Calcium-Dependent Solvation of the Myristoyl Group of Recoverin†

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ABSTRACT: Recoverin is an *N*-myristoylated calcium-binding protein present in the photoreceptor cells of the mammalian retina. It is believed to function as a calcium sensor in visual signal transduction by coupling the kinetics of the recovery phase of the photoresponse to changes in the levels of intracellular Ca^{2+}. Upon binding Ca^{2+}, recoverin undergoes a conformational change that allows it to associate with membranes in a manner that requires *N*-myristoyl modification. It has been proposed that, in the Ca^{2+}-free conformation, the myristoyl group is sequestered in a hydrophobic part of the protein, and in the Ca^{2+}-bound conformation, the myristoyl group is exposed to solution. The crystal structure of Ca^{2+}-bound recoverin reveals an exposed cluster of hydrophobic residues, raising the possibility that residues in this region may function as part of an intramolecular myristoyl binding site. Fluorescence spectroscopy analysis of interactions between recoverin and 1-anilinonaphthalene-8-sulfonate (ANS) shows that an increase in solvent-accessible hydrophobic surface accompanies Ca^{2+} binding. ^1H nuclear magnetic resonance (NMR) spectra of myristoyl protons show dispersed chemical shifts in the Ca^{2+}-free conformation that become relatively uniform upon the addition of Ca^{2+}. Two-dimensional nuclear Overhauser effect (NOE) spectra of Ca^{2+}-free recoverin show NOE contacts between myristoyl protons and aromatic ring protons. Tryptophan fluorescence quenching by acrylamide indicates that the myristoyl group is in proximity to a tryptophan residue only in the Ca^{2+}-free conformation. These results indicate that the myristoyl group is in contact with residues in the hydrophobic cluster in Ca^{2+}-free recoverin and that it is exposed to solution in the Ca^{2+}-bound conformation.

Recoverin, an *N*-myristoylated protein present in the rod cells of the mammalian retina, has been demonstrated to play a role in regulating cellular responses to changes in levels of intracellular Ca^{2+} (Gray-Keller *et al.*, 1993; Kawamura, 1993; Kawamura *et al.*, 1993). Recoverin is a member of the EF-hand-containing superfamily of Ca^{2+}-binding proteins whose members include calmodulin, troponin C, and calbindin D_{9K}. Proteins in this superfamily bind Ca^{2+} via the EF-hand motif, a helix−loop−helix structure which ligates a Ca^{2+} ion with a series of conserved oxygen-containing amino acid side chains (Strynadka & James, 1989). Recoverin itself defines a distinct subfamily of Ca^{2+}-binding proteins that share a number of structural features (Nakayama & Kretsinger, 1994). These proteins are largely comprised of two EF-hand pairs, and in all cases where it has been investigated, the N-terminal glycine residue is covalently linked to a myristoyl or other type of short-chain fatty acyl residue (Dizhoor *et al.*, 1992; Kobayashi *et al.*, 1993a). Members of this subfamily are expressed in nervous tissues of both vertebrates and invertebrates and include frequenin, neurocalcin, and hippocalcin (Nakano *et al.*, 1992; Kobayashi *et al.*, 1993b; Pongs *et al.*, 1993; Teng *et al.*, 1994).

A general property of members of the recoverin subfamily proteins is their Ca^{2+}-dependent association with membranes. Recoverin reversibly binds to rod outer segment (ROS)[1] membranes and to phospholipid vesicles in the presence of Ca^{2+} but not in the presence of EGTA. This property requires *N*-myristoylation (Dizhoor *et al.*, 1993; Kobayashi *et al.*, 1993a; Ladant, 1995). *In vivo*, this would presumably result in a Ca^{2+}-dependent change in the affinity of recoverin for membranes and possibly would affect the relative distribution of recoverin in the membrane and cytoplasmic cellular compartments in a manner responsive to fluctuations in the levels of intracellular Ca^{2+}. This property of the protein has been termed a "calcium−myristoyl protein switch" (Zozulya & Stryer, 1992). A model has been proposed for how this switch might operate and is as follows. In the Ca^{2+}-free form of recoverin, the myristoyl moiety is sequestered in a hydrophobic region of the protein and is therefore unavailable for interaction with the membrane. Ca^{2+} binding by recoverin results in a conformational change that moves the N-terminus and the myristoyl group into solution, where it can subsquently promote recoverin−membrane interaction. This model is supported by the observation that the N-terminus of Ca^{2+}-free recoverin is resistant to proteolysis but becomes proteolytically labile in the presence of Ca^{2+} (Dizhoor *et al.*, 1993).

The crystallographic structure of nonmyristoylated recoverin, with a single Ca^{2+} ion bound at EF-hand 3, has recently been reported (Flaherty *et al.*, 1993; see Figure 1). This structure reveals that recoverin is a compact modular protein comprised of two domains connected through a short loop

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[1] Abbreviations: 1D, one-dimensional; 2D, two-dimensional; ROS, rod outer segments; ANS, 1-anilinonaphthalene-8-sulfonate; MOPS, 3-morpholinopropanesulfonic acid; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid; FPLC, fast protein liquid chromatography; HPLC, high-pressure liquid chromatography; NMR, nuclear magnetic resonance; NOE, nuclear Overhauser effect; NOESY, two-dimensional nuclear Overhauser effect spectroscopy; RV, recoverin; MRV, myristoylated recoverin.

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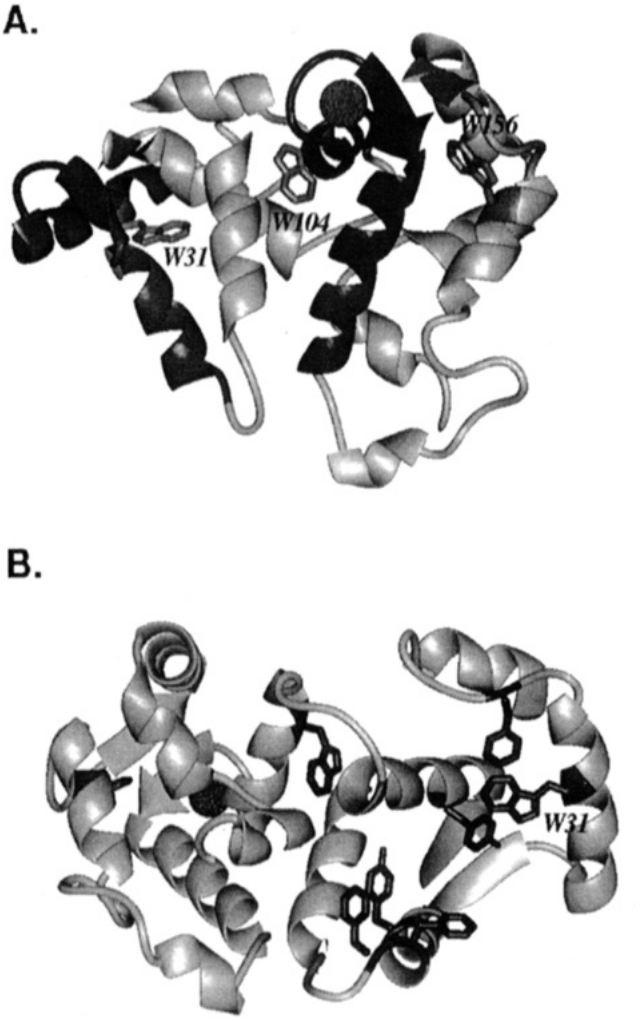

FIGURE 1: Ribbon models of the recoverin crystal structure. Two views of the crystal structure of Ca^{2+}-bound nonmyristoylated recoverin are shown (Flaherty *et al.*, 1993). Panel A shows the four EF-hand consensus motifs alternately shaded darker and lighter. From left to right, they are EF-hands 1, 2, 3, and 4. Side chains of the three tryptophan residues, W31 (in EF-hand 1), W104 (in EF-hand 3), and W156 (in EF-hand 4), are displayed. The Ca^{2+} ion bound at EF-hand 3 is also shown. Panel B displays the aromatic side chains present in the hydrophobic cluster (F23, W31, Y53, F56, F57, and Y86) as well as the three tryptophans. This view is rotated approximately 180° about the *y*-axis relative to that view shown in panel A. (Recoverin coordinates were generously provided by D. B. McKay).

region. Each domain contains an EF-hand pair, of which only a single EF-hand appears competent to bind Ca^{2+}. Interestingly, the structure also reveals the presence of a solvent-exposed, concave, hydrophobic surface near the N-terminus. This surface is comprised of residues from EF-hands 1 and 2 and contains a number of aromatic residues (F23, W31, Y53, F56, F57, and Y86). Although only EF-hand 3 is occupied by Ca^{2+} in the crystal, it is reasonable to assume that this structure at least partly represents the Ca^{2+}-bound form of the protein. It is therefore attractive to suggest that residues present in the hydrophobic cleft may interact with the myristoyl group in the Ca^{2+}-free form of recoverin and therefore constitute part of an intramolecular hydrophobic myristoyl-binding site.

In this study, we use fluorescence and NMR spectroscopy to test aspects of the calcium−myristoyl switch model. Ca^{2+}-dependent changes in the interaction of recoverin with the fluorescent dye 1-anilinonaphthalene-8-sulfonate (ANS) indicate that both nonmyristoylated and myristoylated recoverin have a greater solvent-accessible hydrophobic surface area in the Ca^{2+}-bound state as compared to the Ca^{2+}-free state. Fluorescence quenching experiments indicate that the solvent accessibility of tryptophan in recoverin is altered by the myristoyl modification in a Ca^{2+}-dependent manner. Difference NMR spectra of myristoyl protons indicate that the myristoyl group is uniformly exposed to solution in the Ca^{2+}-bound form and in contact with aromatic residues in the Ca^{2+}-free conformation. These observations support the general mechanism originally proposed for the calcium−myristoyl switch and provide evidence for a direct interaction between the myristoyl group and residues in the hydrophobic cleft observed in the crystal structure.

MATERIALS AND METHODS

Purification of Recoverin. Unmodified, myristoylated, and deuteromyristoylated recoverin were expressed and purified by phenyl-Sepharose chromatography essentially as described previously (Ray *et al.*, 1992). Cells used to express the deuteromyristoylated recoverin were induced in the presence of perdeuterated myristic acid (Serdary Research Laboratories, London, Ontario). Nonmyristoylated recoverin used for fluorescence experiments was not purified further. Myristoylated and deuteromyristoylated recoverin used for NMR experiments were purified further as follows. The phenyl-Sepharose EGTA eluate containing recoverin was diluted 5-fold with 10 mM Tris-HCl (pH 8.0), applied to an FPLC Mono-Q column and eluted with a 0−0.5 M NaCl gradient. The recoverin peak fractions were pooled and further purified by reverse-phase HPLC on a C_{18} column using a 5−80% acetonitrile gradient (in the presence of 0.08% trifluoroacetic acid). This final chromatography step separates the unmodified recoverin (approximately 10%) from the modified form. Peaks corresponding to the modified and unmodified forms were identified by electrospray mass spectroscopy analysis. The myristoylated recoverins were lyophilized, redissolved in 10 mM Tris-HCl (pH 8.0), 5 mM EDTA, and 8 M urea, and renatured by dialysis against 10 mM Tris-HCl (pH 8.0), and 5 mM EDTA.

Fluorescence Spectroscopy. Fluorescence spectra were measured on an SLM 8000 (Aminco, Urbana, IL) fluorimeter using a 1 × 0.5 cm cuvette, at 25 °C. Samples (1.0 mL) contained 50 mM MOPS (pH 7.05), 50 mM KCl, 1 mM H-EDTA, 100 μM ANS (Molecular Probes, Eugene, OR), and 3 μM of either unmofidied or myristoylated recoverin. An excitation wavelength of 365 nm was used. Spectra of Ca^{2+}-free recoverin/ANS samples were measured, and 7.5 μL of 1 M $CaCl_2$ was added. After 2 min, Ca^{2+}-bound recoverin/ANS spectra were measured.

Fluorescence quenching experiments were performed on the same instrument using samples essentially the same as those used in the ANS binding experiments. An excitation wavelength of 292 nm was used. Acrylamide was added by serial additions of aliquots of a 5 M acrylamide solution prepared in fluorescence buffer. Fluorescence intensity values were corrected for dilution.

NMR Spectroscopy. Myristoylated and deuteromyristoylated recoverin samples used for NMR investigations were prepared in buffer containing 50 mM Tris-d_{11} and 10 mM dithiothreitol-d_{10} in 99.9% D_2O. The solution pH was adjusted to 8.0 using either 4% NaOD or 2% DCl without

correction for isotope effects. Final recoverin concentrations were approximately 1 mM. Ca^{2+}, when present, was added directly to recoverin samples from a 1.0 M $CaCl_2$ stock solution to a final concentration of 5 mM.

All NMR data were collected on a Bruker DMX-500 spectrometer at 25 °C. Pairs of samples in which the covalently attached myristoyl group was either fully protonated (^{1}H-MRV) or deuterated (^{2}H-MRV) were used to collect one-dimensional (1D) ^{1}H NMR spectra of recoverin in both the Ca^{2+}-free and Ca^{2+}-bound conformations. Each 1D ^{1}H NMR spectrum consists of an average of 1024 transients of 16K data points with a spectral width of 7002 Hz (14 ppm). The resulting 1D spectra of matched recoverin samples were baseline-corrected and normalized relative to the area of aromatic resonances to correct for small concentration differences, and difference spectra (^{1}H-MRV minus ^{2}H-MRV) were calculated using UXNMR software provided by Bruker Instruments, Inc.

All two-dimensional (2D) ^{1}H−^{1}H NOESY spectra (512 t_1 increments by 2048 t_2 data points) were collected in TPPI mode on the Ca^{2+}-free form of myristoylated recoverin using a mixing time of 75 ms and 32 scans/t_1 increment. All 2D data sets were processed using the software package FELIX (Biosym, San Diego, CA). 2D difference spectra were calculated from ^{1}H−^{1}H NOESY spectra of Ca^{2+}-free recoverin covalently modified with either ^{1}H- or ^{2}H-myristate in 50 mM Tris-D_2O buffer at pH 8.0. 2D spectra were normalized relative to the total calculated peak volume in each two-dimensional matrix to correct for small protein concentration differences between recoverin samples before calculation of the NOESY difference spectra.

RESULTS

Interaction of Ca^{2+}-Free and Ca^{2+}-Bound Recoverin with ANS. The calcium−myristoyl switch model proposes that, in Ca^{2+}-free recoverin, the myristoyl moiety is sequestered in a hydrophobic region of the protein and that, in response to Ca^{2+} binding, the N-terminus and myristoyl group moves from this region so as to become available for interaction with membranes and/or other proteins. If this model is correct, one would predict that there would be a Ca^{2+}-dependent increase in the solvent-accessible hydrophobic surface area of recoverin. In order to test this hypothesis, we examined the interaction of Ca^{2+}-free and Ca^{2+}-bound recoverin with the fluorescent dye 1-anilinonaphthalene-8-sulfonate (ANS). The fluorescent emission properties of ANS are known to be affected by the polarity of its local environment; as the polarity of its environment decreases, the quantum yield increases, and the wavelength of its emission maximum decreases (Stryer, 1965).

Figure 2A shows fluorescence spectra of ANS alone and ANS in the presence of the Ca^{2+}-free and Ca^{2+}-bound forms of myristoylated and nonmyristoylated recoverin. In the presence of both forms of Ca^{2+}-free recoverin (and 1 mM EDTA), we observe a modest increase in the ANS quantum yield and a change in the emission maximum from 532 nm (ANS alone) to 501 nm for myristoylated recoverin and 495 nm in the case of nonmyristoylated recoverin. The Ca^{2+}-free nonmyristoylated recoverin supports a greater increase in quantum yield of ANS as compared to the myristoylated protein. Upon addition of Ca^{2+} (to 4 mM), we observe a significant increase in the quantum yield and a change in the emission maximum to 485 nm for both forms of recoverin. These observations are consistent with there being an increase in hydrophobic ANS binding sites on both myristoylated and nonmyristoylated recoverin, accompanying the change from the Ca^{2+}-free to the Ca^{2+}-bound conformation.

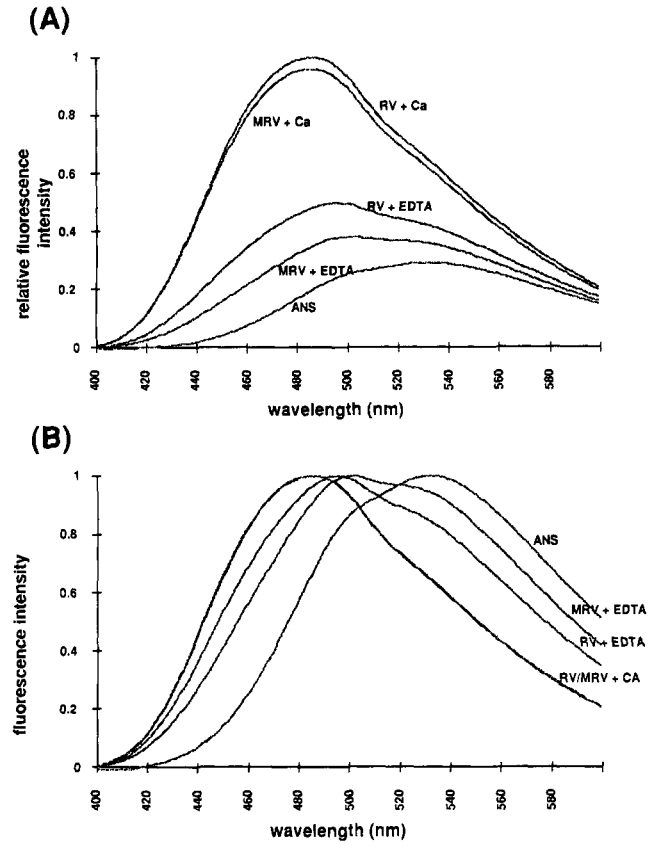

FIGURE 2: Recoverin-ANS fluorescence spectra. Panel A shows the fluorescence emission spectra for ANS alone (ANS), in the presence of 3 μM nonmyristoylated recoverin in the Ca^{2+}-free (RV + EDTA) and Ca^{2+}-bound forms (RV + Ca), and in the presence of 3 μM myristoylated recoverin in the Ca^{2+}-free (MRV + EDTA) and Ca^{2+}-bound forms (MRV + Ca). The increase in ANS fluorescence intensity and decrease in the wavelength of the emission maxima upon addition of Ca^{2+} indicates that an increase in the solvent-accessible hydrophobic surface of recoverin accompanies Ca^{2+} binding. Panel B shows these same spectra normalized relative to their respective emission maxima: 532 nm for ANS alone; 495 nm for Ca^{2+}-free nonmyristoylated recoverin; 501 nm for Ca^{2+}-free myristoylated recoverin; 485 nm for both Ca^{2+}-bound proteins. Comparison of the normalized spectra indicate an equivalence of the Ca^{2+}-bound forms. The Ca^{2+}-free spectra, however, indicate that myristoylated recoverin has less accessible hydrophobic surface than the nonmyristoylated protein. This is also evident in the relative amounts of ANS fluorescence observed for the Ca^{2+}-free recoverins (panel A).

Figure 2B shows the ANS−recoverin spectra from Figure 2A normalized to 1 at their emission maxima. Comparison of the spectral shapes shows that the spectra of the Ca^{2+}-bound forms of myristoylated and nonmyristoylated recoverin are essentially identical. This suggests that, with regard to ANS binding (i.e., polarity and number of ANS binding sites), the two forms of recoverin are highly similar in their Ca^{2+}-bound conformations. In contrast to these, the spectral shapes of the two Ca^{2+}-free proteins markedly differ from one another. Although the two spectra display similar features, the spectrum of the nonmyristoylated recoverin is clearly skewed toward shorter wavelengths. In this regard,

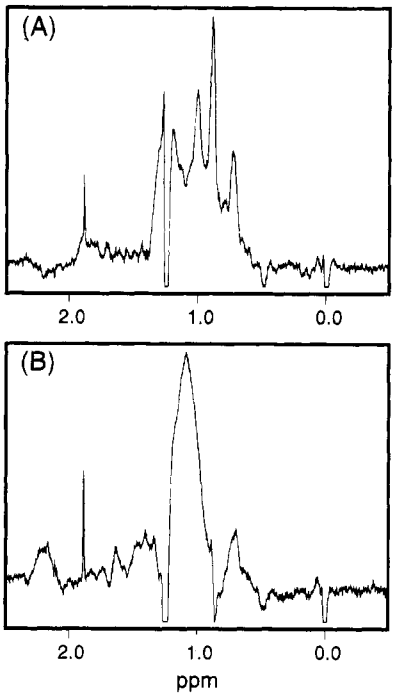

FIGURE 3: ^{1}H NMR difference spectra of myristoyl protons in Ca^{2+}-free and Ca^{2+}-bound recoverin. Panel A shows the NMR difference spectrum resulting from the subtraction of the ^{2}H-MRV spectrum from the spectrum of ^{1}H-MRV under Ca^{2+}-free conditions. The myristoyl methylene protons give rise to a dispersed group of discrete resonances centered around 1.0 ppm. The chemical shift dispersion arises from direct interactions between myristoyl protons with the amino acids in the protein. Panel B shows the same difference spectrum after the addition of Ca^{2+}. The dispersed peaks coalesce into a single broad envelope centered at 1.09 ppm. This observation is consistent with the myristoyl group being relatively solvated in Ca^{2+}-bound recoverin.

its shape is more similar to the Ca^{2+} spectra than is that of the Ca^{2+}-free myristoylated recoverin, indicating that removal of the myristoyl group results in greater exposure of hydrophobic regions of recoverin.

NMR Spectroscopy of ^{1}H- and ^{2}H-Myristoylated Recoverin. In order to investigate the local environment of the myristoyl moiety in the Ca^{2+}-free and Ca^{2+}-bound forms of recoverin, we used 1D and 2D NMR difference spectra to isolate resonances that arise specifically from the aliphatic protons of the myristoyl group. This was accomplished by preparing two myristoylated recoverin (MRV) samples, one with fully protonated myristate (^{1}H-MRV) and the other with uniformly deuterated myristate (^{2}H-MRV). Subtraction of the ^{2}H-MRV spectrum from the ^{1}H-MRV spectrum collected under identical conditions effectively removes overlapping protein resonances to give a difference spectrum containing only resonances which arise from the aliphatic protons of the bound myristoyl group. The resulting 1D difference spectrum for Ca^{2+}-free recoverin is shown in Figure 3A. The difference spectrum clearly shows several discrete myristoyl resonances which are centered around 1.0 ppm. The observed spectral dispersion must arise from direct interactions between the myristoyl group and the protein since the majority of methylene proton resonances are expected to be degenerate for a fully solvated myristoyl moiety. Addition of Ca^{2+} to recoverin results in a loss of the spectral dispersion of myristoyl proton resonances (Figure 3B). The individual peaks coalesce into a single envelope of overlapping myristoyl proton resonances centered around 1.09 ppm. This

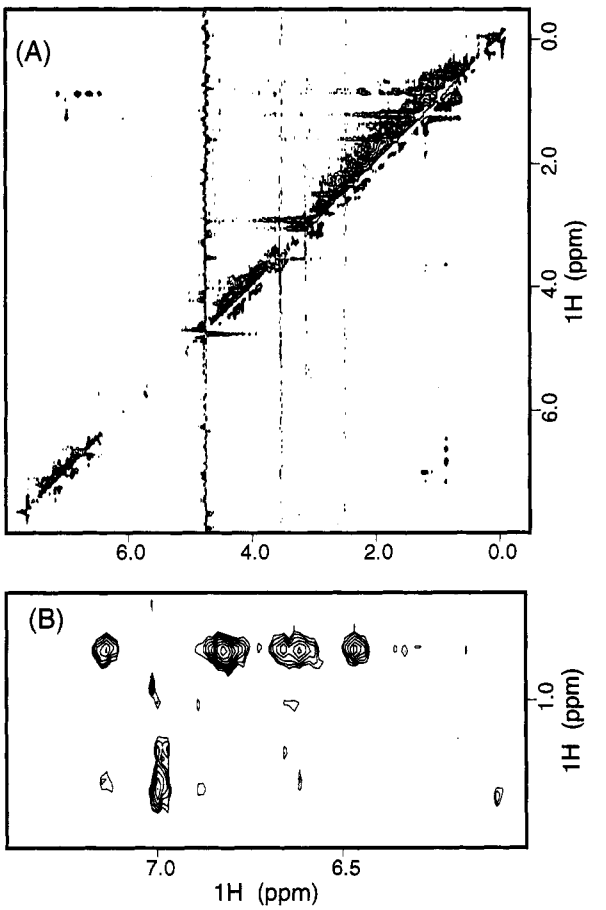

FIGURE 4: ^{1}H-^{1}H 2D NOESY difference spectrum of Ca^{2+}-free myristoylated recoverin. Panel A shows the calculated 2D NOESY difference spectrum generated by subtraction of the NOESY spectrum of Ca^{2+}-free ^{2}H-MRV from a similar spectrum of Ca^{2+}-free ^{1}H-MRV collected under identical conditions. Panel B shows an enlargement of the region between 1.4−0.7 ppm (F1) and 7.3−6.0 ppm (F2) showing strong NOESY cross peaks between myristoyl protons and aromatic side chains.

observation is consistent with the idea that the binding of Ca^{2+} induces a conformational change in recoverin which exposes the myristoyl moiety to the solvent. The line width of the myristoyl ^{1}H resonances is likely broadened by the Ca^{2+}-induced aggregation of recoverin (Kataoka *et al.*, 1993; Ames *et al.*, 1995), which is particularly evident at the protein concentrations required for NMR investigations.

In Ca^{2+}-free recoverin, the myristoyl group is thought to be sequestered in a hydrophobic cleft of the protein which contains a number of aromatic residues (Flaherty *et al.*, 1993). Information on residues that are near each other in the tertiary structure of the protein may be obtained using 2D homonuclear NOESY. However, the size of MRV (23 kDa) and the large number of aliphatic residues means that resonances which arise from the myristoyl group would be obscured by the large number of overlapping cross peaks present in a conventional homonuclear NOESY spectrum, complicating analysis. Therefore, we used 2D NOE difference spectroscopy to greatly simplify the spectrum and to look for specific interactions between the myristoyl group and aromatic side chains in the Ca^{2+}-free form of recoverin. Figure 4A shows the NOESY difference spectrum calculated by subtraction of the 2D NOESY spectrum of Ca^{2+}-free ^{2}H-MRV from the spectrum of ^{1}H-MRV collected under identical conditions. In such a difference spectrum, all

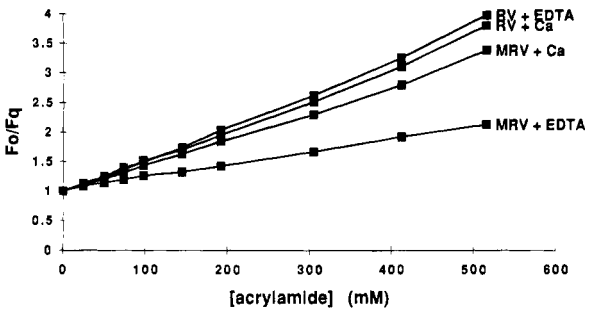

FIGURE 5: Effects of myristoylation and Ca^{2+} on acrylamide quenching of recoverin tryptophan fluorescence. Tryptophan fluorescence quenching curves for myristoylated and nonmyristoylated recoverin in the presence of either EDTA or Ca^{2+} are shown. The fluorescence intensity (at 335 nm) in the absence of acrylamide (F_0) divided by the intensity in the presence of acrylamide at each concentration (F_q) is plotted versus acrylamide concentration. As indicated by the similarity of their slopes, the quenching of tryptophan fluorescence in nonmyristoylated recoverin is relatively efficient and insensitive to the presence (RV + Ca) or absence (RV + EDTA) of Ca^{2+}. Solvent accessibility of tryptophan is therefore not significantly different in the Ca^{2+}-bound and Ca^{2+}-free conformations of nonmyristoylated recoverin. In contrast to this, the solvent accessibility of tryptophan is significantly reduced in the Ca^{2+}-free form of myristoylated recoverin (MRV + EDTA) relative to that in the Ca^{2+}-bound form (MRV + Ca). Note also that the slope of the quenching curve for the Ca^{2+}-bound form of myristoylated recoverin is similar to those of the nonmyristoylated proteins.

intraprotein cross peaks will be suppressed, leaving only cross peaks originating from myristoyl protons. Figure 4 shows there are several NOESY cross peaks between the aliphatic myristoyl protons, identified in the 1D difference spectrum of Figure 3A, and aromatic side chains. These cross peaks are clearly evident on both sides of the diagonal. The region between 6.0−7.3 ppm (*F*2) and 0.7−1.4 ppm containing well-defined cross peaks is shown enlarged in Figure 4B. These data show that the covalently bound myristoyl group is within NOE contact distance (≤5 Å) of a number of aromatic side chains in the Ca^{2+}-free form of recoverin.

Effects of Myristoylation and Ca^{2+} on Tryptophan Fluorescence Quenching. The NOESY difference spectrum clearly demonstrates interactions between myristoyl protons and aromatic residues in Ca^{2+}-free recoverin. This observation is consistent with the model that, in the Ca^{2+}-free conformation of MRV, the myristoyl group interacts with residues in the hydrophobic cleft. The proposed myristoyl-binding region contains a number of aromatic residues including F23, Y53, F56, F57, Y86, and W31 (Figure 1B). If the myristoyl group contacts this part of the protein in the Ca^{2+}-free conformation, then myristoylation of recoverin should also influence the solvent accessibility of W31 in a Ca^{2+}-dependent manner. We looked for evidence for such an interaction by examining the effect of Ca^{2+} on the acrylamide quenching of tryptophan fluorescence of myristoylated and non-myristoylated recoverin.

Figure 5 shows acrylamide quenching curves for myristoylated and non-myristoylated forms of recoverin in the presence of either EDTA or Ca^{2+}. The tryptophan fluorescence of non-myristoylated recoverin is efficiently quenched by acrylamide in the presence of either EDTA or Ca^{2+}. Since the slopes of the quenching curves are relatively similar in the Ca^{2+}-free and Ca^{2+}-bound forms of non-myristoylated recoverin, we conclude that the solvent accessibilities of the tryptophans are not significantly different in the two conformations. In contrast to this, we observe a significant difference in quenching between the Ca^{2+}-free and Ca^{2+}-bound forms of the myristoylated protein. In the presence of EDTA, the quenching is relatively inefficient. The quenching curve for the Ca^{2+}-bound form is similar to those observed for nonmyristoylated recoverin. Since recoverin contains three tryptophan residues (at positions 31, 104, and 156), it is not possible to assign unequivocally the quenching phenomenon to a particular residue or residues in the wild-type protein. However, the crystal structure of recoverin shows that W31 is situated near the amino terminus (and therefore near the myristoyl group) in the solvent-exposed hydrophobic cleft (Figure 1). Since Ca^{2+} binding alone does not significantly affect tryptophan quenching in the absence of myristoylation, this suggests a direct interaction between the myristoyl group and a tryptophan residue in Ca^{2+}-free recoverin. Structural considerations would therefore strongly indicate that W31 paricipates in this interaction.

DISCUSSION

In this study, we have used several lines of investigation to examine the calcium−myristoyl switch model for the Ca^{2+}-dependent conformational change and membrane association of bovine recoverin. On the basis of earlier studies of the Ca^{2+}-dependent proteolytic susceptibility and membrane localization of recoverin, it has been proposed that, in Ca^{2+}-free recoverin, the myristoyl group is sequestered in a hydrophobic part of the protein, and in the Ca^{2+}-bound form, the myristoylated N-terminus is positioned in such a way as to allow myristoyl−membrane interactions. A crystal structure of nonmyristoylated recoverin with a single Ca^{2+} bound at EF-hand 3 has recently been reported (Flaherty *et al.*, 1993). An interesting feature revealed in this structure is the presence of a large surface cluster of hydrophobic residues in the N-terminal domain of the protein (see Figure 1). This raises the possibility that this hydrophobic surface may function as a myristoyl binding site in Ca^{2+}-free recoverin. We have tested this hypothesis using fluorescence and NMR spectroscopy.

Fluorescence spectroscopy analysis of the interaction of Ca^{2+}-free and Ca^{2+}-bound recoverin with ANS demonstrates that Ca^{2+} binding results in a significant increase in the amount of solvent-accessible hydrophobic surface on both myristoylated and nonmyristoylated recoverin. Comparison of the ANS spectra of myristoylated and nonmyristoylated recoverin in the Ca^{2+}-free conformation indicates that both the unmodified N-terminus and the myristoylated N-terminus are able to protect hydrophobic residues from solution but that the myristoylated N-terminus confers a greater degree of protection. The difference in ANS binding by the Ca^{2+}-free forms of recoverin can be seen with respect to both the relative quantum yields and the wavelength of the emission maxima. Furthermore, these data show that the N-terminus is able to occupy the hydrophobic region in the absence of lipid modification. This is in agreement with the observation that the Ca^{2+}-dependent tryptic proteolysis of the N-terminus of recoverin is not noticeably affected by myristoylation, nor is the Ca^{2+}-dependent interaction of recoverin with phenyl-Sepharose (Dizhoor *et al.*, 1993; C.-K. Chen and J. B. Hurley, unpublished observations). Upon addition of Ca^{2+}, we observe an increase in ANS quantum yield and a shift in the fluorescent emission maximum toward shorter wavelengths. Since the ANS spectra of the Ca^{2+}-bound forms of

myristoylated and nonmyristoylated recoverin are extremely similar, we conclude that the Ca^{2+}-dependent exposure of hydrophobic surface is not grossly affected by the presence or absence of myristoylation and that the conformations of these two species are, with respect to the surface hydrophobicity of the protein, essentially identical. These data are consistent with the myristoyl group being in solution, rather than associated with the protein, in the presence of Ca^{2+}.

Solution ^1H NMR difference spectra of myristoyl protons provide further support for these conclusions. In Ca^{2+}-free recoverin, the 1D difference spectrum shows a number of discrete resonances for the myristoyl protons (Figure 3A). This is consistent with individual myristoyl methylene protons occupying unique chemical environments as a result of being packed against the protein. This is confirmed by the 2D NOESY difference spectrum (Figure 4), which shows several direct interactions between myristoyl protons and aromatic residues in the Ca^{2+}-free conformation of recoverin. When Ca^{2+} is added to these samples, the disperse peaks coalesce into a single envelope of peaks centered at 1.09 ppm (Figure 3B). This is most likely due to the movement of the myristoyl group into solution, where individual methylene proton resonances are expected to be equivalent.

Since W31 is located in the solvent-exposed hydrophobic cleft (Figure 1), we investigated the possibility of an interaction between tryptophan and the myristoyl group in the Ca^{2+}-free form of recoverin by examining the effects of both Ca^{2+} binding and myristoylation on the quenching of tryptophan fluorescence by acrylamide. These results indicate that a solvent-accessible tryptophan is protected from quencher by the myristoylation, but only in the Ca^{2+}-free conformation. This is in agreement with earlier observations of recoverin tryptophan fluorescence that were suggestive of a direct myristoyl−tryptophan interaction (Ray *et al.*, 1992). Briefly, these observations are as follows. The tryptophan fluorescence emission maximum of myristoylated recoverin is 333 nm in the Ca^{2+}-free form and 339 nm in the presence of Ca^{2+}. The emission maximum for the nonmyristoylated recoverin is 339 nm in either conformation. This indicates that the myristoyl group is, in some way, providing a nonpolar environment for a tryptophan (or tryptophans) in the Ca^{2+}-free conformation. The Ca^{2+} and myristoyl dependence of this fluorescence change suggest that the tryptophan is protected from solvent by the myristoyl group in the Ca^{2+}-free form and is deprotected in the Ca^{2+}-bound form.

Although these quenching data alone do not allow us to specify which tryptophan (or tryptophans) in myristoylated recoverin is undergoing a Ca^{2+}-dependent change in quenching, W31 would seem the the most likely on the basis of the following considerations. Recoverin contains three tryptophan residues (W31, W104, and W156). In the Ca^{2+}-bound structure, W104 and W156 are relatively buried in the interior of the C-terminal domain, while W31 is solvent-exposed in the hydrophobic cluster in the N-terminal domain (see Figure 1). Since efficient acrylamide quenching requires solvent accessibility of the fluorophore, W31 would be expected to interact more efficiently with quencher than either W104 or W156. There is, however, the possibility that a Ca^{2+}-binding-induced conformational change in the C-terminus could itself significantly alter the solvent accessibility of W104 and W156. If this were the case, we would expect to observe significant differences in the quenching of the Ca^{2+}-free and Ca^{2+}-bound forms of nonmyristoylated recoverin. We do not observe this, but rather we find that the change in quenching requires myristoylation. Furthermore, circular dichroism studies on myristoylated and nonmyristoylated recoverin show that the secondary structural change that accompanies Ca^{2+} binding is not affected by myristoyl modification (Kataoka *et al.*, 1993). Finally, consideration of the relative distances between the N-terminus and W31, W104, and W158 (along with their relative solvent accessibilities) would indicate that an interaction between the myristoyl group and W31 would require the least dramatic changes in conformation relative to that which is observed in the crystal structure (Figure 1). Our data do not allow us to assign specifically a direct interaction between the myristoyl group and W31 in the hydrophobic cleft of Ca^{2+}-free recoverin. An alternative possibility is that binding of the myristoyl group in the hydrophobic cleft serves to stabilize an interaction between N-terminal residues and W31, resulting in the protection of W31 from the bulk solvent. This model is supported by the observation that the nonacylated N-terminus provides partial inhibition of ANS binding to the hydrophobic cleft in the Ca^{2+}-free form of recoverin (Figure 2). Furthermore, NMR data indicate that, in solution, the N-terminal helix is longer in Ca^{2+}-free myristoylated recoverin as compared to that of Ca^{2+}-bound nonmyristoylated crystal form, in which residues 2−8 are disordered (Ames *et al.*, 1994; Flaherty *et al.*, 1993). In any case, this model would place the myristoyl group in proximity to, or in direct contact with, W31.

The data presented in this study are in agreement with the general features of the calcium−myristoyl switch model originally proposed to account for recoverin's Ca^{2+}-dependent membrane association. Our observations provide strong evidence that the surface cluster of hydrophobic residues present on the N-terminal domain play a role in the sequestration of the myristoyl group in the Ca^{2+}-free conformation of recoverin. Furthermore, we provide evidence that the myristoyl group is in solution in the Ca^{2+}-bound conformation. The position of the myristoyl group in these two conformations can account for the Ca^{2+}-dependent membrane association by a mechanism which involves a reversible availability of the myristoyl group for interaction with the lipid bilayer. Figure 6 presents a model to account for our observations. Figure 6A represents the Ca^{2+}-free form of nonmyristoylated recoverin. The N-terminus (cylinder) partially occupies the hydrophobic cluster (shaded oval) but does not protect W31 (open indole ring) from solution (W104 and W156 are shown as shaded indole rings). Figure 6B represents the Ca^{2+}-free form of myristoylated recoverin. This is essentially the same as shown in Figure 6A, but the myristoyl group excludes additional solvent from the hydrophobic cluster and contacts W31. Figure 6C represents the Ca^{2+}-bound form of myristoylated recoverin. The myristoylated N-terminus is no longer sequestered in the hydrophobic binding site and the myristoyl group is exposed in solution where it can interact with membrane or another protein.

NOTE ADDED IN PROOF

During the course of this study, the solution structure of Ca^{2+}-free myristoylated recoverin was determined by NMR spectroscopy (Tanaka *et al.*, 1995). This structure shows that the myristoyl group is sequestered in a hydrophobic

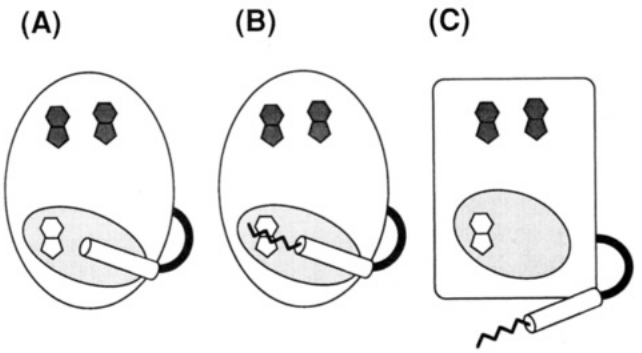

FIGURE 6: Cartoon of Ca^{2+}-free and Ca^{2+}-bound conformations of recoverin. A model, consistent with our observations, for the Ca^{2+}- and myristoyl-dependent occupancy of the hydrophobic cluster is presented. (A) represents the Ca^{2+}-free state of non-myristoylated recoverin. The N-terminus (cylinder) occupies part of the hydrophoic cluster (shaded oval) but does not directly protect W31 (open indole) from solvent (W104 and 156 are represented as shaded indoles). (B) representing Ca^{2+}-free myristoylated recoverin, is similar to (A) but indicates the additional presence of the myristoyl group in the hydrophobic cluster that results in enhanced exclusion of ANS and protection of W31 from solvent. (C) represents myrisotylated recoverin in the Ca^{2+}-bound state. In this conformation, the myristoylated N-terminus is removed from the hydrophobic cluster and the myristoyl group is exposed to solution where it can interact with membrane or other proteins. The Ca^{2+}-dependent movement of the N-terminus from the hydrophobic cluster does not require myristoylation [figure modified from Dizhoor *et al.* (1993)].

pocket formed mainly by residues in the N-terminal domain. The tertiary structure of this hydrophobic pocket, however, is significantly different from that observed in the crystal structure. A large number of NOE contacts between hydrophobic residues and the myristoyl group are observed. Five of the six aromatic residues present in the hydrophobic pocket of Ca^{2+}-bound recoverin, including W31, are in NOE contact with myristoyl protons. This structure is therefore in agreement with our general conclusions regarding the location of the myristoyl group in Ca^{2+}-free recoverin.

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