluorescence of the dye in the presence of the protein was almost the same as that of free dye.

| Table I | Thermodynamic parameters for GdmCl-induced unfolding of myristoylated (Myr) and non-myristoylated (nonmyr) NCS-1 in the presence or absence of calcium monitored by fluorescence |
|---------|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
|         | Apo-Myr-NCS-1 | Holo-Myr-NCS-1 | Apo-nonmyr-NCS-1 | Holo-nonmyr-NCS-1 |
| $\Delta G^0_1$ | 2.42 ± 0.2681 | 0.3468 ± 0.05776 | 2.983 ± 0.3251 | 1.013 ± 0.2255 |
| $m_1$     | 4.758 ± 0.5176 | 2.32 ± 0.3935 | 5.789 ± 0.6185 | 9.888 ± 3.484 |
| $\Delta G^0_2$ | 5.967 ± 0.7895 | 3.355 ± 0.8915 | 4.542 ± 0.4481 | 1.311 ± 0.5633 |
| $m_2$     | 1.937 ± 0.2553 | 2.397 ± 0.6271 | 1.556 ± 0.1528 | 1.507 ± 0.6243 |
| $\Delta G^0_3$ | 7.301 ± 2.24  | 6.658 ± 0.7134 | 5.41 ± 1.545  | 4.264 ± 0.2441 |
| $m_3$     | 1.156 ± 0.3601 | 2.636 ± 0.2748 | 0.8563 ± 0.2537 | 1.752 ± 0.09903 |
| $\Delta G^0_4$ | 8.769 ± 0.6982 | 8.769 ± 0.6982 | 7.922 ± 0.6986 |
| $m_4$     | 1.614 ± 0.1278 | 1.614 ± 0.1278 | 1.491 ± 0.131 |
| Global $\Delta G^0_{N-D}$ | 15.68 ± 1.45 | 19.14 ± 1.85 | 12.93 ± 0.71 | 14.51 ± 1.6 |

**Fig. 5.** Distribution of the native, intermediate, and unfolded states of NCS-1 as a function of GdmCl concentration. The fractions of the native ($f_n$), first intermediate ($f_1$), second intermediate ($f_2$), third intermediate ($f_3$), and unfolded ($f_d$) states were calculated using parameters deduced from the fits of the unfolding transitions. a, apo non-myristoylated NCS-1; b, apo myristoylated NCS-1; c, holo non-myristoylated NCS-1; d, holo myristoylated NCS-1.
The Unfolding Process Is Reversible in the Presence of Calcium—We investigated whether the unfolding process is reversible by allowing the protein to refold. Refolding was monitored by the change in fluorescence emission wavelength maxima. The unfolding process was found to be completely reversible when calcium was present, as seen in the emission maximum, which was 336 nm (Fig. 9a). In the absence of calcium, protein was partially refolded because the emission maximum remained at 342 nm when protein was refolded back (Fig. 9c).

When refolded data were plotted as fluorescence intensity, their intensity increased with the decrease of GdmCl concentration, at a concentration of <0.1 M, the intensity decreased, and the refolded plot looks almost similar to unfolded data. Here, we also noticed clear variations in the unfolding transitions of myristoylated and non-myristoylated NCS-1 (Fig. 9). These experiments indicate that transitions occurring in the presence of calcium are readily reversible, whereas transitions in the absence of calcium occurring at lower concentrations of GdmCl are not fully reversible. The effect of myristoylation was more prominently visible during refolding in the presence of calcium.

**Fig. 6.** Change in far-UV CD spectra of (a) myristoylated and (b) non-myristoylated NCS-1 with varying concentrations of GdmCl in the presence of calcium. The experiment was performed in 50 mM Tris, pH 7, containing 100 mM KCl in the presence of 5 mM CaCl₂. The numbers 0, 1, 2, 3, 4, and 5 in a represent 0, 0.02, 1, 2.8, 3.5, and 6 M GdmCl, respectively. The numbers 0, 1, 2, 3, 4, and 5 in b represent 0, 0.05, 1, 1.8, 3.2, and 6 M GdmCl, respectively.

**Fig. 7.** Percentage change in ellipticity at 222 nm with GdmCl in the presence of calcium. a, wild type myristoylated and non-myristoylated NCS-1. Inset in a shows the percentage change in ellipticity at 222 nm with subdenaturing concentrations (0–2 M) of GdmCl. b, E120Q mutants of myristoylated and non-myristoylated NCS-1. Protein concentration was 18 μM.

**Fig. 8.** Change in ANS fluorescence intensity (at 487 nm) during unfolding of myristoylated and non-myristoylated NCS-1 in the presence (a) and absence (b) of calcium. The experimental conditions were the same as those described in the Fig. 1 legend. Protein concentration was 36 μM. ANS concentration was 120 μM. Solid lines, myristoylated NCS-1; dotted lines, non-myristoylated NCS-1.
calcium, as seen in the difference of the fluorescence intensity plots (Fig. 9).

Calcium Binding Increases the Rigidity and Protects the Protein from Limited Proteolysis—Limited proteolysis by trypsin has been applied to investigate the conformational flexibility of proteins and the effect of calcium and myristoylation. NCS-1 possesses many lysine and arginine residues, and many of them are located at the N terminus and C terminus. In the presence of calcium, trypsin digestion yields two major bands of about 18 and 16 kDa even when myristoylated and non-myristoylated proteins were digested for 2 h at room temperature (Fig. 10, a and b). However, in the absence of calcium, we do not see any of these bands, and the protein is completely digested within a few minutes of incubation with trypsin (Fig. 10c). This suggests that there were subtle changes in the domain conformation, and the protein gained rigidity upon calcium binding. These two bands were also seen when up to 500 mM GdmCl was present (Fig. 11) and start disappearing when the denaturant concentration was increased to 1 M, suggesting that protein rigidity is lost or that domains start “melting” when GdmCl is present. This might be happening due to the loss of calcium binding during unfolding. The intensity of these two bands is much lower in the case of E120Q mutants, suggesting increased susceptibility of mutant proteins to trypsin.

DISCUSSION

A combination of fluorescence and circular dichroism spectroscopy was used to investigate the equilibrium unfolding of myristoylated and non-myristoylated NCS-1 induced by GdmCl in the presence and absence of calcium. To our knowledge, this is the first protein of the calcium sensor subfamily whose unfolding is described. Unfolding transitions were used to ascertain the impact of N-terminal myristoylation, calcium, and EF3 on the structural and functional consequences of NCS-1. It has been shown in the case of recoverin and NCS-1 that N-terminal myristoylation induces cooperativity for calcium binding and hence influences the local or global conformation (10, 23). One of these aspects is possibly interrelated to the calcium-myristoyl switch, at least in recoverin (23).

The unfolding of a protein of the size of NCS-1 is expected to be complex. Large proteins are not likely to follow all of the folding principles established for smaller proteins. They probably have more complex pathways of folding so that models based on studies of small globular proteins may not fully explain the folding and assembly of large proteins (24), as seen in the case of NCS-1. We observed that both myristoylated and non-myristoylated NCS-1 followed five-state transitions in the presence of calcium. In the absence of calcium, unfolding followed a four-state transition, and the protein is unfolded at much lower GdmCl concentrations, suggesting an important role played by calcium in providing conformational stability. Calcium is known to enhance the conformational stability of calcium-binding proteins, and a similar extrinsic stabilization effect is also seen in NCS-1. NCS-1 in the presence of excess Ca?+ requires a higher concentration of GdmCl (6 M) for complete unfolding than it does in the absence of calcium (3.5 M GdmCl), suggesting a very important role for calcium in protein conformational stability.
The unfolding transitions of the E120Q mutant of myristoylated NCS-1 are identical in the presence or absence of calcium, suggesting that calcium does not enhance the conformational stability of this mutant, although the mutant binds calcium, albeit with lower affinity (10). These data suggest that inactivating EF3 has a profound effect on the equilibrium transitions and conformational stability of the protein. Calcium or myristoylation does not appear to have any influence on the unfolding of myristoylated and non-myristoylated E120Q mutant. The data suggest that EF3 plays a major role in defining myristoylation-related changes in NCS-1, and if this motif is disabled, myristoylation no longer influences the structure or conformation. The equilibrium unfolding of both forms of wild type protein in the absence of calcium is similar to that of the E120Q mutant, suggesting that (i) inactivating site 3 has a serious impact on the unfolding and stability of NCS-1 and (ii) the presence of calcium does not influence the unfolding transitions of the E120Q mutant, even though the E120Q mutant binds calcium (probably via other EF-hand motifs).

The existence of unfolding intermediates was also revealed by overlapping of unfolding transitions obtained by CD and fluorescence. Because they did not superimpose, this showed that equilibrium intermediates are formed during unfolding. We observed four and five states of transitions in apo and holo NCS-1, respectively. It is expected for a protein of this size to have multistate transitions. The picture that emerges from this study is that the presence of calcium dictates the formation of equilibrium intermediates. Although myristoylation does not affect the number of transitions, it does influence the free energy change of unfolding ($\Delta G^\circ$) and the fraction of intermediates populated, suggesting that there are changes in the conformational stabilities of myristoylated and non-myristoylated NCS-1. ANS was used to follow unfolding of the holo protein. The ANS fluorescence intensity was increased in myristoylated and non-myristoylated NCS-1, suggesting the change in the ANS-binding pockets. When we compared the change in the fluorescence intensity of the dye ANS in the presence of calcium, it was significantly higher in the case of non-myristoylated protein than in myristoylated NCS-1, suggesting that a higher degree of apparent hydrophobicity is exposed during intermediate states in non-myristoylated protein than in myristoylated NCS-1. However, this was not seen in the case of the E120Q mutant, implying that mutating this EF-hand not only decreased the stability but also changed the pattern of unfolding transitions.

The influence of myristoylation on protein structure is best seen during unfolding of NCS-1 by far-UV CD. At subdenaturing and even lower concentrations of GdmCl, the CD signal is increased by 25% in the case of non-myristoylated NCS-1. The apparent increase in the ellipticity value at subdenaturing
concentrations of GdmCl appears to be related to the changes in dihedral angles (27), which might happen due to the binding of denaturant (28). It has been pointed out previously that even small alterations in backbone dihedral angles are known to change optical activity (27, 29, 30) due to rigidity or stiffening of protein structure, perhaps locally (31). Protein stiffening is described as likely changes in phi-psi backbone dihedral angles, which comes at the cost of decreasing entropy or spatial freedom (31, 32). This may not, however, mean a real change in the secondary structure content (27). Based on the substantial increase in CD signal in the case of non-myristoylated NCS-1, we suggest that there are larger changes in the phi-psi dihedral angles (more stiffening) in non-myristoylated protein than in myristoylated NCS-1. We therefore suggest that myristoylation reduces this stiffening in the protein in the presence of calcium, probably by influencing the local or global structure.

We next examined whether the unfolding was reversible; the proteins were subjected to refolding. In the presence of calcium, wild type proteins were completely refolded as seen by Trp fluorescence because the wavelength maximum reverts back to that of native protein. In the absence of calcium, they are partially refolded, suggesting a role of calcium in the refolding process. We also noticed differences in the Trp fluorescence pattern of refolding of myristoylated and non-myristoylated NCS-1 in the presence of calcium, again suggesting that the role of myristoylation depends on the presence of cations.

In conclusion, these results revealed an important role of N-terminal myristoylation and calcium binding in the stability and equilibrium unfolding of NCS-1. Calcium (used in excess for saturating all sites) exerts a significant extrinsic stabilisation effect on the proteins and modulates unfolding transitions. Our data suggest that myristoylation, in fact, affects the equilibrium transitions or unfolding, although it does not influence the number of unfolding intermediates. Myristoylation was found to reduce the stiffening of the protein at subdenaturing concentrations of GdmCl. The influence of myristoylation on unfolding was more prominent when calcium was present. This suggests that there is an interplay between calcium and myristoylation. Whether myristoylation directly affects the structure of NCS-1 is not yet known because the three-dimensional structure of the myristoylated form has not been solved yet. We are currently attempting to solve the structure of the protein. Disabling EF3 by a single mutation drastically affects the conformational stability, and calcium does not influence the unfolding transitions of both forms of the mutant, suggesting that an important role is played by the third EF-hand. We postulate that other proteins of the superfamily might exhibit similar unfolding transitions and that calcium and myristoylation would play a very significant role in conformational stability and unfolding transitions.

REFERENCES
1. Pongs, O., Lindemeier, J., Zhu, X. R., Theil, T., Endelkamp, D., Krah-Jentgens, I., Lambrecht, H. G., Koch, K. W., Schwemmer, J., Rivescrucci, R., Mallart, A., Galceran, J., Canal, I., Barbas, J. A., and Ferrus, A. (1998) Neuron 11, 15–28
2. Cox, J. A., Durussel, I., Comte, M., Nef, S., Nei, P., Lenz, S. E., and Gundelfinger, E. D. (1994) J. Biol. Chem. 269, 32907–32913
3. Burgoyne, R. D., and Weiss, J. L. (2001) Biochem. J. 355, Pt 1, 1–12
4. Zoualya, S., and Streyer, L. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 11569–11573
5. Ames, J. B., Ishima, R., Tanaka, T., Gordon, J. I., Stryer, L., and Ikura, M. (1997) Nature 389, 198–202
6. Farazi, T. A., Waksman, G., and Gordon, J. L. (2001) J. Biol. Chem. 276, 39501–39504
7. Burgoyne, R. D., O’Callaghan, D. W., Hasdemir, B., Haynes, L. P., and Tepikin, A. V. (2004) Trends Neurosci. 27, 203–209
8. Hwang, J. Y., and Koch, K. W. (2002) Biochem. Biophys. Acta 1600, 111–117
9. Hilfiker, S. (2003) Biochem. Soc. Trans. 31, Pt 4, 828–832
10. Jeromin, A., Muralidhar, D., Parmesanwaran, M. N., Roder, J., Fairwell, T., Scarlata, S., Dowal, L., Mustafi, S. M., Chary, K. V., and Sharma, Y. (2004) J. Biol. Chem. 279, 27158–27167
11. Hendricks, K. B., Wang, B. Q., Schnieders, E. A., and Thorner, J. (1999) Nat. Cell Biol. 1, 234–241
12. Guo, W., Malin, S. A., Johns, D. C., Jeromin, A., and Nerbonne, J. M. (2002) J. Biol. Chem. 277, 26436–26443
13. Sippy, T., Cruz-Martin, A., Jeromin, A., and Schweizer, F. E. (2003) Nat. Neurosci. 6, 1031–1038
14. Ames, J. B., Hendricks, K. B., Strahl, T., Huttner, I. G., Hamasaki, N., and Thorner, J. (2000) Biochemistry 39, 12149–12161
15. Bourne, Y., Dannenberg, J., Pollmann, V., Marchot, P., and Pongs, O. (2001) J. Biol. Chem. 276, 11949–11955
16. Fisher, J. B., Sharma, Y., Juliane, S., Picciotti, R. A., Krylov, D., Hurley, J., Roder, J., and Jeromin, A. (2000) Protein Expression Purif. 20, 66–72
17. Hung, H. C., Chen, Y. H., Liu, G. Y., Lee, H. J., and Chang, G. G. (2003) Bull. Math. Biol. 65, 553–570
18. Dignam, J. D., Qu, X., and Chaires, J. B. (2001) J. Biol. Chem. 276, 4028–4037
19. Patel, S., Chaffotte, A. F., Gobard, F., and Pauthe, E. (2004) Biochemistry 43, 1724–1735
20. Akaike, H. (1973) in Second International Symposium on Information Theory (Petrov, B. N., and Csaki, F., eds) pp 281–291, Akademia Kiado, Budapest, Hungary
21. Stryer, L. (1965) J. Mol. Biol. 13, 482–485
22. Samistotonov, G. V., Rodionova, N. A., Razuuvayev, O. I., Uversky, V. N., Grigas, A. F., and Gilmanashin, R. I. (1991) Biopolymers 31, 119–128
23. Ames, J. B., Poremb, T., Tanaka, T., Ikura, M., and Stryer, L. (1995) J. Biol. Chem. 270, 4526–4533
24. Neet, K. E., and Timm, D. E. (1994) Protein Sci. 3, 2167–2174
25. Hamasaki-Katagiri, N., Molchanova, T., Takeda, K., and Ames, J. B. (2004) J. Biol. Chem. 279, 12744–12754
26. O’Callaghan, D. W., and Burgoyne, R. D. (2004) J. Biol. Chem. 279, 14347–14354
27. Hirst, J. D., Bhattacharjee, S., and Onufriev, A. V. (2003) Faraday Discuss. Chem. Soc. 122, 253–267, discussion, 269–282
28. Makhatadze, G. I., and Privalov, P. L. (1992) J. Mol. Biol. 226, 491–505
29. Wilson, G., Hecht, L., and Barron, L. D. (1996) Biopolymers 35, 12518–12525
30. Park, S. H., Shalongo, W., and Stellwagen, E. (1997) Protein Sci. 6, 1694–1700
31. Bhuyan, A. K. (2002) Biochemistry 41, 13386–13394
32. Kumar, R., Prabhu, N. P., Yadaiah, M., and Bhuyan, A. K. (2004) Biophys. J. 87, 2656–2662