Recoverin, a member of the neuronal calcium sensor branch of the EF-hand superfamily, serves as a calcium sensor that regulates rhodopsin kinase (RK) activity in retinal rod cells. We report here the NMR structure of Ca\textsuperscript{2+}-bound recoverin bound to a functional N-terminal fragment of rhodopsin kinase (residues 1–25, called RK25). The overall main-chain structure of recoverin in the complex is similar to structures of Ca\textsuperscript{2+}-bound recoverin in the absence of target (\textsigma;1.8 \text\AA~root-mean-square deviation). The first eight residues of recoverin at the N terminus are solvent-exposed, enabling the N-terminal myristoyl group to interact with target membranes, and Ca\textsuperscript{2+} is bound at the second and third EF-hands of the protein. RK25 in the complex forms an amphipathic helix (residues 4–16). The hydrophobic face of the RK25 helix (Val-9, Val-10, Ala-11, Ala-14, and Phe-15) interacts with an exposed hydrophobic groove on the surface of recoverin lined by side-chain atoms of Trp-31, Phe-35, Phe-49, Ile-52, Tyr-53, Phe-56, Phe-57, Tyr-86, and Leu-90. Residues of recoverin that contact RK25 are highly conserved, suggesting a similar target binding site structure in all neuronal calcium sensor proteins. Site-specific mutagenesis and deletion analysis confirm that the hydrophobic residues at the interface are necessary and sufficient for binding. The recoverin-RK25 complex exhibits Ca\textsuperscript{2+}-induced binding to rhodopsin immobilized on concanavalin-A resin. We propose that Ca\textsuperscript{2+}-bound recoverin is bound between rhodopsin and RK in a ternary complex on rod outer segment disk membranes, thereby blocking RK interaction with rhodopsin at high Ca\textsuperscript{2+}.

Calcium ion (Ca\textsuperscript{2+}) in retinal rod cells plays a critical role in regulating the phototransduction cascade in vision (1–3). Recoverin, a 23 kDa Ca\textsuperscript{2+}-binding protein and member of the EF-hand superfamily, serves as a Ca\textsuperscript{2+} sensor in retinal rods (4, 5). Recoverin prolongs the lifetime of photoexcited rhodopsin by inhibiting rhodopsin kinase only at high Ca\textsuperscript{2+} levels (5–8). Hence, recoverin makes the desensitization of rhodopsin responsive to Ca\textsuperscript{2+}, and the shortened lifetime of photoexcited rhodopsin at low Ca\textsuperscript{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 segment (9). Upon light activation, 98% of recoverin is detected in the rod inner segment, whereas in the dark more than 10% of recoverin returns to the outer segment (10), consistent with the conventional role of recoverin in the inhibition of RK.

Recoverin contains four EF-hand Ca\textsuperscript{2+} binding motifs and a myristoyl or related fatty acyl group covalently attached at the N terminus (11). The cooperative binding of two Ca\textsuperscript{2+} to the second and third EF-hands (EF-2 and EF-3) induces the binding of myristoylated, but not unmyristoylated recoverin to rod outer segment disc membranes (12, 13). The three-dimensional structures of myristoylated recoverin with 0, 1, and 2 Ca\textsuperscript{2+} bound have been determined by NMR spectroscopy (14–16). In the Ca\textsuperscript{2+}-free state, the myristoyl group is sequestered in a deep hydrophobic cavity inside the protein. The binding of two Ca\textsuperscript{2+} leads to the extrusion of the myristoyl group and exposure of hydrophobic residues. The Ca\textsuperscript{2+}-induced exposure of the myristoyl group, termed Ca\textsuperscript{2+}-myristoyl switch, enables recoverin to bind to membranes only at high Ca\textsuperscript{2+}. Ca\textsuperscript{2+}-bound recoverin on the membrane surface also binds to rhodopsin kinase (RK) (17) and inhibits the kinase activity only at high Ca\textsuperscript{2+} levels (6, 18). By contrast, the Ca\textsuperscript{2+}-free form of recoverin does not bind and inhibit RK. Ca\textsuperscript{2+}-bound recoverin interacts with RK exclusively at residues 1–25 (19), and deletion of these residues also abolishes the kinase interaction with light-excited rhodopsin (20). According to surface plasmon resonance measurements, the N-terminal RK peptide binds to recoverin with micromolar affinity and only in the presence of Ca\textsuperscript{2+} (19, 20).

Recoverin is structurally similar to other NCS proteins (Fig. 4A): Frq1 (21), frequenin (22), neurocalcin (23), GCAP2 (24), GCAP3 (25), and KChIP1 (26). The common structural features of NCS proteins are an ~200-residue chain containing four EF-hand motifs, the sequence CP\textalpha\textomega\textomega\textomega, which in the first EF-hand that markedly impairs its capacity to bind Ca\textsuperscript{2+}, and an N-terminal myristoylation consensus sequence. In all NCS proteins, the four EF-hands form two domains packed in a globular
arrangement that contrasts with the dumbbell-shaped arrangement of EF-hand domains seen in calmodulin (27) and troponin C (28). A striking feature of the NCS structures is a solvent-exposed groove lined by hydrophobic residues, Phe-23, Trp-31, Phe-35, Phe-49, Ile-52, Tyr-53, Phe-56, Tyr-86, and Leu-90 in the N-terminal domain of recoverin that remain invariant in all other NCS proteins (Fig. 1, highlighted in boldface). These hydrophobic residues in GCAP2, recoverin, and KChIP1 have been implicated previously in target recognition (26, 29, 30).

We report here the NMR-derived structure of Ca\(^{2+}\)/H11001-bound recoverin in solution bound to a functional fragment of rho-dopsin kinase (residues 1–25, hereafter referred to as RK25). This is the first atomic resolution structure of a Ca\(^{2+}\)/H11001-myristoyl switch protein bound to a functional target protein. The structure reveals that RK25 forms a long amphipathic helix, whose hydrophobic surface interacts with the hydrophobic groove of recoverin described above. We propose that recoverin binding to this N-terminal helix might block rhodopsin binding to the kinase active site, because deletion of the N-terminal RK residues abolishes interaction with light-excited rhodopsin (20). The structural interaction of RK25 with recoverin reported here is somewhat different from structures of target complexes seen previously with other EF-hand proteins like calmodulin (31), KChIP1 (26), calcineurin B (32), and troponin C (33) and may offer new insight into the structural basis of target specificity.

**EXPERIMENTAL PROCEDURES**

** Protein Preparation —** To prepare recombinant bovine unmyristoylated recoverin uniformly labeled with nitrogen-15 and carbon-13, the protein was expressed in *Escherichia coli* strain BL21(DE3) carrying the pET11a vector (Novagen) harboring the recoverin coding sequence grown in M9 minimal medium containing \([1^{15}N]NH_4Cl\) and \([1^{13}C_6]glucose\) according to well-established procedures (34–36). Labeled recoverin was purified from the soluble fraction of bacterial cell lysates using phenyl-Sepharose hydrophobic interaction chromatography as described previously (12, 37). Peak fractions were then applied to an anion-exchange column (50-ml bed, DEAE-Sepharose equilibrated in buffer A (1 M EGTA, 1 mM MgCl\(_2\), 10 mM Tris-HCl, pH 7.5) and eluted with a linear salt gradient (0–0.2 M KCl) at a flow rate of 2 ml min\(^{-1}\) over the course of 180 min. Peak fractions were concentrated to 5 ml, and final purity was greater than 98%, as judged by SDS-PAGE.
A functional polypeptide fragment of RK (residues 1–25, named RK25) uniformly labeled with $^{15}$N and/or $^{13}$C and tagged with a C-terminal His$_6$ tract was expressed in E. coli strain BL21(DE3)-RIL (Stratagene) carrying the pET31b vector (Novagen) harboring the RK25 coding sequence grown in M9 medium containing $[^{15}$N]NH$_4$Cl and $[^{13}$C]glucose (34–36). Labeled RK25 fused with ketosteroid isomerase was isolated from the insoluble fraction of bacterial cell lysates dissolved in 8 M urea buffer and purified using Ni$^{2+}$-chelate affinity chromatography on a nitrilotriacetate resin (Ni-NTA, Qiagen), according to the manufacturer's instructions. Peak fractions were then dialyzed extensively against 4 liters of distilled H$_2$O to remove urea. After dialysis the insoluble fusion protein was then isolated by centrifugation and cleaved by cyanogen bromide according to the manufacturer's instructions. The cleaved RK25-His$_6$ peptide was then dissolved in 8M urea buffer and purified again using Ni$^{2+}$-chelate affinity chromatography (Qiagen). The purified RK25 peptide was dialyzed against acetate buffer (10 mM sodium acetate, pH 5.0) to remove urea and concentrated about 10-fold to a final concentration of 0.5 mM used in NMR experiments.

The recoverin-RK25 complex used in NMR experiments was prepared by mixing a relatively dilute stock solution of Ca$^{2+}$-bound recoverin (0.05 mM recoverin, 10 mM Tris, 2 mM CaCl$_2$, and 5 mM dithiothreitol, pH 7.0) to 1 eq of RK25 (0.05 mM RK25 in 1 mM sodium acetate, 2 mM CaCl$_2$, and 5 mM dithiothreitol at pH 5.0). The dilute complex was then concentrated 20-fold to generate a final sample used in NMR studies (0.3 ml of 1 mM recoverin-RK25 at pH 7.0).

**Pulldown Binding Assay**—100 μl of Ni-NTA resin was equilibrated in batch mode with a 1:1 mixture of RK25-His$_6$ and recoverin in the presence and absence of saturating Ca$^{2+}$ and N-terminal myristoylation. The equilibrated beads were washed twice to remove any unbound recoverin. The washed beads were treated with 10 μl of 1% SDS sample buffer and analyzed using SDS-PAGE (Fig. 2A). Similar pulldown assays using delipidated rhodopsin immobilized on concanavalin-A-Sepharose (38) were performed to measure the binding of recoverin and RK25-His$_6$ to rhodopsin.

**Isothermal Titration Calorimetry (ITC)**—ITC experiments were performed using a MicroCal VP-ITC microcalorimeter as described previously (39). Samples of RK25 and Ca$^{2+}$-bound recoverin were dialyzed overnight against high Ca$^{2+}$ buffer (10 mM Tris, 2 mM CaCl$_2$, and 1 mM dithiothreitol, pH 7.0). A sample of the dialysis buffer was placed in the reference cell, and RK25 was placed in the sample cell. Experiments were performed at 25 °C with an RK25 concentration of 70 μM. 25 injections of 1 mM Ca$^{2+}$-bound recoverin were made in 10-μl aliquots. An injection delay of 240 s was utilized to allow the baseline to return after each injection. A blank titration into dialysis buffer was subtracted from the data to correct for heat of dilution. Analysis of the ITC data were performed as described previously (39, 40).

**NMR Spectroscopy**—Samples for NMR analysis consisted of $^{15}$N-labeled or $^{13}$C/$^{15}$N-labeled recoverin (1.0 mM) bound to 1 eq of unlabeled RK25 in 0.3 ml of a 95% H$_2$O, 5% [3H]H$_2$O solution containing 10 mM Tris and 2 mM CaCl$_2$, pH 7.0). Reverse-labeled samples (i.e. $^{15}$N- or $^{13}$C/$^{15}$N-labeled RK25 bound to 1 eq of unlabeled recoverin) were also prepared for the NMR experiments. All NMR experiments were performed at 37 °C on a Bruker DRX-500 or DRX-600 spectrometer equipped with a 4-channel interface and triple-resonance probe with triple-axis-pulsed field gradients and a DRX-600 spectrometer equipped with an Ultrashield Bruker magnet, a three-channel interface, and cryo-probe with z-axis pulsed field gradients. The $^1$H,$^1$N HSQC spectra (see Fig. 3) were recorded on a sample of $^{15}$N-labeled recoverin bound to unlabeled RK25 (Fig. 3A) and $^{15}$N-labeled RK25 bound to unlabeled recoverin (Fig. 3B) in 95% H$_2$O, 5% $^2$H$_2$O. The number of complex points and acquisition times were: 256, 180 ms ($^{15}$N (F$_1$)); 512,
64 ms ($^{1}H (F_{2})$). The $^{13}C(F_{1})$-edited, $^{13}C(F_{3})$-filtered NOESY-heteronuclear multiple quantum coherence spectra were recorded on a sample of unlabeled recoverin protein bound to $^{13}C$-labeled RK25 (41) as well as $^{13}C$-labeled recoverin bound to unlabeled RK25 (data not shown). Intermolecular NOESY experiments were performed as described previously (42). Stereospecific assignments of chiral methyl groups of valine and leucine were obtained by analyzing $^{13}C,^{1}H$ HSQC experiments performed on a sample that contained 10% $^{13}C$ labeling in either recoverin or RK25 (43). All triple-resonance experiments were performed, processed, and analyzed as described (44, 45) on a sample of $^{13}C,^{15}N$-labeled recoverin-RK25 (in 95% $H_{2}O$, 5% $D_{2}O$) with the following number of complex points and acquisition times: HNCO ($^{15}N (F_{1})$ 32, 23.7 ms; $^{13}CO (F_{2})$ 64, 42.7 ms; $^{1}H (F_{3})$ 512, 64 ms); HNCACB ($^{15}N (F_{1})$ 32, 23.7 ms; $^{13}C (F_{2})$ 48, 6.3 ms; $^{1}H (F_{3})$ 512, 64 ms); CBCACONNH ($^{15}N (F_{1})$ 32, 23.7 ms; $^{13}C (F_{2})$ 48, 6.3 ms; $^{1}H (F_{3})$ 512, 64 ms); CBCACOCAHA ($^{13}C (F_{1})$ 52, 6.8 ms; $^{13}CO (F_{2})$ 64, 42 ms; $^{1}H (F_{3})$ 384, 64 ms); HBHACONNH ($^{13}N (F_{1})$ 32, 23.7 ms; $^{1}H_{ab} (F_{2})$ 64 21 ms; $^{1}H (F_{3})$ 512, 64 ms).

The triple resonance and NOESY spectra measured above were assigned and analyzed to determine secondary and tertiary structure in the recoverin-RK25 complex. The chemical shift index (see Wishart et al. (46) for a detailed description), $^{3}J_{NH_{2}}$ coupling constants, and NOE connectivity patterns for each residue were analyzed and provided a measure of the overall secondary structure. Small $^{3}J_{NH_{2}}$ coupling constants (<5 Hz), strong NOE connectivities (NN(i,i+1) and αN(i,i+3)), and positive chemical shift index are characteristic of residues in an α-helix. Conversely, large $^{3}J_{NH_{2}}$ coupling constants (>8 Hz), strong αN(i,i+1) and weak NN(i,i+1) NOE connectivities, and a negative chemical shift index are characteristic of residues in a β-strand. The results of the secondary structure analysis of recoverin-RK25 complex are summarized schematically in Fig. 1.

**Structure Calculation**—Backbone and side-chain NMR resonances of recoverin-RK25 complex were assigned as described previously (45) and will be deposited into the BioMagRes NMR Data bank before publication. Structure calculations were performed using the YASAP protocol within X-PLOR (47), as described previously (48). A total of 2592 interproton distance constraints were obtained as described (45) by analysis of $^{13}C$-edited and $^{15}N$-edited NOESY-HSQC spectra (100-ms mixing time) of $^{13}C,^{15}N$-labeled recoverin bound to unlabeled RK25 and $^{13}C,^{15}N$-labeled RK25 bound to unlabeled recoverin. In addition to the NOE-derived distance constraints, the following additional constraints were included in the structure calcu-
In recoverin bound to RK25 by NMR, we first developed a recombinant RK25 construct. To determine the atomic-resolution structural interaction of recoverin bound to RK25, residues of RK (called RK25) (19, 20). To determine the atomic-resolution structural interaction of recoverin bound to RK25, we have shown that recoverin interacts with the N-terminal 25 residues of RK (called RK25) (19, 20). To determine the atomic-resolution structural interaction of recoverin bound to RK25 by NMR, we first developed a recombinant RK25 construct expressed in E. coli that produces milligram amounts of isotope-labeled RK25 and without interference from glutathione S-transferase or other fusion partners (see "Experimental Procedures"). To verify that our bacterially expressed RK25 sample is functionally intact, we performed a series of pulldown assays (Fig. 2A) showing that it interacts with both myristoylated and unmyristoylated forms of Ca2+-bound recoverin and does not interact with Ca2+-free myristoylated recoverin (Fig. 2A). Ca2+-bound recoverin and not the Ca2+-free form also interacts with delipidated rhodopsin coupled to concanavalin A-Sepharose (38, 49) (Fig. 2A), suggesting that Ca2+ induces recoverin to form a ternary complex with both RK25 and rhodopsin. Indeed, RK25 alone does not bind to immobilized rhodopsin (data not shown), but the recoverin-RK25 complex binds intact to the rhodopsin resin, consistent with a ternary interaction. The energetics of recoverin binding to RK25 was determined using ITC (Fig. 2B). The calorimetric titration was conducted at 25 °C as described under “Experimental Procedures.” Titration of Ca2+-bound recoverin into RK25 resulted in a simple monophasic isotherm. Analysis of the ITC data using a "1 site" model (MicroCal Origin Software) revealed that RK25 binds to Ca2+-bound recoverin with a 1:1 stoichiometry, a dissociation constant of 1.4 μM, and a binding enthalpy of −1.3 kcal/mol.

### RESULTS

#### Recoverin Binding to RK25 and Rhodopsin

Previous studies have shown that recoverin interacts with the N-terminal 25 residues of RK (called RK25) (19, 20). To determine the atomic-resolution structural interaction of recoverin bound to RK25 by NMR, we first developed a recombinant RK25 construct expressed in E. coli that produces milligram amounts of isotope-labeled RK25 and without interference from glutathione S-transferase or other fusion partners (see “Experimental Procedures”). To verify that our bacterially expressed RK25 sample is functionally intact, we performed a series of pulldown assays (Fig. 2A) showing that it interacts with both myristoylated and unmyristoylated forms of Ca2+-bound recoverin and does not interact with Ca2+-free myristoylated recoverin (Fig. 2A). Ca2+-bound recoverin and not the Ca2+-free form also interacts with delipidated rhodopsin coupled to concanavalin A-Sepharose (38, 49) (Fig. 2A), suggesting that Ca2+ induces recoverin to form a ternary complex with both RK25 and rhodopsin. Indeed, RK25 alone does not bind to immobilized rhodopsin (data not shown), but the recoverin-RK25 complex binds intact to the rhodopsin resin, consistent with a ternary interaction. The energetics of recoverin binding to RK25 was quantified using ITC (Fig. 2B). The calorimetric titration was conducted at 25 °C as described under “Experimental Procedures.” Titration of Ca2+-bound recoverin into RK25 resulted in a simple monophasic isotherm. Analysis of the ITC data using a "1 site" model (MicroCal Origin Software) revealed that RK25 binds to Ca2+-bound recoverin with a 1:1 stoichiometry, a dissociation constant of 1.4 μM, and a binding enthalpy of −1.3 kcal/mol.

### Structural Statistics for the Recoverin-RK25 Complex

| Parameter                                | Recoverin | RK25 |
|------------------------------------------|-----------|------|
| NMR restraints                           | 848       | 105  |
| Short to medium range (residue i to i+j, j = 1–4) | 440       | 0    |
| Long range (residue i to i+j, j > 4)      | 124       | 18   |
| Hydrogen bond                            | 12        | 20   |
| Calcium bound at EF2 and EF3             | 205       | 55   |
| Intermolecular distance restraints        |           |      |
| Average root mean square deviation (Å) among 15 refined structures | 0.67 ± 0.08 | 0.8 ± 0.09 |
| Backbone of structured regions%          | 78        | 64   |
| Heavy atoms of structured regions%        | 1.15 ± 0.06 | 1.25 ± 0.1 |
| Backbone of all residues%                | 0.9 ± 0.09 | 1.1 ± 0.09 |
| Heavy atoms of all residues%             | 1.5 ± 0.1  | 1.6 ± 0.1  |
| Deviations from idealized geometry        |           |      |
| Bond lengths (Å)                         | 0.0082 ± 0.0001 | 0.0098 ± 0.0001 |
| Bond angles (°)                          | 2.09 ± 0.009 | 2.11 ± 0.009 |
| Improper (°)                             | 0.90 ± 0.005 | 0.99 ± 0.005 |
| Ramachandran statistics of 20 structures |           |      |
| Most favored regions (%)                 | 78        | 64   |
| Additional allowed regions (%)           | 17        | 20   |
| Generously allowed regions (%)           | 4         | 12   |
| Disallowed regions (%)                   | 1         | 4    |

*Residues were selected on the basis of 15N backbone dynamics. Recoverin, residues 9–17, 25–37, 43–45, 46–56, 62–73, 80–82, 83–92, 101–109, 116–118, 119–132, 148–157, 166–168, 169–177; RK25, residues 4–16.

NMR Structural Analysis of Recoverin-RK25 Complex

The 1H,15N HSQC NMR spectrum of 15N-labeled recoverin exhibited spectral changes in slow exchange that saturated upon the addition of 1 eq of unlabeled RK25 (Fig. 3A and supplemental Fig. 1), confirming that recoverin binds to RK25 in a 1:1 complex under NMR conditions. The 1H,15N HSQC spectrum of 15N-labeled recoverin in the complex exhibited the expected number of backbone amide resonances (195) with uniform intensity, indicating that recoverin in the complex is structurally homogeneous and stably folded. Pulsed field gradient NMR diffusion studies (50) and dynamic light scattering analysis confirmed that the complex is globular and monomeric under NMR conditions.

The 1H,15N HSQC NMR spectrum of 15N-labeled RK25 bound to 1 eq of unlabeled recoverin exhibited highly resolved peaks with uniform intensity, suggesting that RK25 adopts well-defined secondary structure and is stably bound to recoverin in the complex (Fig. 3B). By contrast, the spectrum of RK25 in the absence of recoverin showed much narrower chemical shift dispersion, indicative of a random coil secondary structure (NMR data not shown). Some of the RK25 residues in the complex (M1–F3 and 19–25) exhibited very broad resonances, indicating that these residues are dynamically disordered. The RK25 residues in the complex that exhibit sharp NMR peaks (G4–A18) adopt α-helical secondary structure (Fig. 1B) as determined by analyses of J-coupling, chemical shift index, hydrogen-deuterium exchange, and sequential NOE patterns (see supplemental Fig. 2).

More than 90% of the observed NMR resonances and NOE data for the recoverin-RK25 complex were assigned as described under “Experimental Procedures,” which then served as the basis for determining the structural constraints in Table 1. The chemical shift assignments and structural constraints
have been deposited into the Biological Magnetic Resonance Data Bank data base repository (accession number 7293). The final structures derived from the NMR data in Table 1 are illustrated in Fig. 4 (RCSB Protein Data Bank code 2I94). The overall structure of recoverin-RK25 is a 1:1 complex with overall dimensions, 46 Å (length) by 28 Å (height) by 31 Å (depth). The final NMR-derived structures (15 lowest energy structures of a total of 30) were superimposed, and the root mean square deviation relative to the mean structure was calculated to be 0.6 Å for main-chain atoms and 1.18 Å for all heavy atoms in regions of regular secondary structure. The average main-chain structure of the complex in solution is represented as a ribbon diagram in Fig. 4, A and B.

The structure of recoverin in the complex (Fig. 4) is similar to that of Ca\(^{2+}\)-bound recoverin alone in solution (root mean square deviation = 1.8 Å) (16) and the Ca\(^{2+}\)-bound forms of other NCS proteins (22, 26, 51, 52). The unmyristoylated N-terminal region of recoverin (residues 2–9) is solvent-exposed and structurally disordered as it is in all other Ca\(^{2+}\)-bound NCS proteins and in contrast to the highly sequestered N-terminal region of Ca\(^{2+}\)-free myristoylated recoverin (14). A total of 11 α-helices and 4 β-strands are observed in recoverin: H1 (residues 9–17), H2 (residues 25–37), H3 (residues 46–56), H4 (residues 62–73), H5 (residues 83–92), H6 (101–109), H7 (residues 119–132), H8 (residues 134–138), H9 (residues 148–157), H10 (residues 169–178), H11 (residues 182–188), S1 (residues 43–45), S2 (residues 80–82), S3 (residues 116–118) and S4 (residues 166–168) (Fig. 1A). Recoverin contains two domains comprised of four EF-hands: EF1 (green) and EF2 (red) are linked and form the N-terminal domain. EF3 (cyan) and EF4 (yellow) form the C-terminal domain. EF2 and EF3 interact and form a cleft at the interface between the two domains. Each EF-hand consists of a helix-turn-helix structure very similar to the structure of Ca\(^{2+}\)-occupied EF-hands seen in previous x-ray structures of calmodulin (27) and troponin C (28). The four EF-hands of recoverin are arranged in a tandem array and form a globular structure with a concave hydrophobic groove on the surface of the N-terminal domain (highlighted in yellow in Fig. 4, C and D).

The structure of RK25 in the complex consists of an amphipathic α-helix (residues 4–16, Fig. 1B). The hydrophobic surface of the RK25 helix (Leu-6, Val-9, Val-10, Ala-11, Phe-15) interacts with the exposed hydrophobic groove on recoverin (Trp-31, Phe-35, Phe-49, Ile-52, Tyr-53, Phe-56, Phe-57, Tyr-86, and Leu-90). Dipolar residues on the opposite face of the RK25 helix (Ser-5, Thr-8, Asn-12, Ile-16) are solvent-exposed. The helical structure of RK25 in the complex is stabilized by hydrophobic intermolecular interactions with recoverin, as free RK25 in solution is completely unstructured (supplemental Fig. 3).

**Intermolecular Structural Interactions**—Analysis of isotope-filtered NMR data selectively probed intermolecular contacts involving amino acid residues of recoverin located less than 5 Å away from residues of RK25 located at the binding interface (C–D). Bound calcium ions are colored orange. The EF-hands and RK25 helix in panel A are colored as defined in Fig. 1. Exposed hydrophobic residues forming a groove on the surface of recoverin are highlighted yellow in C and D.

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**FIGURE 4.** NMR-derived structures of recoverin-RK25 target complex. The average main-chain structure of 15 NMR-derived structures (Protein Data Bank code 2I94) is depicted as ribbon diagrams (A–B) and space-filling models (C–D). Bound calcium ions are colored orange. The EF-hands and RK25 helix in panel A are colored as defined in Fig. 1. Exposed hydrophobic residues forming a groove on the surface of recoverin are highlighted yellow in C and D.
The four EF-hands form two domains connected by a U-shaped myristoyl group. Ca$^{2+}$-free state, the covalently attached myristoyl group sequesters the N-terminal hydrophobic groove and covers up the target binding site. In short, Ca$^{2+}$-induced extrusion of the myristoyl group of recoverin causes exposure of residues that bind and inhibit RK. Physiologically, this Ca$^{2+}$-induced inhibition of RK by recoverin provides a mechanism that helps explain light adaptation. In the dark or under dim illumination Ca$^{2+}$ levels in retinal rods are maintained high (54, 55) and Ca$^{2+}$-bound recoverin inhibits RK, which prolongs the lifetime of light-excited rhodopsin (R*) and maximizes sensitivity of the rod cell. Under bright illumination cytosolic Ca$^{2+}$ levels decrease ~10-fold (56), resulting in the dissociation of Ca$^{2+}$-free recoverin from RK (17) and activation of RK (18), thus promoting phosphorylation of R*. The phosphorylated receptor then binds to arrestin, causing inactivation of R* and desensitization of the rod cell.

Rhopodin kinase binding to recoverin is structurally related to target binding seen in other EF-hand proteins including calmodulin and KChIP1 (Fig. 6). EF-hand proteins generally form a stable domain of two interacting EF-hands that create an exposed crevice to interact with a helical segment of target proteins (colored magenta in Fig. 6). In calmodulin, the two N-terminal EF-hands interact with one side of a target helix peptide (M13) derived from myosin light-chain kinase (Fig. 6A) (31). N-terminal EF-hands of KChIP1 interact in a related fashion with an α-helix derived from the T1 domain of Kv4.2 channels (Fig. 6B) (26). The orientation of the RK25 helix bound to recoverin (Fig. 6C), however, is somewhat different from that of calmodulin and KChIP1. The RK25 helix lies nearly parallel to the helices of EF2 (magenta), whereas the M13 helix is positioned almost perpendicular to the EF2 helices of CaM. The parallel orientation of the target helix with respect to EF2 would cause steric clashes in the CaM complex due to bulky side chains on M13 (Phe-17 and Ile-20) that interact with EF2. In addition, the M13 helix contains a number of lysine residues (Fig. 5). The hydrophobic face of the RK25 helix (colored magenta in Fig. 5) interacts with many aromatic residues in the exposed hydrophobic groove of recoverin (highlighted in yellow in Fig. 5). Most striking are intermolecular contacts involving RK25 residues Val-9, Val-10, Ala-11, Ala-14, and Phe-15. The γ1-methyl group of Val-9 (RK25) contacts the γ2-methyl of Ile-52 and the aromatic ring of Tyr-53 and Phe-56 (recoverin). The side-chain methyl groups of Val-9 (RK25) are flanked on both sides by aromatic rings of Phe-56 and Phe-57 (recoverin). The side-chain methyl groups of Val-10 (RK25) contact the δ1-methyl group of Ile-52 and aromatic rings of Phe-49 and Tyr-86 from recoverin. The β-methyl group of Ala-11 (RK25) contacts both the δ1- and δ2-methyl groups of Leu-90 and aromatic ring protons (HH2 and HZ3) of Trp-31. The β-methylene group of S13 (RK25) makes contact with the δ1- and γ2-methyl groups of Ile-52 and aromatic ring protons of Phe-56. The side-chain methyl group of Ala-14 (RK25) is spatially close (<5 Å) to the aromatic side chains of Phe-35 and Tyr-86 from recoverin. The aromatic ring of Phe-15 (RK25) makes contact with the aromatic ring of Trp-31 and Phe-35. The intricate network of intermolecular hydrophobic interactions illustrated in Fig. 5 accounts for the high binding affinity ($K_d = 1.4 \mu M$) and favorable enthalpy (Fig. 2B). Indeed, a recent site-directed mutagenesis analysis of RK25 demonstrated that Val-9, Val-10, and Phe-15 are each essential for the binding interaction with recoverin (20).
Structure of Recoverin Bound to Rhodopsin Kinase

![Diagram of recoverin bound to rhodopsin kinase]

**FIGURE 6. Target binding to various EF-hand proteins.** Shown are calmodulin (CaM)/M13 peptide derived from MLCK (PDB code 1CDL) (A), KChIP1/Kv4.2N30 peptide derived from T1 domain of A-type voltage-gated K⁺ channels (PDB code 1S6C) (B), and recoverin-RK25 complex from this study (C).

**FIGURE 7. Schematic model of Ca²⁺-induced inhibition of rhodopsin kinase.** Myristoylation (red) targets Ca²⁺-bound recoverin to the membrane surface, where it interacts with rhodopsin. Recoverin also interacts with the N-terminal helix of rhodopsin kinase (magenta), forming a ternary complex on the membrane surface that blocks phosphorylation of rhodopsin. Light activation leads to a lowering of cytosolic Ca²⁺, causing conformational changes in recoverin that sequester the covalently attached myristoyl group and disrupt the interaction with rhodopsin kinase. Ca²⁺-free recoverin then dissociates from the membrane surface, allowing RK to phosphorylate the C-terminal tail of light-excited rhodopsin.

(Lys-6, -18, and -19) that form ionic interactions with CaM, in contrast to the RK25 helix that forms mainly hydrophobic contacts with recoverin. Finally, the RK25 helix (Gly-7, Ala-11, and Phe-15) makes important contacts with Trp-31 in recoverin that are not conserved in either KChIP1 or CaM. These different intermolecular structural contacts along with the altered orientation of the target helix with respect to EF-2 provide a structural basis for target specificity.

The structure of the recoverin-RK25 complex combined with a recent deletion and mutagenesis analysis of RK (20) provides insight into the mechanism of calcium-induced inhibition of rhodopsin kinase by recoverin (Fig. 7). Our structure of the recoverin-RK25 complex reveals an exposed N-terminal myristoyl group that we propose may recruit the recoverin-RK complex to the membrane surface in close proximity to rhodopsin. In addition, we presented data in this study suggesting that Ca²⁺-bound recoverin may form a ternary complex with RK and rhodopsin (Fig. 2A). On the basis of these observations, we propose the following molecular mechanism for kinase inhibition. In the dark, when Ca²⁺ levels are high, Ca²⁺-bound recoverin is at the membrane surface, where it interacts on one side with rhodopsin and on the other side with the N-terminal helix of RK. In essence, recoverin is sandwiched between rhodopsin and RK and thereby blocks their interaction, which may explain in part the kinase inhibition. One consequence of the ternary interaction is that recoverin is able to hold rhodopsin and RK in close proximity, so that they could find one another very quickly once the inhibition is removed. Light activation of retinal rods leads to a lowering of cytosolic Ca²⁺ (54, 57), which causes Ca²⁺ dissociation from recoverin, leading to large conformational changes that cause the myristoyl group to become sequestered inside the protein. The sequestration of the myristoyl group accomplishes two things. First, it causes recoverin to dissociate from the membrane surface because the sequestered myristoyl group is no longer accessible to serve as a membrane anchor. Second, the sequestered myristoyl group covers the hydrophobic residues (Trp-31, Phe-35, Phe-49, Ile-52, Tyr-53, Phe-56, and Leu-90) that cause the myristoyl group to become sequestered inside the protein. The sequestration of the myristoyl group abolishes RK binding to recoverin, leading to large conformational changes that cause the myristoyl group to become sequestered inside the protein. The sequestration of the myristoyl group therefore should facilitate R⁺-free recoverin from both rhodopsin and RK clearing a path so that RK can now quickly access R⁺. The N-terminal RK helix has been implicated in substrate recognition, because deletion of the N-terminal helix abolishes RK binding to R⁺ (20). The dissociation of recoverin from the N-terminal helix, therefore, should facilitate R⁺ binding to RK and thereby promote phosphorylation. Future structural studies on the full-length RK enzyme are now needed at the atomic level to further test and refine our proposed regulatory mechanism.

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