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Nuclear Magnetic Resonance Evidence for Ca^{2+}-induced Extrusion of the Myristoyl Group of Recoverin*

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Recoverin, a recently discovered member of the EF-hand protein superfamily, serves as a Ca^{2+} sensor in vision. A myristoyl or related *N*-acyl group covalently attached to the amino terminus of recoverin enables it to translocate to retinal disc membranes when the Ca^{2+} level is elevated. Two-dimensional ^{1}H-^{13}C shift correlation NMR spectra of recoverin containing a ^{13}C-labeled myristoyl group were obtained to selectively probe the effect of Ca^{2+} on the environment of the attached myristoyl group. In the Ca^{2+}-free state, each pair of methylene protons bonded to carbon atoms 2, 3, 11, and 12 of the myristoyl group gives rise to two peaks. The splittings, caused by nonequivalent methylene proton chemical shifts, indicate that the myristoyl group interacts intimately with the protein in the Ca^{2+}-free state. By contrast, only one peak is seen for each pair of methylene protons in the Ca^{2+}-bound state, indicating that the myristoyl group is located in an isotropic environment in this form. Furthermore, the ^{1}H-^{13}C shift correlation NMR spectrum of Ca^{2+}-bound recoverin is very similar to that of myristic acid in solution. ^{1}H-^{13}C shift correlation NMR experiments were also performed with ^{13}C-labeled recoverin to selectively probe the resonances of methyl groups in the hydrophobic core of the protein. The spectrum of Ca^{2+}-bound myristoylated recoverin is different from that of Ca^{2+}-free myristoylated recoverin but similar to that of Ca^{2+}-bound unmyristoylated recoverin. Hence, the myristoyl group interacts little with the hydrophobic core of myristoylated recoverin in the Ca^{2+}-bound state. Three-dimensional ($^{13}C/F_1$)-edited ($^{13}C/F_3$)-filtered heteronuclear multiple quantum correlation-nuclear Overhauser effect spectroscopy spectra of recoverin containing a ^{13}C-labeled myristoyl group were obtained to selectively probe protein residues located within 5 Å of the myristoyl group. The myristoyl group makes close contact with a number of aromatic residues in Ca^{2+}-free recoverin, whereas the myristoyl group makes no observable contacts with the protein in the Ca^{2+}-bound state. These NMR data demonstrate that the binding of Ca^{2+} to recoverin induces the extrusion of its myristoyl group into the solvent, which would enable it to interact with a lipid bilayer or a hydrophobic site of a target protein.

Recoverin, a retinal 23-kDa calcium-binding protein, serves as a Ca^{2+}-sensor in vision (1–3). The protein modulates the Ca^{2+}-sensitive deactivation of rhodopsin (4–7). The x-ray crystal structure of unmyristoylated recombinant recoverin with a single bound Ca^{2+} showed that the protein contains four EF-hand motifs arranged in a compact linear array (8). EF-2 and EF-3 but not EF-1 and EF-4 can bind Ca^{2+} (8, 9). Retinal recoverin contains a covalently attached NH_2-terminal myristoyl or related fatty acyl group (10). The binding of two Ca^{2+} to myristoylated but not unmyristoylated recoverin induces its translocation to disc membranes (11, 12). What is the molecular mechanism of the calcium-myristoyl switch mediating this Ca^{2+}-induced membrane interaction? One possibility *a priori* is that the myristoyl group of recoverin is sequestered in the Ca^{2+}-free state and extruded in the Ca^{2+}-bound state (12). Alternatively, the binding of Ca^{2+} could lead to the exposure of a hydrophobic surface, which would be stabilized by a persistently buried myristoyl group. Structural information is needed to distinguish between these possibilities.

We have recently solved the structure of Ca^{2+}-free myristoylated recoverin in solution by multidimensional NMR spectroscopy (13). The myristoyl group is sequestered in a deep hydrophobic pocket formed by aromatic and other nonpolar groups contributed by five flanking helices. The lower solubility and aggregation of the Ca^{2+}-bound form of the myristoylated protein has thus far precluded a complete determination of its structure by NMR methods. However, we succeeded in carrying out NMR studies that were designed to answer two questions. 1) Does the binding of Ca^{2+} to myristoylated recoverin induce the extrusion of the myristoyl group, or does it remain sequestered? The preparation of recoverin bearing a ^{13}C-labeled myristoyl group enabled us to specifically probe the environment of the methylene protons of the fatty acyl group. 2) What is the effect of Ca^{2+} binding on the hydrophobic core of the protein? The environment of the methyl groups of isoleucine, leucine, and valine residues of Ca^{2+}-free and Ca^{2+}-bound myristoylated recoverin and of Ca^{2+}-bound unmyristoylated recoverin was specifically probed to learn about the interaction of the core with the myristoyl group.

EXPERIMENTAL PROCEDURES

Sample Preparation—Recombinant myristoylated recoverin with uniformly ^{15}N- or ^{13}C-labeled protein and ^{13}C-labeled myristoyl group was expressed in overproducing *Escherichia coli* strains pTrec2/pBB131/DH5αF′ grown in M9 minimal medium (14) and purified as described previously (12). ^{13}C-labeled myristic acid (99 atom %, Isotec, Inc., Miamisburg, Ohio) was added (5 mg/liter) 1 h before induction of *N*-myristoyl-CoA transferase. Uniformly ^{15}N-/^{13}C-labeled unmyristoy-

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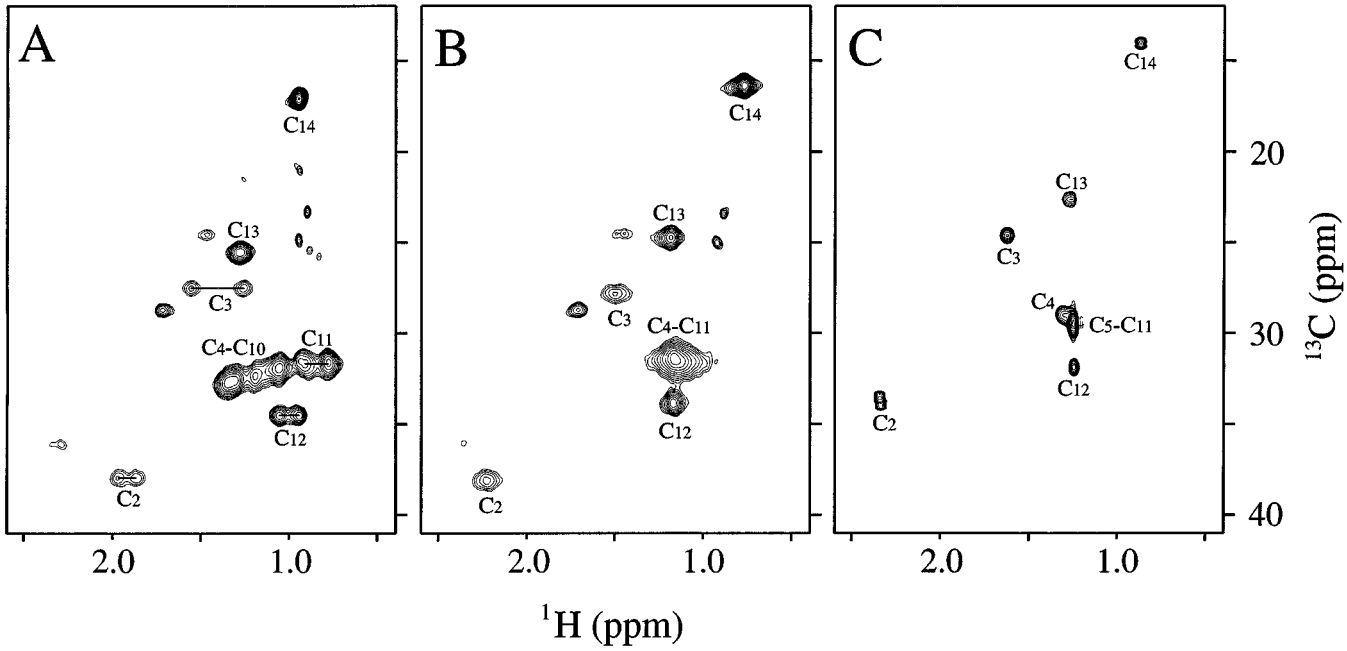

FIG. 1. **Two-dimensional NMR spectra showing that the environment of the attached myristoyl group becomes isotropic when recoverin binds Ca^{2+}.** Two-dimensional ^{1}-^{13}C HMQC NMR spectra of Ca^{2+}-free (*A*), Ca^{2+}-bound myristoylated recoverin (*B*), and free myristic acid dissolved in chloroform (*C*). The attached myristoyl group was labeled with ^{13}C (99%), and the protein was labeled with ^{15}N (>95%).

lated recoverin was expressed in *E. coli* strain pTrec2/DH5α and purified by the procedure used for myristoylated recoverin except that the phenyl-Sepharose chromatography step was replaced by an ammonium sulfate precipitation step in which 70% $(NH_4)_2SO_4$ was added slowly to the bacterial extract to precipitate contaminating proteins. The soluble fraction was then extensively dialyzed to remove $(NH_4)_2SO_4$ and finally purified twice by ion exchange chromatography on Q-Sepharose as described previously (12).

Recoverin samples for NMR experiments were prepared by dissolving lyophilized protein (1 mM) in 99.996% $^{2}H_2O$ (total volume, 500 μl) containing 0.1 M KCl and 10 mM $^{2}H_{10}$-dithiothreitol. The pH was adjusted to 7.0 using 4% NaO^{2}H without correcting for isotope effects. Calcium-bound recoverin samples were produced by adding 10 mM $CaCl_2$. ^{13}C-Myristic acid was dissolved in 500 μl of deuterated chloroform (4 mg/ml).

NMR Spectroscopy—All NMR experiments were performed, unless otherwise noted, at 30 °C on a UNITY-plus 500 spectrometer equipped with a four-channel NMR interface and a triple resonance probe with an actively shielded z gradient together with a pulse field gradient accessory. Two-dimensional ^{1}H-^{13}C HMQC[1] (15) and ^{1}H-^{13}C CT-HSQC (16) spectra were recorded with the following numbers of complex points and acquisition times: ^{13}C (F_1) 512, 51 ms; ^{1}H (F_2) 416, 52 ms (32 transients) for HMQC and ^{13}C (F_1) 128, 26 ms; ^{1}H (F_2) 416, 52 ms (32 transients) for CT-HSQC. A three-dimensional proton-carbon-proton correlation-carbon correlation spectroscopy (17) spectrum of Ca^{2+}-free myristoylated recoverin (the protein is labeled with ^{15}N and the myristoyl group is labeled with ^{13}C) was recorded to assign ^{1}H and ^{13}C resonances of the myristoyl group. The numbers of complex points and acquisition times were: ^{1}H (F_1) 96, 27 ms; ^{13}C (F_2) 22, 7 ms; and ^{1}H (F_3) 416, 52 ms (16 transients). Well resolved ^{1}H and ^{13}C resonances at the positions 2, 3, and 11–14 were unambiguously assigned by proton-carbon-proton correlation using carbon correlation spectroscopy. The other resonances were assigned by careful inspection of the nuclear Overhauser effect patterns observed in the (^{13}C/F_1)-edited (^{13}C/F_3)-filtered HMQC-NOESY (18) spectrum and by the analysis of the NMR-derived structures that were calculated using distance restraints from unambiguously assigned myristoyl signals (13). The numbers of complex points and acquisition times for (^{13}C/F_1)-edited (^{13}C/F_3)-filtered HMQC-NOESY experiment were: ^{1}H (F_1) 64, 15 ms; ^{13}C (F_2) 32, 9 ms; and ^{1}H (F_3) 516, 53 ms (16 transients). Two-dimensional ^{1}H-^{13}C HMQC and incredible natural abundance double quantum transfer experiment (19) spectra of myristic acid were recorded at 25 °C on a standard UNITY-plus 500 spectrometer and used to assign the ^{1}H and ^{13}C resonances of myristic acid. The following numbers of complex points and acquisition times were used: ^{13}C(F_1) 128, 29 ms; ^{1}H(F_2) 256, 128 ms (8 transients) for ^{1}H-^{13}C HMQC and ^{13}C(F_1) 256, 32 ms; ^{13}C(F_2) 512, 128 ms (32 transients) for incredible natural abundance double quantum transfer experiment (19) spectrum. All data sets were processed and analyzed on a Sun Sparc20 workstation using the software nmrPipe and nmrDraw (20).

RESULTS

The environment of the amino-terminal myristoyl group of recoverin in the Ca^{2+}-free and Ca^{2+}-bound states was examined using NMR spectroscopy. Two-dimensional ^{1}H-^{13}C HMQC spectra of recoverin containing a ^{13}C-labeled myristoyl group are shown in Fig. 1, (*A* and *B*). Because this experiment selectively probes protons that are covalently attached to ^{13}C, only the methylene and methyl proton resonances of the myristoyl group are expected to appear in these spectra. Weak extraneous peaks observed near 0.9, 1.5, and 1.7 ppm in the ^{1}H dimension are due to natural abundance ^{13}C signals from the protein. Assignments of the myristoyl group resonances for the Ca^{2+}-free state shown in Fig. 1*A* were derived from proton-carbon-proton correlation using carbon correlation spectroscopy and ^{13}C-filtered NOESY measurements. Methylene protons at positions 2, 3, 11, and 12 exhibit doublet peaks in the ^{1}H dimension. This splitting is not temperature-dependent in the range 25–35 °C and most likely arises from the protein environment of the highly sequestered myristoyl group. A deep, hydrophobic protein surface surrounds the myristoyl group in the solution structure of Ca^{2+}-free myristoylated recoverin (13). Proton resonances at positions 2, 11, and 12 are upfield shifted for Ca^{2+}-free recoverin relative to those of Ca^{2+}-bound recoverin and free myristic acid (Table I). The upfield shifts seen in the spectra of Ca^{2+}-free recoverin are due to aromatic ring-current effects caused by residues (Trp^{31}, Tyr^{32}, Phe^{49}, Tyr^{53}, Phe^{56}, Phe^{57}, Phe^{83}, Tyr^{86}, and Trp^{104}) lining the myristoyl group binding pocket (13).

The addition of Ca^{2+} to recoverin leads to striking spectral changes in the HMQC spectrum (Fig. 1*B*). The chemical shifts of the myristoyl group resonances of Ca^{2+}-bound recoverin are almost identical with those of myristic acid dissolved in chloroform (Fig. 1*C* and Table I). The assignments for free myristic

[1] The abbreviations used are: HMQC, heteronuclear multiple quantum correlation; CT-HSQC, constant-time heteronuclear single quantum correlation; NOESY, nuclear Overhauser effect spectroscopy.

acid can therefore be extended to the myristoyl group of Ca^{2+}-bound recoverin. In Ca^{2+}-bound recoverin, all of the myristoyl group resonances become singlet peaks, indicating that the myristoyl group is located in an isotropic chemical environment in this form. The intensity of the myristoyl group signals were similar before and after the addition of Ca^{2+}, suggesting that these signals were produced by all the recoverin in the sample. Furthermore, the spectral similarity between the myristoyl group of Ca^{2+}-bound recoverin and free myristic acid suggests that the myristoyl group becomes solvent exposed when two Ca^{2+} bind to recoverin.

Fig. 2 presents ^{1}H-^{13}C CT-HSQC spectra of Ca^{2+}-free and Ca^{2+}-bound myristoylated recoverin and Ca^{2+}-bound unmyristoylated recoverin. Aggregation of Ca^{2+}-free unmyristoylated recoverin (21) precluded obtaining a satisfactory spectrum of this form. The spectra shown in Fig. 2 depict the resonances of methyl groups of Ile, Leu, and Val that are highly upfield-shifted (−0.5–0.6 ppm). These methyl groups reside in the hydrophobic core, where they are affected by aromatic ring currents. The peaks having ^{13}C chemical shifts in the range 6–20 ppm are assigned to the methyl groups of Ile, and those having ^{13}C shifts greater than 20 ppm are assigned to the methyl groups of Leu and Val. The spectra of the Ca^{2+}-free (Fig. 2*A*) and Ca^{2+}-bound (Fig. 2*B*) forms of myristoylated recoverin are quite different, suggesting that the binding of Ca^{2+} to myristoylated recoverin may induce conformational changes in its hydrophobic core. Indeed, large structural differences have been observed between the Ca^{2+}-free myristoylated (13) and single Ca^{2+}-bound unmyristoylated (8) forms. By contrast, the spectra of the Ca^{2+}-bound forms of myristoylated (Fig. 2*B*) and unmyristoylated (Fig. 2*C*) recoverin are strikingly similar. Hence, the binding of Ca^{2+} makes the core of the myristoylated protein like that of the unmyristoylated protein. The absence of an effect of the myristoyl group on the protein core in the Ca^{2+}-bound state provides further support for Ca^{2+}-induced extrusion of the fatty acyl chain.

Fig. 3 presents selected slices from a three-dimensional ($^{13}C/F_1$)-edited ($^{13}C/F_3$)-filtered HMQC-NOESY spectrum recorded on Ca^{2+}-free and Ca^{2+}-bound recoverin containing a ^{13}C-labeled myristoyl group. These spectra selectively probe atoms of protein residues that are located within 5 Å of the myristoyl methyl group. The spectrum of Ca^{2+}-free recoverin exhibits nuclear Overhauser effect cross-peaks between the methyl resonance of the myristoyl group and many resonances from aromatic residues (Fig. 3*A*). In contrast, no such cross-peaks were observed in the spectrum of Ca^{2+}-bound recoverin (Fig. 3*B*). The loss of observable nuclear Overhauser effect cross-peaks between the myristoyl group and the protein in the Ca^{2+}-bound state provides additional support for Ca^{2+}-induced extrusion of the myristoyl group.

TABLE I

^{1}H and ^{13}C assignments of myristoyl resonances

^{1}H chemical shifts (ppm) are followed by ^{13}C chemical shifts (ppm) in parentheses.

Position	Ca^{2+}-free recoverin[a]	Ca^{2+}-bound recoverin[a]	Myristic acid[b]
C-2	1.86, 1.97 (38.0)	2.23 (38.1)	2.34 (33.7)
C-3	1.26, 1.55 (27.5)	1.50 (27.8)	1.62 (24.6)
C-4	0.8, 1.1 (32)[c]	1.0–1.3 (30–33)[c]	1.30 (29.0)
C-5–C-10	1.0–1.4 (31–33)[c]	1.0–1.3 (30–33)[c]	1.2–1.3 (29–30)[c]
C-11	0.77, 0.91 (31.6)	1.0–1.3 (30–33)[c]	1.2–1.3 (29–30)[c]
C-12	0.94, 1.05 (34.5)	1.17 (33.9)	1.25 (31.9)
C-13	1.28 (25.5)	1.18 (24.7)	1.26 (22.6)
C-14	0.95 (17.1)	0.77 (16.4)	0.87 (14.1)

[a] Recoverin samples were in 0.1 M KCl, 10 mM [$^{2}H_{10}$]dithiothreitol, pH 7.0, at 30 °C. Chemical shift reference is 3-(trimethylsilyl)propionate, sodium salt.

[b] ^{13}C-Myristic acid was dissolved in deuterated chloroform (4 mg/ml) at 25 °C. Chemical shift reference is tetramethyl silane.

[c] Precision in chemical shifts limited due to peak overlap.

DISCUSSION

Our recent NMR determination of the three-dimensional structure of Ca^{2+}-free myristoylated recoverin (13) showed that the myristoyl group is sequestered in a hydrophobic niche. The goal of the present study was to determine the fate of the myristoyl group in the Ca^{2+}-bound form of the protein. ^{1}H-^{13}C shift correlation spectroscopy probed the environment of the

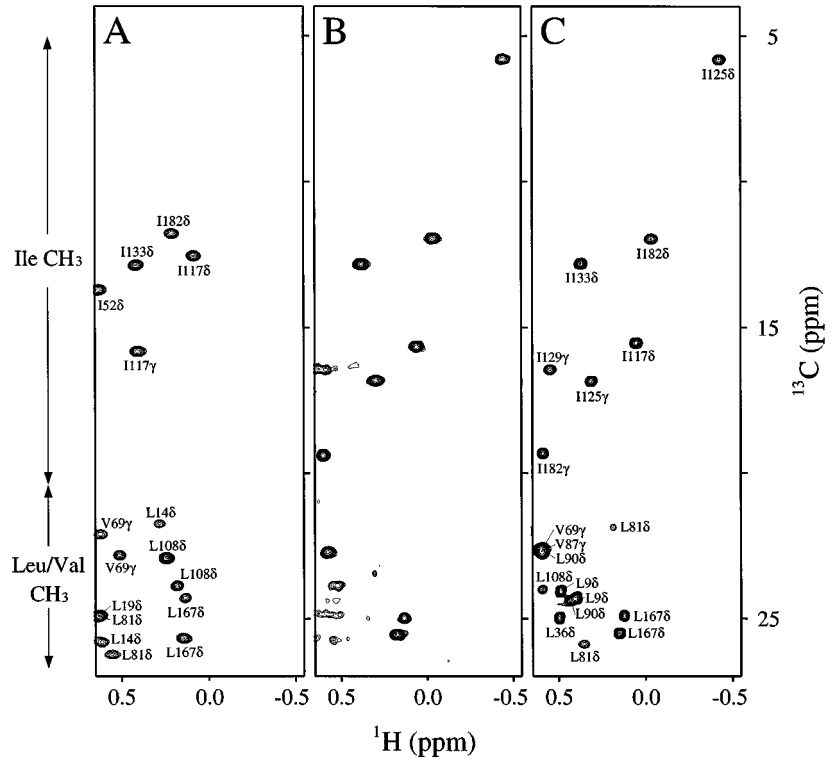

FIG. 2. **Two-dimensional NMR spectra showing that the hydrophobic core of myristoylated recoverin becomes similar to that of unmyristoylated recoverin when Ca^{2+} binds to the protein.** ^{1}H-^{13}C CT-HSQC NMR spectra of Ca^{2+}-free myristoylated (*A*), Ca^{2+}-bound myristoylated (*B*), and Ca^{2+}-bound unmyristoylated recoverin (*C*). The protein was uniformly labeled with ^{13}C (>95%). Sequence-specific assignments are indicated for Ca^{2+}-free myristoylated (13) and Ca^{2+}-bound unmyristoylated recoverin. For the Ca^{2+}-bound myristoylated protein, complete sequence-specific assignments have not been obtained due to a dynamic equilibrium between monomeric and multimeric forms (21). Resonances of many residues in the NH_2-terminal domain are unobservable due to exchange broadening. In contrast, resonances from the COOH-terminal domain are not broadened for Ca^{2+}-bound myristoylated recoverin, and the resonance frequencies are almost identical to those of Ca^{2+}-bound unmyristoylated recoverin.

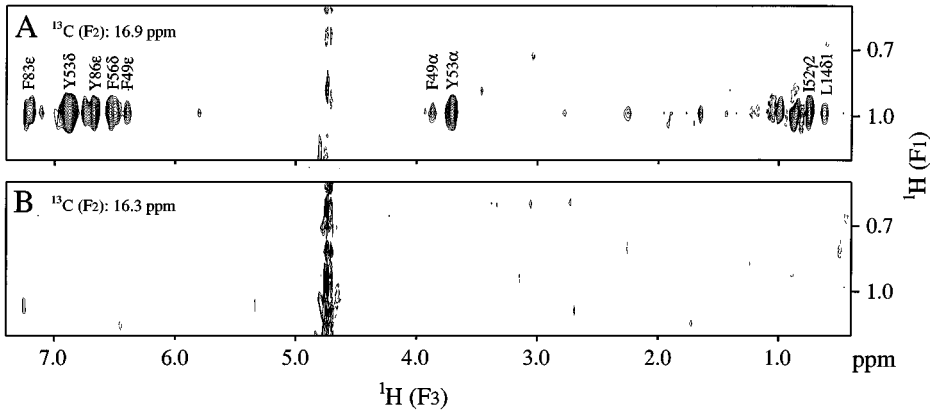

FIG. 3. **Three-dimensional (^{13}C/F$_1$)-edited (^{13}C/F$_3$)-filtered HMQC-NOESY spectra of Ca^{2+}-free (*A*) and Ca^{2+}-bound myristoylated recoverin (*B*).** The attached myristoyl group was labeled with ^{13}C (99%), and the protein was labeled with ^{15}N (>95%). Selected slices at F_2 = 16.9 and 16.3 ppm are shown to specifically probe the myristoyl methyl group.

NH_2-terminal fatty acyl group. These NMR spectra showed that the myristoyl group moves from a sequestered to a solvent-exposed environment when recoverin binds Ca^{2+}. Furthermore, the similarity of the ^{1}H-^{13}C CT-HSQC spectra of Ca^{2+}-bound myristoylated and Ca^{2+}-bound unmyristoylated recoverin indicates that the myristoyl group does not markedly influence the structure of the hydrophobic core of the protein when Ca^{2+} is bound. In contrast, the myristoyl group is essential for the structural integrity of recoverin in the Ca^{2+}-free state. These NMR data demonstrate that the binding of Ca^{2+} leads to the extrusion of the myristoyl group, as proposed by Zozulya and Stryer (12).

Ca^{2+}-induced exposure of the myristoyl group nicely accounts for several previous experimental observations: 1) rod outer segment membranes bind Ca^{2+}-bound myristoylated recoverin but not unmyristoylated or Ca^{2+}-free myristoylated recoverin (11, 12); 2) myristoylated recoverin aggregates in the Ca^{2+}-bound state, whereas it is monomeric in the Ca^{2+}-free state (21); 3) the NH_2-terminal region of Ca^{2+}-bound recoverin can be proteolytically cleaved by trypsin, whereas the Ca^{2+}-free form is resistant (11); and 4) in aqueous solution, myristoylated recoverin has 100-fold lower affinity for Ca^{2+} than does unmyristoylated recoverin (9). The lower Ca^{2+} binding affinity is partly due to the work needed to extrude the myristoyl group from a hydrophobic niche into the aqueous exterior. The 10-fold greater Ca^{2+} binding affinity of myristoylated recoverin in the presence of rod outer segment membranes (5, 7, 9, 12) probably arises from an energetically favorable interaction of the extruded myristoyl group.

A concerted allosteric model has been proposed by Ames *et al.* (9) in which cooperative binding of two Ca^{2+} to recoverin drives the protein from the T state (cytosolic) to the R state (membrane bound). The solution structure of Ca^{2+}-free recoverin, with the myristoyl group buried in a hydrophobic pocket (13), explains why the T state does not bind to membranes. We presented here direct experimental evidence that the myristoyl group is extruded to the protein exterior when two Ca^{2+} bind (the R state). In the rod outer segment, the extruded myristoyl group is almost certainly not in contact with water. Rather, it is inserted either into a lipid bilayer or it is bound to a nonpolar niche of a membrane protein. Positively charged residues located near the NH_2 and COOH termini may also help to promote membrane binding (22, 23).

A series of homologs of recoverin, termed the neurocalcins and hippocalcin, have recently been discovered in the nervous system (24–26). Their amino acid sequences are moderately to highly identical with that of recoverin, and they are acylated at their NH_2 termini. Several of these homologs have been shown to possess Ca^{2+}-myristoyl switches (24, 25). These proteins, like recoverin, may make the deactivation of G protein-coupled receptors Ca^{2+}-sensitive (3, 5, 7). A number of aromatic and other hydrophobic residues lining the myristoyl binding site of recoverin are conserved among these homologs (13), suggesting that the myristoyl group binds to essentially the same site in the Ca^{2+}-free state of all family members. The Ca^{2+}-induced extrusion of the myristoyl group seen in recoverin probably also occurs in the homologs. This can be tested by carrying out ^{1}H-^{13}C shift correlation spectroscopy on homologs containing a ^{13}C-labeled myristoyl group. This NMR method of probing the environment of the myristoyl group at the atomic level is a powerful means of dissecting the structural mechanism of myristoyl switches generally (23).

A number of membrane-binding proteins are modified by post-translational attachment of C_{15} (farnesyl), C_{16} (palmitoyl), or C_{20} (geranyl) acyl groups to cysteine residues. Palmitoylation of various seven-helix receptors and G protein α-subunits is enzymatically reversible; agonist-enhanced turnover of covalently attached palmitate may play a dynamic role in regulating various signal transduction events (27, 28). In contrast, co-translational myristoylation of recoverin and other proteins is a permanent modification. As demonstrated here for recoverin, ligand-induced extrusion of the myristoyl group is one means of achieving reversible membrane targeting. Dynamic attachment and removal of acyl groups from cysteine residues serves as an additional mechanism. Proteins such as nonreceptor tyrosine kinases (Fyn, Lck, Yes, Fgr, and Hck (29, 30)) and some G protein α-subunits ($G_{ol\alpha}$ and $G_{il\alpha}$ (28, 31)) are both myristoylated and palmitoylated. The myristoyl switch in combination with reversible palmitoylation may serve to modulate the membrane binding affinity and regulate protein-protein interactions on the membrane surface.

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Note Added in Proof—Hughes *et al.* (32) have recently published one-dimensional proton NMR spectra of myristoylated recoverin. Several discrete myristoyl resonances in the Ca^{2+}-free state coalesce into a single envelope of overlapping resonances in the calcium-bound state. They conclude, as we do, that the binding of calcium to recoverin leads to the exposure of the myristoyl group to solvent.

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