A Highly Conserved Cysteine of Neuronal Calcium-sensing Proteins Controls Cooperative Binding of Ca$^{2+}$ to Recoverin*

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Background: Recoverin contains a cysteine (Cys-39) that is highly conserved in neuronal calcium-sensing (NCS) proteins.

Results: The C39A mutation shifts the conformational equilibrium from the R to T state, inducing cooperative calcium binding.

Conclusion: Cys-39 controls the conformational equilibrium of calcium-free recoverin.

Significance: This mutation assigns a previously unknown function to the conserved cysteine in recoverin and possibly all NCS proteins.

Recoverin, a 23-kDa Ca$^{2+}$-binding protein of the neuronal calcium sensing (NCS) family, inhibits rhodopsin kinase, a Ser/Thr kinase responsible for termination of photoactivated rhodopsin in rod photoreceptor cells. Recoverin has two functional EF hands and a myristoylated N terminus. The myristoyl chain imparts cooperativity to the Ca$^{2+}$-binding sites through an allosteric mechanism involving a conformational equilibrium between R and T states of the protein. Ca$^{2+}$ binds preferentially to the R state; the myristoyl chain binds preferentially to the T state. In the absence of myristoylation, the R state predominates, and consequently, binding of Ca$^{2+}$ to the non-myristoylated protein is not cooperative. We show here that a mutation, C39A, of a highly conserved Cys residue among NCS proteins, increases the apparent cooperativity for binding of Ca$^{2+}$ to non-myristoylated recoverin. The binding data can be explained by an effect on the T/R equilibrium to favor the T state without affecting the intrinsic binding constants for the two Ca$^{2+}$ sites.

Recoverin is a small Ca$^{2+}$-binding protein that functions in the phototransduction cascade of vertebrate rod photoreceptor cells (1, 2). Under in vitro conditions, recoverin inhibits rhodopsin kinase (RK) in a Ca$^{2+}$-dependent manner resulting in extended activation of the visual pigment rhodopsin (3, 4). Ca$^{2+}$-bound recoverin binds the N-terminal helix of RK (5, 6), an amphipathic helix also recognized by rhodopsin (7), and thus prevents phosphorylation of activated rhodopsin. When the Ca$^{2+}$ concentration is low, RK is released by recoverin and is then free to phosphorylate rhodopsin in a reaction that helps terminate the photoactivated state (8).

Recoverin is a member of the neuronal calcium sensor (NCS) protein family (9). Like all members of the family, it is a small globular protein with four EF hand motifs and a post-translationally added fatty acyl group (myristoyl or related acyl chain) on the N-terminal glycine residue (Fig. 1A) (10). Two of the EF hands are non-functional in recoverin (Fig. 1B): EF1 as a result of the highly conserved CPXG sequence (residues 39–42) and EF4 as a result of a salt bridge between Lys-161 and Glu-171 (11). Consequently, recoverin binds only two Ca$^{2+}$ ions, one each at EF2 and EF3 (12). The intrinsic affinities of the two sites for Ca$^{2+}$ are different, but the binding is cooperative because Ca$^{2+}$ and the myristoyl chain bind preferentially to two different conformations of the protein (R and T, respectively). The T state is a more compact structure than the R state and sequesters the myristoyl chain within a hydrophobic cavity of recoverin. The binding of two Ca$^{2+}$ ions to recoverin results in the extrusion of the myristoyl chain from the protein interior into the aqueous milieu as recoverin changes from the T to the R state. This conformational transition is known as the “myristoyl switch” (13, 14). In the absence of the acyl chain, non-myristoylated recoverin exists predominantly in the R state, and binding is distinctly non-cooperative, with $K_D$ values reflecting the intrinsic affinities of the two sites for Ca$^{2+}$ ions. Although recoverin clearly binds two Ca$^{2+}$ ions (12), it should be noted that the protein has never been crystallized with Ca$^{2+}$ bound to both sites. In all structures of the Ca$^{2+}$-bound form, only the high affinity EF3 site is occupied (11, 15, 16).

We show here that mutation of the highly conserved Cys-39 (to alanine) within the CPXG motif of EF1 has a dramatic effect on the binding of Ca$^{2+}$ to non-myristoylated recoverin. The mutation increases the apparent cooperativity for binding Ca$^{2+}$ by a mechanism that is most parsimoniously interpreted as an effect on the T/R equilibrium to favor the T state without significantly affecting the intrinsic affinities of EF2 or EF3 for Ca$^{2+}$. We also show from the x-ray crystal structure of wild-type (WT) recoverin under non-reducing conditions that...
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Cys-39 can form a stable sulfenic acid. Cys-39 is the only cysteine residue in recoverin and has been suggested to function as a redox sensor in rod photoreceptor cells through formation of derivatives with higher oxidation states of the sulfur (i.e., sulfenic, sulfinic, or sulfonic acids) (17, 18).

**EXPERIMENTAL PROCEDURES**

Buffers—Buffer A was 50 mM HEPES (pH 7.5), 100 mM NaCl, 1 mM CaCl₂, and 5 mM βME. Buffer B was 50 mM HEPES (pH 7.5), 100 mM NaCl, 5 mM βME, and 50 mM EGTA. Buffer C was 5 mM HEPES (pH 7.5), 100 mM NaCl, and 5 mM βME. Buffer D was 20 mM Tris (pH 8.0) and 3 mM Na₂N₃. Buffer E was 20 mM Tris (pH 8.0), 500 mM NaCl, and 3 mM Na₂N₃. Buffer F was 20 mM Tris (pH 8) and 20 mM imidazole. Buffer G was 20 mM Tris (pH 8) and 500 mM imidazole. Buffer H was 20 mM HEPES (pH 7.5), 120 mM NaCl, 0.1 mM MgCl₂, 3 mM Na₂N₃, 1 mM CaCl₂, and 5 mM βME. Buffer I was 10 mM Tris (pH 7), 100 mM KCl, and 2 mM CaCl₂. Buffer J was 20 mM Tris (pH 8) and 100 mM KCl.

Rv Mutagenesis—Recoverin was cloned into a pET15b vector containing an ampicillin resistance gene. The mutations were introduced using the QuikChange mutagenesis method (Advanced Chromatography Technologies, Aberdale, Scotland) on an Agilent 1100 series HPLC (Agilent Technologies, Berkshire, UK). All mRv preparations used in this study were >95% myristoylated.

Myristoylation of recoverin was also followed by electrospray ionization mass spectrometry (ESI-MS). The LC-MS system consisted of an Agilent 1200 series HPLC connected to an Agilent series 6520 ESI Q-TOF. Protein samples (10 μM) dissolved in a 5% acetonitrile and 0.1% formic acid buffer were separated on a C18 Poroshell 300SB column (1 mm × 75 mm × 5 μm) at 0.5 ml min⁻¹ using a linear gradient of 5–70% acetonitrile in 0.1% formic acid. MS data were collected up to 3000 m/z, and raw spectra were deconvoluted using the maximum entropy algorithm of Agilent Masshunter version B.03.01 software. External mass calibration was performed using a mixture of purine (121 m/z) and HP-0921 (922 m/z) immediately prior to measuring protein samples.
Preparation of Oxidized WT Rv (oxWT Rv)—Cell pellets containing WT Rv were resuspended in Buffer D and sonicated to release the expressed protein. The lysate was clarified by centrifugation and then passed over a Q-Sepharose FF matrix. The column was washed with 5 column volumes of Buffer D, and bound protein eluted with a linear gradient of Buffers D and E in 15 column volumes. Column fractions were analyzed by SDS-PAGE. Fractions containing Rv were concentrated with a YM-10 Centricon and stored at 4 °C in the presence of 10 mM CaCl₂ for 1 month before crystallographic trials.

Preparation of the Regulator of G Protein Signaling (RGS) Domain of RK—RGS is a truncated form of RK in which the catalytic domain is replaced with a small linker peptide (i.e. GSGS) joining residues 1–181 to 512–557 of the kinase (these two regions form the RGS homology domain (22)). RGS is soluble, is expressed well in E. coli, and can be purified by means of a C-terminal His₆ tag. This tag was also used to immobilize RGS for binding assays.

RGS was expressed from the pET28a vector in T7 Express E. coli cells. Cells were grown to A₆₆₀ ≈ 0.6–0.8 at 37 °C with orbital shaking at 220 rpm before induction with 0.25 mM IPTG. Induced cultures were grown an additional 2–3 h before harvesting cells by centrifugation. Cell pellets were resuspended in Buffer F and frozen at −80 °C until purification. RGS was purified from thawed cell suspensions by sonication to release the expressed protein. The lysate was clarified in E. coli, and can be purified by means of a C-terminal His₆ tag. This tag was also used to immobilize RGS for binding assays.

Ca²⁺-binding Assays—Ca²⁺-binding assays were performed at 25 °C according to published protocols (14, 23) using a Hita-chi F-2500 fluorescence spectrometer (Tokyo, Japan) to follow changes in intrinsic tryptophan fluorescence in recoverin. Ca²⁺ standards were prepared by serial dilutions from a 0.1 M CaCl₂ standard (Ricca Chemical Co.). Titration data were fit initially to the Hill equation,

\[ f([Ca^{2+}]) = \frac{[Ca^{2+}]^n}{[Ca^{2+}]^n + K_d^n} \]  

(Eq. 1)

where \( f([Ca^{2+}]) \) is the fraction of sites bound to Ca²⁺, \([Ca^{2+}]\) is the free Ca²⁺ concentration, \( K_d \) is the apparent dissociation constant, and \( n \) is the Hill coefficient. In the non-cooperative model, the \( K_d \) values (\( K_1, K_2 \)) are fit as independent parameters,

\[ f([Ca^{2+}]) = 0.5 \left( \frac{[Ca^{2+}]}{[Ca^{2+}]+K_1} + \frac{[Ca^{2+}]}{[Ca^{2+]}+K_2} \right) \]  

(Eq. 2)

where \( K_1 \) is the first dissociation constant, and \( K_2 \) is the second dissociation constant. Data were also analyzed using Equation 3 for the concerted allosteric model shown in Scheme 1, where \( T \) refers to the conformation of the Ca²⁺-free protein (subscripts indicate the number a Ca²⁺ bound at each site), \( R \) the conformation of the Ca²⁺-bound protein, \( L \) is the T/R ratio, \( K_1 \) and \( K_2 \) are the same as in Equation 2, \( c \) is the ratio of Ca²⁺ affinity for the T and R states (0.003; assumed to be the same for both binding sites), \( \beta \) is equal to \( c + L^{-1} \), and \( \gamma \) is equal to \( c^2 + L^{-1} \) (14, 23).

Titration data were fit to Equations 1–3 using KaleidaGraph 4.1 from Synergy Software (Reading, PA).

RGS-binding Assay—The formation of a complex between RGS and Rv was followed using a Ni-NTA matrix in a type of pulldown assay using spun columns. Typically, 10 nmol of purified RGS was first immobilized on the column. After removing unbound protein with Buffer H, recoverin was added in 1.5-fold molar excess over RGS. Following an incubation period of 30 min on ice, the column was washed again with Buffer H, and the recoverin-RGS complex eluted with 250 mM imidazole in Buffer H. Fractions were analyzed by SDS-PAGE.

The \( K_d \) for the recoverin-RGS interaction was measured using a MicroCal VP-ITC microcalorimeter (GE Healthcare) as described previously (6). WT or C39A Rv was dialyzed against two changes of Buffer I at 4 °C and then loaded into the sample reservoir solution of 50 % glycerol as cryoprotectant. Once initial conditions were identified, subsequent exploration of crystallization space used the hanging drop method. Final crystallization conditions were: C39A, 2.0 M ammonium citrate (pH 7.0); WT, 1.8 M ammonium citrate (pH 7.0); oxWT, 1.8 M ammonium citrate (pH 7.0); and C39D, 2.4 M sodium malonate (pH 7.0). All samples contained 1–5 mM CaCl₂, oxWT Rv was stored for 2–4 weeks at 4 °C in the presence of 10 mM CaCl₂ before use in crystallization trials.

X-ray Data Collection and Analysis—Crystals were soaked in reservoir solution containing 10% glycerol as cryoprotectant before flash freezing in liquid nitrogen. Diffraction data were collected with a Rigaku Raxis Rapid imaging plate detector using a Rigaku rotating anode source operating at 40 kV and 40 mA. Data were processed using the HKL2000 suite of crystallographic programs (24).
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TABLE 1
Data collection and refinement statistics

| Statistics          | WT       | oxWT     | C39A     | C39D     |
|---------------------|----------|----------|----------|----------|
| Data collection     |          |          |          |          |
| Space group         | 14       | 14       | 14       | 14       |
| Resolution range (Å)| 32–1.45  | 42–1.9   | 32–1.5   | 32–1.45  |
| Highest resolution shell (Å) | 1.53–1.45 | 2.0–1.9  | 1.58–1.5 | 1.53–1.45 |
| Unit cell parameters (Å) | a = b = 84.72 | c = 58.85 | a = b = 84.64 | c = 59.48 |
|                     | a = β = γ = 90° | a = β = γ = 90° | a = β = γ = 90° | a = β = γ = 90° |
| Total reflections   | 188,604  | 156,742  | 174,419  | 151,397  |
| Unique reflections  | 36,801   | 16,678   | 32,871   | 36,942   |
| Completeness (%)    | 99.6 (99.9) | 100 (100) | 99.8 (99.2) | 100 (100) |
| Rmerge (%)          | 4.2 (41.1) | 8.5 (45.8) | 8.8 (38.6) | 9.5 (42.1) |
| Rfree (%)           | 17.6 (3.6) | 13.5 (3.7) | 10.1 (2.9) | 9.6 (2.6) |
| Redundancy          | 5.1      | 9.4      | 5.3      | 4.1      |
| Refinement          |          |          |          |          |
| Resolution range (Å)| 32–1.45  | 42–1.9   | 32–1.5   | 30–1.45  |
| Number of reflections | 36,790  | 16,604   | 32,755   | 36,940   |
| Rmerge (%)          | 19.0     | 18.9     | 18.6     | 20.4     |
| Rfree (%)           | 21.2     | 22.1     | 19.7     | 22.2     |
| Protein atoms       | 1,570    | 1,536    | 1,560    | 1,572    |
| Ca2+ atoms          | 1        | 1        | 1        | 1        |
| Water molecules     | 171      | 115      | 164      | 142      |
| r.m.s.d. in bond lengths (Å) | 0.007 | 0.009 | 0.006 | 0.006 |
| r.m.s.d. in bond angles (°) | 1.2 | 1.2 | 1.1 | 1.1 |

*Highest resolution cell values are given parentheses.

Results

In the following presentation, we use mRV to indicate the myristoylated form of recoverin, RV to indicate the non-myristoylated form, and recoverin when we do not want to specify whether or not the protein is acylated.

C39A Mutant—As part of an ongoing effort to study the structure and function of recoverin, we explored a variety of approaches to fluorescently label the protein. Although the single cysteine residue Cys-39 has been labeled with a maleimide derivative of Alexa Fluor 647 (32), the location of this residue in the binding pocket for RK (6) made it non-ideal for a fluorophore label in our studies. To evaluate whether the unique cysteine could be moved to another location in the protein, we first prepared the single mutant C39A to determine the impact of removing this highly conserved cysteine. Coexpression of recoverin and yeast N-myristoyl transferase 1 in E. coli followed by purification on a phenyl-Sepharose column consistently yielded a greater fraction of the C39A mutant in the myristoylated form (75 ± 9.7%) than was observed for WT recoverin (53.8 ± 5.5%), as judged by analysis with reverse-phase HPLC (data not shown). We decided to characterize the C39A mutant further because these data suggested that the mutation might affect the conformation of recoverin. In the following experiments, mRV was purified from contaminating RV using chromatography on Q-Sepharose and then analyzed for purity (generally >95% mRV) by HPLC and ESI-MS (data not shown).

Binding of Ca2+ to the C39A Mutant—The binding of Ca2+ to recoverin was monitored using a method that measures intrinsic tryptophan fluorescence as has been described extensively by Ames and co-workers (14, 23, 33). In agreement with the earlier studies, binding of Ca2+ to WT mRV was cooperative, and the titration data were fit well by the Hill equation (Equation 1) using a Kd of 25.9 ± 0.9 μM and Hill coefficient n = 1.46 ± 0.07 (Fig. 2A). Also in agreement with the earlier studies, binding of Ca2+ to WT RV was non-cooperative, and the titration data were fit well with an equation for two non-identical sites (Equation 2) using dissociation constants of 0.21 ± 0.03 μM (K1) and 2.23 ± 0.33 μM (K2) for the high and low affinity sites corresponding to EF3 and EF2, respectively (Fig. 2A). In stark contrast, C39A RV displayed a much steeper Ca2+ binding curve compared with WT RV, and the data were fit well by the Hill equation for cooperative binding with Kd of 1.33 ± 0.06 μM and Hill coefficient n = 1.23 ± 0.06 (Fig. 2B), suggesting that the effect of mutation was on the cooperative interaction of the two binding sites. Certainly, an n of 1.2 is not large, and the data could also be fit reasonably well with the model for independent

collected at 100 K with beam line 8.2.1 at the Advance Light Source (Lawrence Berkeley National Laboratory, Berkeley, CA) using ADSC Q315R CCD detectors (Area Detector Systems Corporation, San Diego, CA). Data sets were integrated using MOSFLM (24) and scaled using SCALA (25) from the CCP4 software suite v6.3 (26, 27). All structures were solved by the molecular replacement method using PHASER (28) with WT RV (PDB ID code 1OMR (15)) as a search model. Rigid body refinement followed by positional and B-factor refinement was carried out using phenix.refine (29) from the PHENIX software suite v1.8 (30). Manual model building was done using COOT v0.7 (31).

Continuous electron density was observed for residues: 7–197, C39A; 7–198, C39D; 7–198, WT; and 7–194, oxWT. In the oxWT structure, additional 3σ Fo – Fe density was observed at the side chain of residue Cys-39. This density was interpreted to be an oxygen atom of a sulfenic acid side chain and was included from a built-in monomer library of COOT. Water molecules were included in the final refinement after satisfying the criteria of 3σ cutoff Fo – Fe, and 1σ cutoff 2Fo – Fe. Several iterative cycles of refinement were carried out before final submission of data. The data collection and final refinement statistics are given in Table 1. Data sets for C39A RV (PDB ID code 4M20), C39D RV (PDB ID code 4M2P), WT RV (PDB ID code 4MLW), and oxWT RV (PDB ID code 4M2Q) have been submitted to the Protein Data Bank.
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sites (Equation 2) using $K_2$ values for the two sites that were fortuitously identical ($K_1 = K_2 = 2.4 \mu M$) as a result of the mutation. In this case, the mutation would be interpreted to affect the intrinsic affinity of EF3 directly. Although we cannot unequivocally rule out a direct effect of mutation on EF3, we favor the model in which the mutation affects the T/R equilibrium for reasons outlined under “Discussion.”

To explore further the possible effect of the mutation on the cooperative interaction of the two Ca\(^{2+}\)-binding sites, we analyzed the Ca\(^{2+}\) titration data for WT and C39A recoverin using the concerted allosteric model developed by Ames et al. (Scheme 1 and Equation 3) (14). According to this model, the two Ca\(^{2+}\)-binding sites, EF2 and EF3, bind Ca\(^{2+}\) with different intrinsic affinities. Furthermore, the Ca\(^{2+}\)-free protein exists in an equilibrium mixture of R and T states, where the myristoyl chain binds preferentially to the T state and Ca\(^{2+}\) binds preferentially to the R state. Binding of Ca\(^{2+}\) to WT mRv is cooperative because the T state is favored in mRv, and much of the energy from binding Ca\(^{2+}\) to the high affinity EF3 site is utilized in converting the protein into the Ca\(^{2+}\)-binding R state. The R state is favored in WT Rv, and the two sites bind Ca\(^{2+}\) independently. In this model, $K_1$ and $L$ are not independent parameters. Therefore, we fixed $K_2$ and $K_1$ to be values close to those observed for the WT Rv titration and fit the data to determine $L$. As shown in Table 2, the equilibrium ratio of T to R states for the non-myristoylated form of the protein is dramatically shifted from $L = 0.31 \pm 0.14$ in WT Rv to $11.1 \pm 0.8$ in C39A Rv, providing a clear rationale for why this mutant exhibits cooperative interaction of the Ca\(^{2+}\) binding sites in the non-myristoylated protein.

Interestingly, the effect of the C39A mutation is lost in the myristoylated protein. As shown in Fig. 2B, the cooperative binding of Ca\(^{2+}\) to C39A mRv was almost indistinguishable from that of WT mRv, with a $K_D$ of $19.5 \pm 0.4 \mu M$ and Hill coefficient of $n = 1.52 \pm 0.04$. A fit of the data to the concerted allosteric model indicates that the equilibrium constant $L$ changes from 490 \pm 30 in WT mRv to 280 \pm 10 in the mutant (Table 2).

**TABLE 2**

| Protein | Myr | $K_1$ (\(\mu M\)) | $K_2$ (\(\mu M\)) | $L$ |
|---------|-----|-----------------|-----------------|-----|
| WT      |     | 0.15 \(\mu M\)  | 2.4 \(\mu M\)  | 0.31 \(\mu M\) |
| C39A    |     | 0.15 \(\mu M\)  | 2.4 \(\mu M\)  | 11.1 \(\mu M\) |

Errors were calculated using the least squares method ($R^2 > 0.99$).

**FIGURE 2.** Normalized change in fluorescence for Ca\(^{2+}\) binding to myristoylated (open) and non-myristoylated (filled) WT (A) or C39A (B) recoverin. Equation 2 was used to fit titration data for WT mRv, C39A mRv, and C39A Rv. Equation 2 was used to fit the titration data for WT Rv. The dashed lines in B represent the WT titration data from A for comparison.
atom attached to the sulfur (Fig. 6), which we have modeled as a sulfinic acid.

We also prepared the C39D Rv mutant, which was originally created by Permyakov et al. (18) to mimic a higher oxidation state (sulfinic acid) of the Cys-39 sulfur atom. As is shown in Fig. 4B, C39D Rv bound Ca\(^{2+}\) with lower affinity at both sites, shifting the titration curve to the right with little change in the overall slope. Interestingly, C39D Rv was incapable of binding RGS in the pulldown assay shown in Fig. 4C. Because of the disruption to interaction with RGS, it was of interest to determine the crystal structure of the Ca\(^{2+}\)-bound C39D Rv mutant. C39D Rv crystals were grown in 2.4 M sodium malonate (pH 7.0) containing 2 mM CaCl\(_2\) and diffacted to 1.45 Å resolution. The global C39D Rv structure displayed no obvious differences from that of WT Rv, suggesting that the disruption to RGS binding resulted from a direct effect of the mutation in the RGS-binding pocket. Electron density for residue 39 is compared for WT, oxWT, C39A, and C39D Rv in Fig. 6, A–D.

DISCUSSION

The main effect of the C39A mutation on the non-myristoylated form of recoverin is an increase in the observed K\(_D\) for the high affinity EF3 Ca\(^{2+}\)-binding site with little or no change to the low affinity EF2 site (Fig. 2). This result is superficially similar to the effect of the Δ191–202 truncation in the mutant Rv\(^{2–190}\) reported by Koch and co-workers (16) but has been interpreted very differently here. The Δ191–202 truncation affects directly the intrinsic affinity of EF3 for Ca\(^{2+}\), whereas we have interpreted the C39A mutation as directly affecting the T/R equilibrium without significant perturbation of the intrinsic binding constants for either EF2 or EF3.

The Δ191–202 truncation removes a C-terminal helix that is in direct contact with the two helical segments of EF3 in WT recoverin. The two Ca\(^{2+}\) ions bind to Rv\(^{2–190}\) non-cooperatively; the K\(_D\) for EF3 shifts from 0.21 μM in WT to 1.3 μM in the mutant, whereas the K\(_D\) for EF2 is unaltered (6.2 μM) (16). Analysis of the Rv\(^{2–190}\) titration data using the Hill equation yields a Hill coefficient, n, of 0.86 (our analysis of their data), which is consistent with a model for two independent binding sites. Importantly, the effect of the Δ191–202 truncation is preserved in the myristoylated protein; binding of Ca\(^{2+}\) to mRv\(^{2–190}\) is cooperative, as expected, but the affinity (K\(_D\) = 30 μM) is less than for WT mRv (K\(_D\) = 14 μM) as is the Hill coefficient (WT mRv, n = 1.5; mRv\(^{2–190}\), n = 1.3). These results are consistent with a direct effect of the mutation on the intrinsic affinity of EF3 for Ca\(^{2+}\).

In contrast, we have concluded that the apparent decreased Ca\(^{2+}\) affinity in the C39A mutant results from an indirect effect of the mutation on the T/R equilibrium with essentially no change in the intrinsic K\(_D\) of the two binding sites for the following reasons: (i) Cys-39 is located in the EF1 loop, close to the myristoyl-binding pocket and close to the site of other mutations known to affect the T/R equilibrium (e.g., W31K; see

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**FIGURE 3.** Binding assays for WT or C39A Rv to RGS. A, pulldown assay using a Ni-NTA matrix to immobilize RGS as described under “Experimental Procedures.” The RGS-Rv complex was eluted with 250 mM imidazole. Lanes are defined as L, load; FT, flow-through; W, last wash; E, imidazole elution. B, ITC isotherm showing the heat observed upon injecting aliquots of WT Rv (660 μM) into a solution of RGS (60 μM) at 30 °C. C, ITC isotherm showing the heat observed upon injecting aliquots of C39A (340 μM) into a solution of RGS (30 μM) at 30 °C.

**FIGURE 4.** A and B, normalized change in fluorescence for Ca\(^{2+}\)-binding titrations for P40A Rv (A) and C39D Rv (B). Equation 2 was used to fit the titration data. The dashed lines are for WT Rv (from Fig. 2A). C, pulldown assay in which RGS was first immobilized on a Ni-NTA matrix, WT, C39D, or P40A Rv added in the presence of 1 mM CaCl\(_2\), and finally the RGS-Rv complex eluted with 250 mM imidazole. Lanes are defined as L, load; FT, flow-through; W, last wash; E, imidazole elution.
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![Diagram](Fig5A.png)

**FIGURE 5.** A, superposition of the C39A Rv (magenta) structure with that of WT Rv (green). Ca\(^{2+}\) ions are shown as spheres occupying EF3. B, superposition of EF2 (left) and EF3 (right) for C39A and WT Rv. Colors are the same as in A. The red sphere is an axial water molecule.

![Diagram](Fig6.png)

**FIGURE 6.** Electron density at position 39 for WT (A), oxWT (B), C39A (C), and C39D WT (D). The Fourier map (cyan) is shown at 1 σ cutoff for all the atoms. The omit map (red) is shown at 3 σ cutoff for the O atom of sulfenic acid (B) and the side chain of Asp-39 (D).

below), with no obvious connection to EF3 or the C-terminal residues in contact with EF3 in either the Ca\(^{2+}\)-free (34) or Ca\(^{2+}\)-bound (15, 35) proteins. (ii) The structure of Ca\(^{2+}\)-bound EF3 is the same in the C39A mutant as it is in WT Rv (r.m.s.d. = 0.04 Å for residues 110–121; Fig. 5B), suggesting that the mutation affects the Ca\(^{2+}\)-free protein. (iii) The Ca\(^{2+}\)-binding isotherm for C39A Rv is well fit with the Hill equation using a single \(K_d\) and \(n > 1\) (\(n = 1.2\)), indicating that binding of the two Ca\(^{2+}\) ions is cooperative (Fig. 2). (iv) The isotherms for binding of Ca\(^{2+}\) to WT mRv and C39A mRv are indistinguishable within experimental error (Fig. 2), indicating that myristoylation eliminates the effect of the C39A mutation.

Whereas this last result is clearly different from that found for mRv\(^{2–190}\), as discussed above, and inconsistent with a direct effect of the mutation on the binding site in EF3, we must note that a direct effect of mutation on the T/R equilibrium (to favor the T state) would also be expected to express itself in the myristoylated protein. The fact that an effect is not observed in the myristoylated mutant may indicate that the C39A mutation has an adverse effect on sequestration of the myristoyl chain in the T state as well as an effect promoting the T state in the unmyristoylated protein. Although there is an ad hoc element to this suggestion, we note that the site of mutation in the N-terminal domain is close to the myristoyl-binding pocket.

In aggregate, these data are most easily interpreted to indicate that the C39A mutation restores cooperative binding of Ca\(^{2+}\) to Rv because it shifts the T/R equilibrium in favor of T without significantly affecting the intrinsic Ca\(^{2+}\) affinity of EF2 and EF3. Baldwin and Ames (23) have reported two different mutants (W31K and I52A/Y53A) in which the primary effect of the mutations is on the T/R equilibrium. However, in their case the mutations shift the equilibrium in favor of the R state, increasing affinity of the myristoylated protein for Ca\(^{2+}\) with little effect on the non-myristoylated forms. To our knowledge, C39A is the first recoverin mutant for which the binding of Ca\(^{2+}\) to the non-myristoylated protein is cooperative. As such, the mutant should be useful for elucidating the atomic details stabilizing each conformation and thus controlling the position of the T/R equilibrium, especially given the fact that the mutation does not affect downstream interaction of the Ca\(^{2+}\)-bound protein with the RGS domain of RK (Fig. 3).

Finally, the crystal structure of WT Rv under non-reducing conditions shows that the sulfur atom of Cys-39 can oxidize to sulfenic acid (Fig. 6B). These data are of interest because Cys-39 is not only the cysteine residue in recoverin and is strictly conserved among all members of the NCS family. Permyakov et al. showed that recoverin lost the ability to react with the thiol-specific Ellman’s reagent (5,5’-dithiobis-(2-nitrobenzoic acid)) upon incubation under non-reducing conditions, suggesting that the sulfur of Cys-39 underwent oxidation (17). For this reason, noting also that the retina is one of the most vascularized tissues in the body, they further proposed that Cys-39 may function as a redox sensor in rod photoreceptor cells. Trying to mimic higher oxidation states of the sulfur atom, they made the C39D mutant and demonstrated that the Ca\(^{2+}\)-dependent interaction of the mutant mRv with rod outer segment membranes was significantly disrupted by the mutation (18). These data agree well with our results showing that C39D Rv does not bind RGS in a pull-down assay for interaction of the two proteins (Fig. 4C). Further work will be required to understand fully the possible role Cys-39 may play as a redox sensor in photoreceptor cells.
but it is clear at this point that the sulfur atom can sense environmental redox conditions.

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REFERENCES

1. Chen, C. K., Woodruff, M. L., Chen, F. S., Chen, D., and Fain, G. I. (2010) Background light produces a recoverin-dependent modulation of activated-rhodopsin lifetime in mouse rods. J. Neurosci. 30, 1213–1220
2. Chen, C. K., Woodruff, M. L., Chen, F. S., Chen, Y., Cilluffo, M. C., Tranchina, D., and Fain, G. L. (2012) Modulation of mouse rod response decay by rhodopsin kinase and recoverin. J. Neurosci. 32, 15998–16006
3. Klenci, V. A., Calvert, P. D., and Bownds, M. D. (1995) Inhibition of rhodopsin kinase by recoverin: further evidence for a negative feedback system in phototransduction. J. Biol. Chem. 270, 16147–16152
4. Chen, C. K., Inglese, J., Lefkowitz, R. J., and Hurley, J. B. (1995) Ca2+ dependent interaction of recoverin with rhodopsin kinase. J. Biol. Chem. 270, 18060–18066
5. Higgins, M. K., Oprian, D. D., and Schertler, G. F. (2006) Recoverin binds exclusively to an amphipathic peptide at the N terminus of rhodopsin kinase, inhibiting rhodopsin phosphorylation without affecting catalytic activity of the kinase. J. Biol. Chem. 281, 19426–19432
6. Ames, J. B., Levay, K., Wingard, J. N., Lanis, J. D., and Slepak, V. Z. (2006) Structural basis for calcium-induced inhibition of rhodopsin kinase by recoverin. J. Biol. Chem. 281, 37237–37245
7. Palczewski, K., Czuczylko, L., Lebioda, L., Crabb, J. W., and Polans, A. S. (1993) Identification of the N-terminal region in rhodopsin kinase involved in its interaction with rhodopsin. J. Biol. Chem. 268, 6004–6013
8. Maeda, T., Imanishi, Y., and Palczewski, K. (2003) Rhodopsin phosphorylation: 30 years later. Prog. Retin. Eye Res. 22, 417–434
9. Ames, J. B., and Lim, S. (2012) Molecular structure and target recognition of neuronal calcium sensor proteins. Biochim. Biophys. Acta 1820, 1205–1213
10. Dizhoor, A. M., Ericsson, L. H., Johnson, R. S., Kumar, S., Olshevskaya, E., and Koch, K. W. (2003) Impact of N-terminal myristoylation on the Ca2+-dependent conformational transition in recoverin. J. Biol. Chem. 278, 4526–4533
11. Weiergräber, O. H., Senin, I. I., Philippov, P. P., Granzin, J., and Koch, K. W. (2003) Impact of N-terminal myristoylation on the Ca2+-dependent conformational transition in recoverin. J. Biol. Chem. 278, 22972–22979
12. Weiergräber, O. H., Senin, I. I., Zernii, E. Y., Churumova, V. A., Kovalova, N. A., Nazipova, A. A., Perymyakov, S. E., Perymyakov, E. A., Philippov, P. P., Granzin, I., and Koch, K. W. (2006) Tuning of a neuronal calcium sensor. J. Biol. Chem. 281, 37594–37602
13. Perymyakov, S. E., Nazipova, A. A., Denesyuk, A. I., Bakunts, A. G., Zincheko, D. V., Lipkin, V. M., Uversky, V. N., and Perymyakov, E. A. (2007) Recoverin as a redox-sensitive protein. J. Proteome Res. 6, 1855–1863
14. Permyakov, S. E., Zernii, E. Y., Knyazeva, E. L., Denesyuk, A. I., Nazipova, A. A., Klopakova, T. V., Zincheko, D. V., Philippov, P. P., Perymyakov, E. A., and Senin, I. I. (2012) Oxidation mimicking substitution of conservative cysteine in recoverin suppresses its membrane association. Amino Acids 42, 1435–1442
15. Desmeules, P., Penney, S. E., and Salesle, C. (2006) Single-step purification of myristoylated and non-myristoylated recoverin and substrate dependence of myristoylation level. Anal. Biochem. 349, 25–32
16. T. S., Zouzula, S., Niemi, G. A., Flaherty, K. M., Brolley, D., Dizhoor, A. M., McKay, D. B., Hurley, J., and Stryer, L. (1992) Cloning, expression, and crystallization of recoverin, a calcium sensor in vision. Proc. Natl. Acad. Sci. U.S.A. 89, 5705–5709
17. Pace, C. N., Vajdos, F., Fee, L., Grimsley, G., and Gray, T. (1995) How to measure and predict the molar absorption coefficient of a protein. Protein Sci. 4, 2411–2423
18. Singh, P., Wang, B., Maeda, T., Palczewski, K., and Tesmer, J. J. (2008) Structures of rhodopsin kinase in different ligand states reveal key elements involved in G protein-co coupled receptor kinase activation. J. Biol. Chem. 283, 14053–14062
19. Baldwin, A. N., and Ames, J. B. (1998) Core mutations that promote the calcium-induced allosteric transition of bovine recoverin. Biochemistry 37, 17408–17419
20. Leslie, A. G. W., and Powell, H. R. (2007) In Evolving Methods for Macromolecular Crystallography (Read, R. J., and Sussman, J. L. eds) pp. 41–51, Springer, Eric, Italy
21. Evans, P. (2006) Scaling and assessment of data quality. Acta Crystallogr. D Biol. Crystallogr. 62, 72–82
22. Potterton, E., Briggs, P., Turkenburg, M., and Dodson, E. (2003) A graphical user interface to the CCP4 program suite. Acta Crystallogr. D Biol. Crystallogr. 59, 1131–1137
23. Winn, M. D., Ballard, C. C., Cowtan, K. D., Dodson, E. J., Emsley, P., Evans, P. R., Keegan, R. M., Krissinel, E. B., Leslie, A. G. I., McCoy, A., McNicholas, S. J., Murshudov, G. N., Pannu, N. S., Potterton, E. A., Powell, H. R., Read, R. J., Vagin, A., and Wilson, K. S. (2011) Overview of the CCP4 suite and current developments. Acta Crystallogr. D Biol. Crystallogr. 67, 255–242
24. McCoy, A. J., Grosse-Kunstleve, R. W., Adams, P. D., Winn, M. D., Storoni, L. C., and Read, R. J. (2007) PHASER crystallographic software. J. Appl. Crystallogr. 40, 658–674
25. Afonine, P. V., Grosse-Kunstleve, R. W., Echols, N., Headd, J. I., Moriarty, N. W., Mustyakimov, M., Terwilliger, T. C., Urzhumtsev, A., Zwart, P. H., and Adams, P. D. (2012) Towards automated crystallographic structure refinement with phenix.refine. Acta Crystallogr. D Biol. Crystallogr. 68, 352–367
26. Adams, P. D., Afonine, P. V., Bunkóczi, G., Chen, V. B., Davis, I. W., Echols, N., Headd, J. I., Hung, L. W., Kapral, G. J., Grosse-Kunstleve, R. W., McCoy, A. J., Moriarty, N. W., Oefner, R., Read, R. J., Richardson, D. C., Richardson, J. S., Terwilliger, T. C., and Zwart, P. H. (2010) PHENIX: a comprehensive Python-based system for macromolecular structure solution. Acta Crystallogr. D Biol. Crystallogr. 66, 213–221
27. Emsley, P., Lohkamp, B., Scott, W., and Cowtan, K. (2010) Features and development of COOT. Acta Crystallogr. D Biol. Crystallogr. 66, 486–501
28. Gensch, T., Komolov, K. E., Senin, I. I., Philippov, P. P., and Koch, K. W. (2007) Ca2+-dependent conformational changes in the neuronal Ca2+ sensor recoverin probed by the fluorescent dye Alexa 467. Proteins 66, 492–499
29. Ames, J. B., Hamasaki, N., and Molchanova, T. (2002) Structure and calcium-binding studies of a recoverin mutant (E85Q) in an allosteric intermediate state. Biochemistry 41, 5776–5787
30. Ames, J. B., Tanaka, T., Stryer, L., and Ikura, M. (1994) Secondary structure of myristoylated recoverin determined by three-dimensional heteronuclear NMR: implications for the calcium-mysitryl switch. Biochemistry 33, 10743–10753
31. Ames, J. B., Ishima, R., Tanaka, T., Gordon, J. I., Stryer, L., and Ikura, M. (1997) Molecular mechanics of calcium-mysitryl switches. Nature 389, 198–202