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Conformational Dynamics of Recoverin's Ca²⁺-myristoyl Switch Probed by ¹⁵N NMR Relaxation Dispersion and Chemical Shift Analysis

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Abstract

Recoverin, a member of the neuronal calcium sensor (NCS) branch of the calmodulin superfamily, serves as a calcium sensor in retinal rod cells. Ca²⁺-induced conformational changes in recoverin promote extrusion of its covalently attached myristate, known as the Ca²⁺-myristoyl switch. Here we present nuclear magnetic resonance (NMR) relaxation dispersion and chemical shift analysis on ¹⁵N-labeled recoverin to probe main chain conformational dynamics. ¹⁵N NMR relaxation data suggest that Ca²⁺-free recoverin undergoes millisecond conformational dynamics at particular amide sites throughout the protein. The addition of trace Ca²⁺ levels (0.05 equivalents) increases the number of residues that show detectable relaxation dispersion. The Ca²⁺-dependent chemical shifts and relaxation dispersion suggest that recoverin has an intermediate conformational state (I) between the sequestered apo state (T) and Ca²⁺ saturated extruded state (R): T ↔ I ↔ R. The first step is a fast conformational equilibrium ([T]/[I] < 100) on the millisecond time scale (τ_{ex}δω < 1). The final step (I ↔ R) is much slower (τ_{ex}δω > 1). The main chain structure of I is similar in part to the structure of half-saturated E85Q recoverin with a sequestered myristoyl group. We propose that millisecond dynamics during T ↔ I may transiently increase the exposure of Ca²⁺-binding sites to initiate Ca²⁺ binding that drives extrusion of the myristoyl group during I ↔ R.

Keywords

recoverin; NMR; calcium; Ca²⁺-myristoyl switch; NMR relaxation dispersion analysis; vision; phototransduction; EF-hand

INTRODUCTION

Calcium ion (Ca²⁺) in retinal rod cells plays a critical role in regulating phototransduction in vision ^{1–3}. Recoverin, a 23 kDa Ca²⁺-binding protein and member of the EF-hand superfamily, serves as a Ca²⁺ sensor in retinal rods ^{4,5}. Recoverin prolongs the lifetime of photoexcited rhodopsin by inhibiting rhodopsin kinase only at high Ca²⁺ levels ^{5–8}. Hence, recoverin makes the desensitization of rhodopsin responsive to Ca²⁺, and the shortened

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SUPPORTING INFORMATION AVAILABLE

Supporting information includes three tables for selection of the residues and fit parameters; and seven Figures that discuss the fitting with the R₂⁰ constraint, the three-site exchange model, NMR titration at trace Ca²⁺ levels, χ²/n map of the domain fit, ¹⁵N NMR ZZ-exchange data, computer simulation of observed NMR line shapes, and ¹⁵N Chemical shift comparison between the E85Q mutant and wild-type recoverin.

lifetime of photoexcited rhodopsin at low Ca^{2+} levels may promote visual recovery and contribute to the adaptation to background light. More recently, recoverin was shown to have a different role in synaptic termini and was found localized in the rod inner segment⁹. Upon light activation (at low Ca^{2+} levels), 98% of recoverin is detected in the rod inner segment, whereas in the dark (high Ca^{2+} levels), more than 10% of recoverin returns to the outer segment¹⁰. This light-dependent protein translocation is likely facilitated by a Ca^{2+} -myristoyl switch mechanism described below.

Recoverin belongs to a family of neuronal calcium sensor (NCS) proteins¹¹, including NCS-1¹², neurocalcin¹³, and hippocalcin¹⁴ (Fig. 1). The common structural features of NCS proteins are an approximately 200-residue chain containing four EF-hand motifs, the sequence CPXG (where X is a non-conserved residue) in the first EF-hand that markedly impairs its capacity to bind Ca^{2+} , and an amino-terminal myristoylation consensus sequence. Recoverin binds to two Ca^{2+} at the second and third EF-hands (EF2 and EF3 in Fig. 1)¹⁵ that induces the binding of myristoylated, but not unmyristoylated, recoverin to rod outer segment disc membranes^{16,17}. The three-dimensional structures of myristoylated recoverin with 0 and 2 Ca^{2+} bound have been determined by NMR spectroscopy^{18–20}. In the Ca^{2+} -free state, the myristoyl group is sequestered in a deep hydrophobic cavity inside the protein, forming a compact structure called the T state (Fig. 2A). The binding of two Ca^{2+} causes a large protein conformational change that leads to extrusion of the N-terminal myristoyl group, forming an elongated form of the protein called the R state (Fig. 2B). The Ca^{2+} -induced exposure of the myristoyl group, termed Ca^{2+} -myristoyl switch, enables recoverin to bind to membranes only at high Ca^{2+} ^{21,22}. Although much is known about the structures of NCS proteins, very little is known about the dynamics of the Ca^{2+} -myristoyl switch.

We report here a ^{15}N NMR relaxation dispersion and chemical shift analysis on recoverin to probe the dynamics of Ca^{2+} -induced conformational changes. Our NMR analysis reveals backbone conformational dynamics on the millisecond timescale for residues in both the N-domain and C-domain, particularly in the helices at the domain interface. The Ca^{2+} -induced conformational changes do not proceed through a simple two-state mechanism as initially suggested¹⁵, but rather the Ca^{2+} -free sequestered form of recoverin (T^0) converts into a structurally distinct intermediate state (I). In the absence of Ca^{2+} , the equilibrium ratio $[\text{T}]/[\text{I}]$ is estimated to be ≤ 100 . We suggest that the main chain structure of I (inferred from $\delta\omega$ for each residue) may be somewhat similar to that of half saturated E85Q recoverin¹⁹ that contains a sequestered myristoyl group. We propose that rapid Ca^{2+} binding to the intermediate state ($\text{T} \leftrightarrow \text{I}$) is coupled to slow extrusion of the myristoyl group during $\text{I} \leftrightarrow \text{R}$.

MATERIALS AND METHODS

Expression and purification of myristoylated recoverin

Per-deuterated and ^{15}N -labeled myristoylated recoverin was expressed in *E. coli* strain, DH5 α , co-transfected with plasmids, pTREC2 and pBB131 as described previously¹⁶. The cells were grown in M9-broth in 90% $^2\text{H}_2\text{O}$ at 32° C until the cell culture reached an optical density of 0.7–0.8 at 600 nm. The myristoyl-CoA transferase expression was induced by adding IPTG (0.5 mM). Recoverin expression was induced 30 min. later by rapidly raising the temperature to 42° C and growing the cells for an additional 4 hrs. The protein purification procedure was similar to that used previously^{19,23}. Briefly, the cells were disrupted using a French Pressure Cell (Spectronic Instruments, Inc. USA) and the extract was clarified by centrifugation for 1.5 hrs at 4° C. The crude extract was treated with CaCl_2 (3 mM), adsorbed onto a Phenyl Sepharose CL-4B (Pharmacia Biotech) column at 4° C and washed thoroughly using high Ca^{2+} buffer. The Ca^{2+} -free protein was eluted using Ca^{2+} -free buffer containing EGTA. The protein was further purified using Q-Sepharose

chromatography at room temperature and then concentrated to a final concentration of 10 mg/ml using a Centricon YM-10 microconcentrator (Millipore Corp., USA). The overall yields were 10 mg from 1 L cultured cells. Proteins were aliquoted, frozen by liquid nitrogen and stored at -80°C . Protein concentrations in this study were determined by measuring the optical density at 280 nm and using a molar absorption coefficient, $\epsilon = 23,950\text{ cm}^{-1}\text{ M}^{-1}$. The final purified recoverin sample was more than 98% pure as judged by SDS-PAGE, and more than 95% myrisoylated as confirmed by reverse-phase HPLC and mass spectrometry analysis.

NMR Spectroscopy

Two samples of recoverin were prepared for NMR experiments: one without Ca^{2+} and the other with a slight concentration of Ca^{2+} (0.05 equivalents to the protein concentration, denoted 5% Ca^{2+}). The protein for the NMR experiments consisted of per-deuterated and ^{15}N -labeled protein (0.5 mM) in 0.3 ml of a 95% H_2O /5% $^2\text{H}_2\text{O}$ solution containing either 10 mM sodium phosphate (pH 7.0) and 1 mM EGTA for the Ca^{2+} -free sample or 10 mM Tris- d_{11} (pH 7.0) for the 5% Ca^{2+} sample. The choice of buffer (10 mM phosphate or Tris) had no effect on the NMR spectrum of Ca^{2+} -free recoverin. The Ca^{2+} -free NMR sample was confirmed to be >99.9% in the apo-state by monitoring a characteristic NMR resonance at 10.0 ppm (assigned to G115 that represents Ca^{2+} -free recoverin with signal-to-noise ratio of 1000) and the lack of any detectable NMR intensity at 10.72 ppm (assigned to G115 that represents Ca^{2+} -bound recoverin). The NMR samples (both Ca^{2+} -free and at 5% Ca^{2+}) were confirmed to be monodisperse and contain only monomeric protein as judged by dynamic light scattering and pulsed-field gradient diffusion measurements described by ²⁴. All NMR experiments were performed at 30°C on Bruker Avance III 600 or 800 MHz spectrometers equipped with a four-channel interface and triple-resonance cryogenic-probe (TCI) with pulsed field gradients.

^{15}N R_1 , R_2 , and NOE Experiments

^{15}N longitudinal relaxation rates (R_1) and transverse relaxation rates (R_2) of recoverin in the absence and presence of 5% Ca^{2+} were measured using pulse schemes described previously at 81.08 MHz ^{15}N resonance frequency ²⁵. $\{^1\text{H}\}$ - ^{15}N hetero-nuclear Overhauser enhancements (NOE) was measured for recoverin in the presence and absence of 5% Ca^{2+} . Relaxation delays for R_1 experiments were 0.016, 0.16, 0.32, 0.48, 0.65, 0.8, and 0.96 s, and for R_2 experiments 4.8, 9.6, 19.2, 38.4, 57.6, 76.8, and 105.6 ms. The 180° pulses for Carr-Purcell-Meiboom-Gill (CPMG) sequence were applied every 1.0 ms. Uncertainty of R_1 and R_2 values were determined by Monte-Carlo error simulation using signal intensities that contain experimental noise fluctuation. Uncertainty of the NOE experiments was estimated from the signal-to-noise ratio. We chose to compare relaxation rates and NOE values directly, rather than values derived by analyses such as the model-free approach, which can be difficult to analyze in the presence of conformational exchange.

R_2 dispersion experiments

Constant-time relaxation-compensated CPMG experiments were performed both at 60.82 and 81.08 MHz ^{15}N resonance frequencies in the absence and presence of 5% Ca^{2+} ²⁶. These experiments were conducted with the following conditions: a constant time of 40 ms, 180° pulse duration of 90 μs , and various half durations between 180° pulses, τ_{cp} , of 5, 2.5, 1.67, 1.25, 0.833, 0.625, 0.417, and 0.312 ms. Here, τ_{cp} is a half duration between CPMG 180° pulses: the values correspond to the effective field strength, $\nu_{\text{CP}} (1/4\tau_{\text{cp}})$ of 50, 100, 150, 200, 300, 400, 600, and 800 Hz.

R₂ dispersion data analysis

The R₂ dispersion data were analyzed to obtain three parameters that describe the exchange equilibria: 1) the relative chemical shift difference between the major and minor species ($\delta\omega/2\pi$), 2) the correlation time for exchange (τ_{ex}), and 3) the relative populations (p_a and $p_b = 1 - p_a$) as well as the intrinsic relaxation rate (R_2^0) assuming the two-site exchange model. First, the CPMG NMR data sets that exhibit significant R₂ dispersion profiles were selected for the analysis. To quantify the extent of R₂ dispersion, a root-mean-square-deviation of R₂ value (R₂ RMSD) within each set of an R₂ dispersion profile was calculated as described by ²⁷. We chose to use the R₂ RMSD to quantify the R₂ dispersion, because it represents all the obtained R₂ points ²⁷. When there is no chemical exchange around the examined ν_{CP} range, the R₂ values should be uniform as a function of ν_{CP} . On the other hand, an elevated R₂ is expected for the residues that undergo dynamics on the chemical shift time scale as a function of ν_{CP} . In addition to this criterion, residues that exhibit large inphase ¹⁵N transverse relaxation rate ($R_2 > 30 \text{ s}^{-1}$) were also chosen for the dispersion analysis. Overall, the R₂ RMSD and large R₂ were used as primary indicators to select residues whose R₂ dispersion are analyzed: residues that satisfy (1) RMSD of R₂ in the R₂ disp $> 2 \text{ s}^{-1}$ or (2) $R_2 > 30 \text{ s}^{-1}$ in either at 60.82 MHz or 81.08 MHz data were tentatively selected for optimization of chemical exchange parameters (Table S1).

The optimization of the parameters was performed by minimizing the target χ^2 -function for R₂ dispersion given in Equation (S1) in the Supporting information, section [4]. First, the minimization was performed for each residue (i.e., total 16 R₂ values) using the data sets recorded at two magnetic field strengths. Here, five unknown parameters (p_a , τ_{ex} , $\delta\omega/2\pi$, R_2^0 at 60.82 MHz ($R_2^0_{61 \text{ MHz}}$), and R_2^0 at 81.08 MHz ($R_2^0_{81 \text{ MHz}}$)) were optimized. The R₂ dispersion in the target χ^2 -function was calculated by solving the Bloch-McConnell equation assuming a two-site exchange model (Equation S2). Since there may be systematic errors (such as modulation of antiphase components during CPMG), we accepted the fits with the χ^2 normalized with respect to the number of degrees of freedom ($\chi^2/n < 4$) as a confidence limit. This assumes an experimental error that is twice as large as that derived from the signal-to-noise ratio of the individual fits. Uncertainties of the optimized parameters were estimated by repeating the optimization for 50 datasets simulated by the Monte Carlo method.

Next, optimization of the parameters for a group of residues in each domain (termed “domain-fit”) was performed including the residues that were fit by the “individual residue” analysis. In the domain-fit, a set of p_a and τ_{ex} was determined as global parameters for each domain by a grid search ²⁷. At each grid point, χ^2 -function was minimized with respect to local parameters, $\delta\omega/2\pi$, $R_2^0_{61 \text{ MHz}}$ and $R_2^0_{81 \text{ MHz}}$. Uncertainties of the optimized global parameters were estimated from the confidence region corresponding to values of χ^2/n larger than the fitted minimum. Finally, it was tested whether one set of p_a and τ_{ex} can be used to explain the observed conformational exchange of the entire protein (called “whole protein-fit”) using the same data sets as those used for the domain-fit. Using the pre-determined global parameters (described in the Results), the local parameters were optimized. These fits are not analyzed by F-test because uncertainties of the dispersion parameters are not necessarily explained by a Gaussian distribution ²⁷.

Optimization of the exchange parameters were performed by assuming the upper limits of R_2^0 , 27 and 31 s^{-1} for the datasets recorded at 60.82 and 81.08 MHz, respectively (Supporting Information, section [4]). The upper limit values of R_2^0 were determined to be more than one-standard deviation unit larger than the entire average of the dispersion R₂ values. This is sufficiently larger than the apparent R₂ value for the dispersion, in which ¹H R₁ in the NH antiphase relaxation was estimated to be less than 2 s^{-1} (assuming 15% residual protonated α and β protons in per-deuterated protein) ²⁸. These limitations are used

to reduce the searching parameter space, but do not necessarily improve the results (Supporting Information, section [4]). Chemical shift changes are shown in Hz units at 60.82 MHz resonance frequency.

RESULTS

Overall ^{15}N NMR relaxation profile of recoverin

^{15}N NMR relaxation data for individual amide resonances of Ca^{2+} -free recoverin are presented in Fig. 3 and indicate an overall rigid and globular fold. Average ^{15}N R_1 and R_2 values of all observed residues were $0.79 (\pm 0.06) \text{ s}^{-1}$ and $19 (\pm 5) \text{ s}^{-1}$, respectively. A rotational correlation time estimated based on the R_1/R_2 ratio was $15 (\pm 0.3) \text{ ns}$, consistent with a protein monomer under NMR conditions. For the most part, residues in the EF-hand regions containing regular secondary structure have R_1 and R_2 values very close to the mean values, consistent with the overall globular fold in Fig. 2A. By contrast, R_2 values greater than the average were observed for residues in helix A, the loop between helices A and B (colored dark blue in Fig. 2B), helix D, inter-domain linker, and helix J. The increased R_2 values here suggest some type of conformational exchange dynamics in these regions that will be analyzed in more detail in the R_2 dispersion analysis. Because chemical exchange broadening was observed for so many residues, quantitative analysis of the fast internal motion and detailed analysis of the rotational correlation time was not performed here. The last 10 residues at the C-terminus (residues 191 – 202) have R_2 values less than the average, R_1 values greater than the average, and heteronuclear NOE values less than 0.65 (Fig. 3), indicating that the last 10 residues at the C-terminus are dynamically disordered.

Ca^{2+} -saturated myristoylated recoverin, in contrast to the Ca^{2+} -free state above, tends to aggregate under NMR conditions due to the Ca^{2+} -induced exposure of the myristoyl group²⁰, making it impossible to perform NMR relaxation experiments on Ca^{2+} saturated protein. A polar myristic acid analog (13-oxa tetradecanoic acid) attached to Ca^{2+} -saturated recoverin was shown previously to prevent this aggregation and allowed us to determine the NMR structure of the Ca^{2+} -saturated protein²⁰. Unfortunately, the polar myristoyl group becomes somewhat destabilized when sequestered inside the hydrophobic core of Ca^{2+} -free recoverin, and therefore perturbs both the thermodynamics and kinetics of the Ca^{2+} -myristoyl switch, making the polar fatty acyl analog unsuitable for the current dynamics studies. Thus, we chose to study the dynamics of Ca^{2+} -induced conformational changes in recoverin by simply adding a trace amount of Ca^{2+} (0.05 equivalents of Ca^{2+} referred to in this study as 5% Ca^{2+}) to native myristoylated recoverin. This trace addition of Ca^{2+} (up to 10%, see Supporting Information section [6]) nudges the equilibrium toward the Ca^{2+} -saturated (R) state and causes detectable Ca^{2+} -induced spectral changes (Fig. 4) but does not cause any protein aggregation at $\leq 5\%$ Ca^{2+} as judged by light scattering analysis. Therefore, under the 5% Ca^{2+} condition, it is possible to detect Ca^{2+} -induced effects on the NMR relaxation data (see R_2 dispersion analysis section below) that report on millisecond conformational dynamics of the Ca^{2+} -myristoyl switch.

The addition of 5% Ca^{2+} did not change the overall R_1 and R_2 relaxation profiles (Figs. 3A and 3B), and did not cause any protein aggregation as monitored by dynamic light scattering measurements (see Materials and Methods). The average R_1 and R_2 values of all the observed residues were $0.79 (\pm 0.07) \text{ s}^{-1}$ and $21 (\pm 7) \text{ s}^{-1}$, respectively, and the rotational correlation time estimated based on the R_1/R_2 ratio was $16 (\pm 0.3) \text{ ns}$. NOE values were also high (> 0.8 , Fig. 3C) in regions of regular secondary structure, demonstrating that the protein does not aggregate and remains in a globular fold in the presence of 5% Ca^{2+} . The heteronuclear NOE values recorded at 60.82 MHz in the 5% Ca^{2+} condition were almost identical to those in the absence of Ca^{2+} (data not shown). Overall, the 5% Ca^{2+} -condition does not perturb the sub-nanosecond dynamics of the major species of the protein. However,

the 5% Ca^{2+} condition has a rather profound effect on the R_2 dispersion data (and hence conformational dynamics) as discussed in the next section.

R_2 dispersion analysis of Ca^{2+} -free recoverin

The unusually high R_2 values (Fig. 3) measured for particular residues (helix α_A , loop between α_A and α_B , α_D , inter-domain linker, and α_I) suggest conformational exchange dynamics in these regions. ^{15}N relaxation dispersion measurements were performed on Ca^{2+} -free recoverin (see Materials and Methods) to quantitatively analyze the exchange equilibrium in terms of exchange rate (τ_{ex}) and equilibrium populations (p_a and p_b). Representative residues that exhibit significant R_2 dispersion curves are illustrated in Fig. 5. To quantify the extent of R_2 dispersion, a root-mean-square-deviation of R_2 value (R_2 RMSD) was calculated within each set of an R_2 dispersion profile as described by ²⁷. The R_2 RMSD for each residue in the Ca^{2+} -free state is shown in Fig. 6A. The residues that exhibit rather large R_2 RMSD were used in the fitting analysis below (Table 1) and report on millisecond exchange events. A quantitative analysis of the R_2 dispersion data for individual residues in the Ca^{2+} -free state was performed assuming a two-site exchange model using a set of R_2 dispersion data recorded at 60.82 and 81.08 MHz ^{15}N resonance frequencies (Supporting Information, section [5]). Twelve residues that have significant R_2 RMSD were selected for the optimization: E15, L17, Q18, T21, F23, S41, H91, M92, and S94 in the N-terminal domain and I173, K180, and I186 in the C-terminal domain (Table 1 and Table S2). The average exchange rate is 0.23 ms and 0.54 ms for residues in the N-domain and C-domain, respectively. The relative population of T^0 in these fits varies somewhat in the range, 0.52 to 0.994.

Although the equilibrium ratio of $[T^0]/[R^0]$ has been measured previously to be 400 ¹⁵, the dispersion data as a whole could not be fit with such a skewed population ratio (Table 1). In the case of a “fast-exchange” equilibrium, the magnitude of chemical exchange contribution to R_2 , R_{ex} , is approximately given by $p_a p_b (\delta\omega)^2 \tau_{\text{ex}}$. If the population ratio is 1:400, the $p_a p_b$ term must be too small to provide significant magnitude of chemical exchange in the range of ν_{CP} examined in our dispersion experiments. In the case of a “slow-exchange” equilibrium, the magnitude of R_{ex} is approximately given by p_b / τ_{ex} . If $p_b = 1/400$, τ_{ex} has to be smaller than 0.5 ms to observe the significant magnitude of R_{ex} , which would result in $\delta\omega/2\pi$ values greater than 500 Hz for all the residues, which is not observed. Overall, the fact that R_2 dispersion was observed under our experimental conditions for the Ca^{2+} -free form itself indicates that the observed exchange equilibrium ratio is much smaller than the predicted ratio, $[T^0]/[R^0] = 400$ for the simple $T^0 \leftrightarrow R^0$ transition. On the basis of the R_2 dispersion data, the equilibrium population ratio of the major form (T^0) and minor species is ≤ 100 .

R_2 dispersion analysis at 5% Ca^{2+}

The addition of 5% Ca^{2+} has a profound effect on the R_2 dispersion profile, showing increased R_2 dispersion in Fig. 5. Also, a number of additional residues now exhibit significantly high R_2 RMSD (Fig. 6B) that can be included in the fitting analysis. Residues N3, G7, K11, I13, Q18, T21, F23, T24, S29, R46, K63, Q67, F73, T80, L81, H91, M92, S94, A95, and N99 in the N-terminal domain and residues Y109, D110, G115, I117, E121, K131, I133, S134, T147, W156, F158, L167, T168, E169, F172, I173, E174, T176, L177, K180, E181, L183, and E189 in the C-terminal domain were fit individually (Table 1 and Table S2). A comparison of the R_2 RMSD profiles in the Ca^{2+} -free state (Fig. 6A) and at 5% Ca^{2+} (Fig. 6B) reveals that the R_2 dispersion for the exchange residues are dramatically enhanced by the addition of 5% Ca^{2+} . This Ca^{2+} -dependent dispersion suggests that the exchange equilibrium may involve a minor species coupled to a Ca^{2+} -dependent pathway. Indeed, Figure 7A shows a linear correlation between the R_2 RMSD profiles at 5% Ca^{2+}

versus 0% Ca^{2+} , demonstrating a correlation of the two R_2 RMSD profiles (Fig. 6A and 6B). This correlation suggests that the exchange equilibrium examined by the dispersion experiments probes a minor species that is Ca^{2+} -dependent and that the 5% Ca^{2+} may push this equilibrium along the Ca^{2+} -myristoyl switch pathway.

Using the residues that were fit by the two-site exchange model individually, we tested whether the R_2 dispersion data at 5% Ca^{2+} can be explained assuming a set of global parameters for each domain. The p_a values of the two domains were also similar to each other, 0.978 and 0.960, respectively (Table 2, and Supporting Information section [7]). These values are slightly lower than those in the Ca^{2+} -free state (0.988 and 0.992, respectively), consistent with the idea that 5% Ca^{2+} pushes the equilibrium forward along the Ca^{2+} -myristoyl switch pathway. The exchange time scale obtained by the domain-fits is relatively uniform, $\tau_{\text{ex}} = 0.6$ ms (with asymmetric uncertainties from 0.25 to 0.9 ms) in the N-terminal domain and 0.89 ms (with asymmetric uncertainties from 0.466 to 1.5 ms) in the C-terminal domain (Table 2, and Supporting Information section [7]). Overall, the populations and exchange rates in the two domains were optimized to relatively similar values, suggesting that the domains undergo similar conformational exchange dynamics consistent with a concerted conformational change.

Observation of three protein states ($T \leftrightarrow I \leftrightarrow R$)

The R_2 dispersion analysis above indicates the presence of a minor equilibrium species whose population is greater than 1% even in the absence of Ca^{2+} . A simple two-state model ($T \rightarrow R$ transition, see ¹⁵) can not account for this observed >1% population, because the equilibrium ratio, $[T^0]/[R^0]$ has been measured to be 400 ± 50 from previous Ca^{2+} binding studies ¹⁵. To understand the origin of the minor species, we wanted to know how this species is thermodynamically coupled to the T and R states by analyzing the effects of Ca^{2+} on ^{15}N - ^1H HSQC spectra and ZZ-exchange data as described below.

Highly sensitive ^{15}N - ^1H HSQC spectra were recorded as a function of Ca^{2+} concentration from 0 to 10% (Supporting information, section [6]). The spectrum recorded under the 5% Ca^{2+} condition exhibits a set of very weak, Ca^{2+} -induced peaks (Fig. 4). The Ca^{2+} -induced peaks were not observed in the spectrum of the Ca^{2+} -free form (with or without 1 mM EGTA), indicating that the presence of the minor peaks were generated by the addition of 5% Ca^{2+} and were titrated as a function of Ca^{2+} concentration ((Supporting information, section [6]). The Ca^{2+} -induced NMR peaks have chemical shifts that are almost identical to those of Ca^{2+} -saturated recoverin, on average less than 20 Hz deviation in ^{15}N dimension (labeled in Fig. 6B) ²⁰. For example, Ca^{2+} -induced weak peaks that appear at 10.72, 9.13, 8.92, 8.53 and 8.22 ppm are assigned to G115, Y65, I173, N120 and I186, respectively, in the Ca^{2+} -bound state on the basis of the known chemical shift assignments for Ca^{2+} -saturated recoverin ²⁰ (Figs. 4A and 4B). The sharpness of the NMR signals for the Ca^{2+} -saturated R-state (highlighted red in Fig. 4) indicates that R is in a slow equilibrium, consistent with the “slow exchange profile” observed in previous NMR spectra that monitor a Ca^{2+} titration (Supporting information section [5]) ¹⁵. Titrating trace amounts of Ca^{2+} (0, 1, 2, 5 and 10% Ca^{2+}) caused an increased intensity of these peaks vs. Ca^{2+} (but did not cause any change in peak position), confirming that the weak Ca^{2+} -induced peaks are due to the Ca^{2+} -saturated R-state (Supporting information, section [6]). We further confirmed that the Ca^{2+} -induced weak peaks represent the R-state by recording ZZ-exchange experiments (Supporting information, section [8]). These spectra demonstrate that the weak Ca^{2+} -induced amide resonances exhibit a ZZ-exchange crosspeak with their corresponding resonance in the Ca^{2+} -free state. Thus, formation of the Ca^{2+} saturated R state must involve a relatively slow final step.

The HSQC spectrum under the 5% condition also showed small but detectable changes in chemical shifts of the main peaks that monitor Ca^{2+} -free recoverin in a Ca^{2+} -dependent equilibrium (Fig. 4). A number of residues in the Ca^{2+} -free state (e.g. G7, S10, S41, I44, T45, L81, M92, G96, W104, I117, S134 and K162) exhibit small changes in ^{15}N chemical shift ranging from 0 – 17 Hz (on average 2.54 Hz) upon adding 5% Ca^{2+} (referred to as $\Delta_{\text{cs}}^{\text{f-5\%Ca}}$ in Fig. 8A). Additional residues not shown in Fig. 4 also exhibit Ca^{2+} -induced shifts. The residues that exhibit detectable peak shifts at 5% Ca^{2+} also exhibit much larger chemical shift differences when comparing the Ca^{2+} -free and fully Ca^{2+} saturated forms (referred to as $\Delta_{\text{cs}}^{\text{f-bound}}$ in Fig. 8B). The profile of $\Delta_{\text{cs}}^{\text{f-5\%Ca}}$ (Fig. 8A) suggests that the observed conformational exchange is fast (Table 2) and amplified by the addition of 5% Ca^{2+} (Fig. 7A). Overall, the correlation of the R_2 RMSD (Fig. 7A) and the Ca^{2+} -dependent shift of the main peaks at 5% Ca^{2+} (Fig. 8) demonstrate that Ca^{2+} -free recoverin (T^0 state) must be in a fast equilibrium with an intermediate state (I) that is distinct from the Ca^{2+} -saturated R-state.

In summary, the above ^{15}N chemical shift analysis detects at least three-distinct protein conformational states ($T \leftrightarrow I \leftrightarrow R$): (1) the Ca^{2+} -free protein (T^0 state) is represented by the major peaks in both Ca^{2+} -free and the 5% Ca^{2+} -condition; (2) the Ca^{2+} -saturated protein (R^2 state) is represented as a set of weak Ca^{2+} -induced peaks in “slow exchange” at 5% Ca^{2+} ; and (3) there must be an intermediate state (I) that is in a “fast exchange” equilibrium with T^0 (causing Ca^{2+} -dependent shifts of the main peaks), but in slow exchange with R^2 (causing sharp, Ca^{2+} -induced peaks at 5% Ca^{2+}).

R_2 dispersion data fit by a global set of parameters

Since p_a and τ_{ex} in the fitting analysis above were found to be similar in both domains, the R_2 dispersion profiles were fit assuming a set of global exchange parameters (p_a and τ_{ex}) for the entire protein. We used the two-site exchange model for the $T \leftrightarrow I$ transition because the subsequent $I \leftrightarrow R$ transition is too slow to affect the R_2 dispersion. Indeed, a previous NMR Ca^{2+} titration¹⁵ and kinetic study of the Ca^{2+} -myristoyl switch in recoverin²⁹ indicate that the time scale of R-state formation is much slower than the NMR chemical shift time scale (Supporting information, section [5]). Based on the results of the domain-fit in Table 2, all the data were fit (1) assuming $p_a = 0.99$ and $\tau_{\text{ex}} = 0.25$ ms in the Ca^{2+} -free form by optimizing 12 residues (average χ^2/n , 1.24), and (2) assuming $p_a = 0.975$ and $\tau_{\text{ex}} = 0.75$ ms in the 5% Ca^{2+} form by optimizing 44 residues (average χ^2/n , 1.44) (Table S3). In the Ca^{2+} -free condition, based on $p_a = 0.99$, the $[T^0]/[I^0]$ ratio is estimated to be 100. Again, even if some residues that were included in the global-fit undergo local dynamics, the global p_a will not be further skewed than 0.99 as discussed above. To have the significant R_2 RMSD as observed in the profiles (Fig. 6), p_a could even be lower, consistent with the relatively large uncertainty range in the domain fit (Table 2). Therefore, to avoid over-estimating the parameters, we tentatively conclude that the $[T^0]/[I^0] \leq 100$ and $[I^0]/[R^0] \geq 4$ (Supporting Information, section [9]).

In the 5% Ca^{2+} condition, the $\delta\omega/2\pi$ for some residues (I133, S134, E169, and E174) agree quite well with the observed chemical shift differences between T^0 and R^2 (Table S3). However, in many residues, $\delta\omega/2\pi$ is small compared to $\Delta_{\text{cs}}^{\text{f-bound}}$ (Figs. 7B and 7C). In particular, for the residues that have large $\Delta_{\text{cs}}^{\text{f-bound}}$ (V111, G115, and I117, >300 Hz), the optimized $\delta\omega/2\pi$ were for the most part smaller than $\Delta_{\text{cs}}^{\text{f-bound}}$ in Fig. 7 and these residues are all located at the EF-hand 3 site. The fact that their $\delta\omega/2\pi$ is small compared to $\Delta_{\text{cs}}^{\text{f-bound}}$ suggests that the Ca^{2+} -bound EF3 in the intermediate state (I^1) is not in a fully open conformation like it is in the R^2 state. We suggest that the structure of EF3 in the intermediate state may adopt a semi-open conformation like that observed in myosin light chains³⁰ and the C-terminal EF-hand domain of alpha-actinin-2³¹. Small $\delta\omega/2\pi$ values and lack of R_2 dispersion were also observed for N-terminal residues that contact the myristoyl

group, suggesting that the I-state may resemble T^0 and contain a sequestered myristoyl group.

These results demonstrate that the relaxation dispersion data of most residues can be explained by a global conformational equilibrium of the protein in both the Ca^{2+} -free and 5% Ca^{2+} form. However, the interpretation is not straightforward because of internal dynamics on different time-scales or populations other than the Ca^{2+} -dependent conformational equilibrium. In the whole-protein fit, slightly high χ^2/n values were observed for K180 and I186 ($\chi^2/n = 2.65$ and 3.29 , respectively) in the Ca^{2+} -free form, and for T21, L81, S94, W156, K180, and I186 ($\chi^2/n = 3 \sim 3.6$) in the 5% Ca^{2+} form. The relatively high χ^2/n may be due to other local dynamics. In particular, the $\delta\omega/2\pi$ of residues Q18 and T21 were optimized to be >1000 and >800 Hz, respectively in either the Ca^{2+} -free and 5% Ca^{2+} condition (Figs. 7B and 7C). These $\delta\omega/2\pi$ values are larger than a possible $\delta\omega/2\pi$ in a typical diamagnetic protein (presumably < 10 ppm, i.e. 600 Hz, because ^{15}N shift range is ~ 30 ppm). It is reasonable to suppose that these two residues undergo local dynamics with different populations and time scale from those of the global one, and were not fit by assuming a set of global parameters. Since we have data only at the limited frequency ranges, we did not further evaluate those dynamics parameters.

DISCUSSION

The goal of this study was to analyze the conformational dynamics and identify potential intermediates of the Ca^{2+} -myristoyl switch in recoverin using NMR relaxation dispersion and chemical shift analysis. Conformational dynamics of related Ca^{2+} sensor proteins, calmodulin^{32–35} and troponin C^{36,37}, have been studied by NMR, revealing conformational transitions in the EF-hands that take place on the sub-millisecond timescale. We report in this study the first NMR dynamics study of an NCS protein (Fig. 1). ^{15}N NMR relaxation dispersion and chemical shift analysis reveals global Ca^{2+} -induced conformational dynamics in recoverin that occur on the millisecond time scale. We show that Ca^{2+} -free recoverin (T^0) forms a fast equilibrium ($\tau_{ex}\delta\omega < 1$) with a partially opened intermediate species. The binding of two Ca^{2+} by recoverin drives extrusion of the myristoyl group during a final slow step, $I^2 \leftrightarrow R^2$ ($\tau_{ex}\delta\omega > 1$).

Three-state model for the Ca^{2+} -myristoyl switch

Previous Ca^{2+} -binding and structural studies on recoverin suggested a sequential mechanism of the Ca^{2+} -myristoyl switch in which Ca^{2+} binding to the sequestered Ca^{2+} -free state (T) generated an intermediate species (I) with one bound Ca^{2+} that converts into a Ca^{2+} -saturated open state (2 bound Ca^{2+}) with an extruded myristoyl group (R)^{19,20}. The intermediate state was trapped previously by a recoverin mutant (E85Q) that blocked Ca^{2+} binding at EF2 and permitted Ca^{2+} bound solely at EF3¹⁹ (Pdb code: 1LA3). In the current study, we were able to analyze the population and dynamics of this intermediate state in wildtype recoverin without any artificial trapping.

The results of the current NMR study are best explained by a 3-site exchange mechanism (Fig. 9), where three distinct conformational states of the protein are designated as T, I, and R. Since there are two Ca^{2+} -binding sites (0, 1 or 2 Ca^{2+} -bound), the total number of microscopic states are expressed by the 3×3 matrix in Fig. 9. The microscopic Ca^{2+} dissociation constants in Fig. 9 have been measured previously using equilibrium Ca^{2+} binding measurements^{15,19,23}. Each protein conformational state (T, I, and R) has two Ca^{2+} binding sites (EF2 and EF3) defined by microscopic dissociation constants, $K_D(EF2)$ and $K_D(EF3)$. According to the convention of the allosteric model (Monod-Wyman-Changeux³⁸), Ca^{2+} binding is assumed to not change the overall main chain structure of a given conformational state (e.g. the structure of I^0 is similar to that of I^1 and I^2 etc.). The Ca^{2+}

dissociation constants have been measured previously for the R state: $K_d^{R0 \rightarrow R1}(EF3) = 10^{-7}$ M and $K_d^{R1 \rightarrow R2}(EF2) = 10^{-5}$ M^{15,19}. R⁰ binds Ca²⁺ with at least 1000-fold higher affinity than Ca²⁺ binding to T⁰^{15,19,23}, implying that $K_d^{T0 \rightarrow T1}(EF3) = 10^{-4}$ M and $K_d^{T1 \rightarrow T2}(EF2) = 10^{-2}$ M. The equilibrium ratio, $[T^0]/[R^0]$ was measured to be 400 ± 50 from previous equilibrium Ca²⁺ binding studies^{15,19,23}.

Our NMR analysis above has determined the equilibrium ratio, $[T^0]/[I^0]$ to be ≤ 100 , which implies that $[I^0]/[R^0] \geq 4$, since $[T^0]/[R^0]$ is known to be 400¹⁵. Using the NMR-derived value of $[T^0]/[I^0]$ and the principle of microscopic reversibility in Fig. 9, the remaining equilibrium constants for the 3-state model can be estimated as: $[T^1]/[I^1] \sim 3$, $[I^1]/[R^1] \sim 0.13$, $[T^2]/[I^2] \sim 0.1$, and $[I^2]/[R^2] \sim 0.004$. Using these equilibrium constants, the 3-state model (Fig. 9) calculates a fractional saturation curve for Ca²⁺-binding to recoverin that closely matches experimental Ca²⁺-binding data fit previously by the Hill model with an apparent Ca²⁺ dissociation constant of 17 μ M and Hill coefficient of ~ 1.7 ¹⁵. In the presence of saturating Ca²⁺ levels, the 3-state model predicts there will be one dominant binding pathway ($T^0 \rightarrow I^0 \rightarrow I^1 \rightarrow I^2 \rightarrow R^2$) with essentially all of T⁰ converting into R².

Evidence in this study for the existence of the intermediate state (I) is 3-fold: First, the NMR dispersion analysis on Ca²⁺-free recoverin (Tables 1 and 2) indicates an exchange equilibrium between the T-state (T⁰) and some minor species (I⁰) having relative population, $[T^0]/[I^0] < 100$. Previous Ca²⁺-binding studies have shown that $[T^0]/[R^0] = 400$ ¹⁵. Thus, Ca²⁺-free recoverin in the absence of Ca²⁺ is thermally exchanging with a separate species (I⁰) that has much greater population than R⁰. The second supporting evidence for the existence of I can be seen from our spectral analysis of the Ca²⁺-dependent HSQC spectra (Figs. 4 and 8) and the residues that undergo relaxation dispersion (Figs. 2C, 6, and 7). Together, these data reveal that Ca²⁺-free recoverin (T⁰) exists in a fast-exchange equilibrium with some species at 5% Ca²⁺, in contrast to the R-state that exists in a slow-exchange equilibrium. The disconnect between the initial state (T) and final state (R) implies an intermediate state (I) in between T and R. Third, the $\delta\omega$ values determined in the dispersion analysis above (Tables 1 and 2) do not match the $\delta\omega$ values calculated from the known chemical shifts of Ca²⁺-free recoverin¹⁸ and the Ca²⁺-saturated protein²⁰.

Structural insights and dynamics of the Ca²⁺-myristoyl switch

The structures of the initial (T⁰) and final (R²) states of the Ca²⁺-myristoyl switch are both known (see Figs. 2A–B). The next step is to understand how the T and R conformational states are connected structurally to the intermediate species (I) observed in this study. We could not directly determine the three-dimensional structure of the I-state because there is not enough sensitivity in our current dispersion experiments to extract detailed structure as described by³⁹. Instead, we inferred possible conformational changes during $T \leftrightarrow I$ by comparing the structural changes between Ca²⁺-free recoverin (1IKU) and one Ca²⁺ bound E85Q recoverin (1LA3) that may mimic the I-state. The most striking structural difference between 1IKU and 1LA3 can be visualized as a swiveling of the two domains due to a hinge rotation about Gly96 as described previously¹⁹. We propose that a similar domain swiveling may occur during $T^0 \leftrightarrow I^0$ as supported by large R₂ dispersion (Fig. 6A) for residues at the domain interface (e.g. helix A and D) that change solvent accessibility upon domain swiveling. The structure of the I-state in the current study may also contain a sequestered myristoyl group (like in 1LA3) because residues in EF1 that interact most closely with the myristoyl group (e.g. L28, F35, F49, I53, F56) do not exhibit any R₂ dispersion and do not show any chemical shift heterogeneity (Fig. 2C and Table S2). However, the structure of the I-state in this study is not exactly the same as 1LA3, because the $\delta\omega/2\pi$ values observed in the dispersion analysis (Tables S2 and S3) do not match the chemical shift differences

calculated between Ca^{2+} -free recoverin¹⁸ and half-saturated E85Q recoverin (Supporting information [10])¹⁹, particularly for N-domain residues in EF2. We suggest that this discrepancy might be explained by the E85Q mutation in 1LA3 that artificially blocked Ca^{2+} binding at EF2 in contrast to the I-state in wildtype recoverin that binds Ca^{2+} at EF2.

The R_2 dispersion observed for many residues throughout the protein main chain (Fig. 6B) suggests that conformational changes during $T^0 \leftrightarrow I^0$ may take place in both the N-domain and C-domain, consistent with a concerted and global conformational transition. We propose that domain swiveling during $T^0 \leftrightarrow I^0$ causes a *concerted* opening of the protein that may expose *both* Ca^{2+} sites (see arrows in Fig. 9), making EF2 and EF3 more accessible to bind Ca^{2+} . In particular, the EF2 Ca^{2+} -binding loop is partially buried at the domain interface in the T^0 form (Fig. 2A), and EF3 is sterically locked into a Ca^{2+} -free closed conformation in T^0 ¹⁹. Without any domain swiveling, the Ca^{2+} binding sites remain statically locked and unable to bind Ca^{2+} . In essence, the domain swiveling during $T^0 \leftrightarrow I^0$ serves to expose *both* Ca^{2+} -binding sites to facilitate rapid Ca^{2+} binding¹⁵. The concerted structural changes during $T^0 \leftrightarrow I^0$ may also explain the steep Ca^{2+} binding curve observed for myristoylated recoverin with a Hill slope of 1.7¹⁵: In the T-state, both sites are inaccessible, which prevents Ca^{2+} -binding at low Ca^{2+} concentrations; when the Ca^{2+} concentration is raised near the K_d , the protein switches to the I-state, where both sites become exposed (see orange circles in Fig. 9) that promotes abrupt binding of 2 Ca^{2+} and thus may explain the observed Hill slope of 1.7¹⁵.

The binding of two Ca^{2+} to recoverin promotes large conformational changes in the N-domain needed to extrude the myristoyl group during $I^2 \leftrightarrow R^2$ (compare 1LA3 and 1JSA)^{19,20,22}. The dynamics of the large conformational displacements during $I^2 \leftrightarrow R^2$ were not examined in this study, because our R_2 dispersion analysis on Ca^{2+} -free recoverin reports only on the $T \leftrightarrow I$ equilibria. The dynamics of the $I^2 \leftrightarrow R^2$ transition could be determined by performing R_2 dispersion analysis on Ca^{2+} -saturated recoverin. However, the slow exchange rate for the extrusion of the myristate during $I^2 \leftrightarrow R^2$ ²⁹ (inferred by the sharp Ca^{2+} -induced peaks in Fig. 4 and ZZ-exchange crosspeaks in Supporting Information section [8]) indicates that $I^2 \leftrightarrow R^2$ dynamics might be too slow to be studied by conventional relaxation dispersion experiments.

Dynamics of the C-terminal helix and target specificity of NCS proteins

Recoverin and other members of the NCS family all contain a C-terminal helix (J) adjacent to EF4 (Figs. 1 and 2). This helix plays a structural role in controlling target binding to various NCS proteins. Recent structures of recoverin homologs, NCS-1^{12,40,41} and KChIP1⁴² reveal that the corresponding C-terminal helix in these structures is displaced quite far away from a protein crevice between EF3 and EF4 to make room for the binding of a helical target protein. In recoverin the structure of helix J mimics the myosin light chain kinase target helix structural interaction with calmodulin^{43,44}. Our relaxation dispersion data demonstrate that a number of residues in helix J of recoverin exhibit quite large R_2 dispersion (such as K180, L183 and I186 in Table S2) in both Ca^{2+} -free and 5% Ca^{2+} -forms ($\Delta_{CS}^{f-5\%}$ in Fig. 7A), suggesting that helix J might undergo some type of internal motion. Helix J might exhibit a conformational “flickering” or a transient dissociation of this helix away from the protein core, similar to the target-induced displacement of helix J described above for NCS-1 and KChIP1. In recoverin, the helix J binding to the crevice between EF3 and EF4 appears much stronger than it is in NCS-1 or KChIP1, because helix J remains associated with recoverin’s protein core in both Ca^{2+} -free and Ca^{2+} -bound recoverin as well as in the presence of a target helix⁴⁵. However, the helix J association may be weak enough in recoverin to allow transient dissociation on the chemical shift time scale that may explain the observed R_2 dispersion for the C-terminal residues. The amino acid sequence in helix J is highly variable between recoverin, NCS-1 and KChIP1. We suggest that the C-terminal

sequence variability in NCS proteins may serve to fine tune the dynamics of the helix J interaction with the protein core and thus may be important for conferring target specificity among the NCS proteins.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

T⁰	Ca ²⁺ -free closed conformational state with sequestered myristoyl group
I⁰	Ca ²⁺ -free intermediate conformational state
R⁰	Ca ²⁺ -free open state with extruded myristoyl group
I¹	intermediate conformational state with 1 Ca ²⁺ bound
I²	intermediate conformational state with 2 Ca ²⁺ bound
R²	Ca ²⁺ -saturated open state with extruded myristoyl group
HSQC	heteronuclear single quantum coherence
NMR	nuclear magnetic resonance
NOE	nuclear Overhauser effect
R₁	longitudinal relaxation rate constant
R₂	transverse relaxation rate constant
RMSD	root-mean-squared deviation

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Figure 1. Amino acid sequence alignment of bovine recoverin with other NCS proteins. Secondary structure elements (helices and strands), EF-hand motifs (EF1 green, EF2 red, EF3 cyan and EF4 yellow), and millisecond exchange residues (highlighted dark blue) are mapped onto the amino acid sequence of recoverin. Residues that undergo millisecond chemical exchange are highlighted in blue. Swiss Protein Database accession numbers are P21457 (bovine recoverin), P62168 (rat NCS-1), P29554 (bovine neurocalcin), and P32076 (rat hippocalcin).

Figure 2.

Structural changes in Recoverin's Ca²⁺-myristoyl switch (A and B) and conformational fluctuation during T → I (C). Ribbon diagrams show the main chain structures of Ca²⁺-free (A) and Ca²⁺-bound (B) myristoylated recoverin with N-terminal myristoyl group highlighted magenta. Millisecond exchange residues and dynamical hinge region between helix A and B are highlighted dark blue. Secondary structure elements (helices and strands), EF-hand motifs (EF1 green, EF2 red, EF3 cyan and EF4 yellow) are drawn in different colors. In (C), residues that undergo chemical exchange at 5% Ca²⁺ (significant inphase ¹⁵N R₂ or R₂ dispersion, see details in Experimental Procedures) are colored red. The structure of Ca²⁺-bound recoverin (B) shows the entire N-domain (EF1 and EF2) is rotated ~45° with respect to the C-domain (EF3 and EF4) that facilitates exposure of the N-terminal myristoyl group. The ribbon structures were generated using PDB codes 1IKU and 1JSA, respectively.

Figure 3. (A) ^{15}N transverse relaxation rate (R_2), (B) longitudinal relaxation rate (R_1), and (C) $\{^1H\}$ - ^{15}N NOE of recoverin at 81.08 MHz. Filled circles show the data recorded in the absence of Ca^{2+} (NOE is not recorded). Unfilled circles show the data recorded in the 5% Ca^{2+} condition.

Figure 4.

Two representative spectral regions of ^{15}N - ^1H HSQC of recoverin in the Ca^{2+} -free state (black) and in the presence of 5% Ca^{2+} (overlayed in red) are shown in (A) and (B). The insets show Ca^{2+} -induced spectral changes to (A) G115 and (B) S10/I44/I117. The addition of 5% Ca^{2+} causes small chemical shift changes (< 10 Hz) for the main peaks (labeled black: see S41, M92 and Gly115 in (A), and L81, W104, I117 and S134 in (B), and also shown in Fig. 7A) and Ca^{2+} -induced generation of sharp minor peaks (labeled in red). The Ca^{2+} -induced minor peaks have chemical shifts very similar to those of Ca^{2+} -saturated recoverin²⁰ marked by red labels. The intensity of the weak Ca^{2+} -induced peaks was difficult to accurately measure for most residues due to spectral overlap and low signal-to-noise ratio. Nevertheless, a few Ca^{2+} -induced peaks (assigned to G115 and N120) were spectrally distinct and the integrated intensities of these peaks relative to their main peak intensity estimate a relative population of $\sim 1 - 2\%$ for the Ca^{2+} -saturated R state, consistent with a 2:1 Ca^{2+} binding stoichiometry determined previously¹⁵.

Figure 5.

Examples of the R_2 dispersion curves for (A) L81, (B) I117, and (C) E169 of recoverin in the absence (triangles) and presence (circles) of 5% Ca^{2+} . Filled and open circles indicate the data recorded at 60.82 MHz and 81.08 MHz ^{15}N resonance frequencies, respectively. The optimized dispersion curves for the data sets recorded in the presence of 5% Ca^{2+} are shown by dashed lines.

Figure 6. RMSD of R_2 dispersion data recorded at 60.82 and 81.08 MHz ^{15}N resonance frequencies in the (A) absence and (B) presence of 5% Ca^{2+} . In (B), residues that exhibit small Ca^{2+} -saturated signals, a part of which was shown in Fig. 4, are indicated with the residue numbers in the graph. The chemical shift data of the fully Ca^{2+} -saturated form was obtained from a previous study ²⁰.

Figure 7.

(A) Comparison of the R_2 RMSD in the Ca^{2+} -free and the 5% Ca^{2+} condition, and $\delta\omega/2\pi$ values determined assuming a set of global dynamics parameters of the protein in (B) Ca^{2+} -free ($p_a = 0.99$ and $\tau_{\text{ex}} = 0.25$ ms) and (C) 5% Ca^{2+} condition ($p_a = 0.975$ and $\tau_{\text{ex}} = 0.75$ ms). In (A), the linear line indicates the regression line calculated using the data points within two standard deviation from the average. In (C), the $\delta\omega/2\pi$ values are shown for the residues in the N-terminal domain (filled circles) and in the C-terminal domain (unfilled circles), as a function of difference in chemical shifts between the free and fully Ca^{2+} -saturated forms, $\Delta_{\text{CS}}^{\text{f-bound}}$.

Figure 8.

Chemical shift differences (A) of the main peaks between the Ca^{2+} -free and the 5% Ca^{2+} forms, $\Delta_{CS}^{f-5\%Ca}$, and (B) between the Ca^{2+} -free and fully Ca^{2+} -saturated forms, $\Delta_{CS}^{f-bound}$ at 60.82 MHz ^{15}N resonance frequency. The chemical shift data of the fully Ca^{2+} -saturated form was obtained from the previous study²⁰. The uncertainty of chemical shifts calculated from the digital resolution is less than 3 Hz.

Figure 9.

Three State Model of Ca^{2+} -myristoyl Switch. The protein exists in 3 possible conformational states: Sequestered (T), Intermediate (I) and Extruded (R). The protein has two Ca^{2+} binding sites defined by dissociation constants, $K_d^{R0 \rightarrow R1}(\text{EF3}) = 10^{-7} \text{ M}$ and $K_d^{R1 \rightarrow R2}(\text{EF2}) = 10^{-5} \text{ M}$ ¹⁵. Previous Ca^{2+} binding studies¹⁵ determined the equilibrium ratio

$[\text{T}^0]/[\text{R}^0] = 400$ and $c = 0.001$, where $c = \frac{K_d^{R0 \rightarrow R1}(\text{EF3})}{K_d^{T0 \rightarrow T1}(\text{EF3})}$. NMR relaxation dispersion analysis determined $[\text{T}^0]/[\text{I}^0] < 100$ (highlighted red), which implies $[\text{I}^0]/[\text{R}^0] > 4$. The remaining equilibrium constants ($[\text{T}^1]/[\text{I}^1]$, $[\text{I}^1]/[\text{R}^1]$, $[\text{T}^2]/[\text{I}^2]$, and $[\text{I}^2]/[\text{R}^2]$) were calculated using the principle of microscopic reversibility. Solid circles indicate species directly detected by ^{15}N - ^1H HSQC. Dotted circles indicate species inferred from R_2 dispersion analysis.

Table 1

Optimized exchange parameters obtained by fitting residues individually

Conditions		Parameters ^a			
C _α ²⁺	domain ^b	p _a ave (std) ^c	τ _{ex} (ms) ave (std) ^c	R ₂ ⁰ _(61MHz) (s ⁻¹) ave (std) ^c	χ ² /n ave (std) ^c
–	N (10)	0.844 (0.14)	0.23 (0.28)	25.0 (2.5)	2.0 (0.58)
–	C (3)	0.857 (0.23)	0.54 (0.68)	24.1 (4.8)	2.6 (1.03)
+	N (22)	0.825 (0.15)	0.72 (0.72)	25.0 (2.5)	1.9 (1.81)
+	C (27)	0.820 (0.16)	1.0 (0.45)	22.2 (3.0)	1.3 (0.83)

^aR₂ dispersion data recorded at 61 MHz and 81 MHz were used to optimize the parameters for each residue.

^bThe numbers of residues analyzed individually are indicated in the parentheses.

^cAverage and standard deviation for p_a and τ_{ex} values in each domain are described.

Table 2

Optimized exchange parameters obtained by fitting a group of residues with a set of global parameters of p_a and τ_{ex}

Ca ²⁺	Domain ^a	p_a value (range) ^b	τ_{ex} (ms) value (range)	χ^2/n
–	N (9)	0.988 (0.50–0.990)	0.2 (0.04–0.4)	1.21
–	C (3)	0.992 (0.986–0.993)	0.29 (0.21–0.38)	1.95
+	N (19)	0.978 (0.970–0.992)	0.60 (0.25–0.4)	2.34
+	C (23)	0.960 (0.965–0.98)	0.89 (0.47–1.5)	2.05

^aThe numbers of residues analyzed in each domain-fit is indicated in the parenthesis.

^bUncertainties of the values are described. Standard deviation was not used because distributions were asymmetric.