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ORIGINAL ARTICLE

Oxidation mimicking substitution of conservative cysteine in recoverin suppresses its membrane association

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Abstract Recoverin belongs to the family of intracellular Ca^{2+}-binding proteins containing EF-hand domains, neuronal calcium sensors (NCS). In photoreceptor outer segments, recoverin is involved into the recovery of visual cycle via Ca^{2+}-dependent interaction with disk membranes and inhibition of rhodopsin kinase. The function of a conservative within NCS family Cys residue in the inactive EF-loop 1 remains unclear, but previous study has shown its vulnerability to oxidation under mild oxidizing conditions. To elucidate the influence of oxidation of the conservative Cys39 in recoverin the properties of its C39D mutant, mimicking oxidative conversion of Cys39 into sulfenic, sulfinic or sulfonic acids have been studied using intrinsic fluorescence, circular dichroism, and equilibrium centrifugation methods. The C39D substitution results in essential changes in structural, physico-chemical and physiological properties of the protein: it reduces α-helical content, decreases thermal stability and suppresses protein affinity for photoreceptor membranes. The latter effect precludes proper functioning of the Ca^{2+}-myristoyl switch in recoverin. The revealed significance of oxidation state of Cys39 for maintaining the protein functional status shows that it may serve as redox sensor in vision and suggests an explanation of the available data on localization and light-dependent translocation of recoverin in rod photoreceptors.

Keywords EF-hand · NCS family · Vision · Recoverin · Cysteine · Redox regulation

Abbreviations

NCS	Neuronal calcium sensor
ROS	Reactive oxygen species
WT	Recombinant wild-type recoverin
HEPES	*N*-(2-hydroxyethyl)piperazine-*N*′-(2-ethanesulfonic acid)
Tris	Tris(hydroxymethyl) aminomethane
EDTA	Ethylenediaminetetraacetic acid
CD	Circular dichroism
GCAP	Guanylate cyclase activated protein
λ_{max}	Position of fluorescence spectrum maximum
$T_{1/2}$	Mid-transition temperature
FDPB	Finite difference Poisson–Boltzmann method
NMR	Nuclear magnetic resonance

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Introduction

Recoverin is a globular cytosolic calcium sensory protein belonging to the family of neuronal calcium sensor (NCS) proteins of the EF-hand superfamily (for reviews, see Burgoyne 2007; Senin et al. 2002a, b; Chen 2002). Recoverin contains four potential EF-hand type Ca^{2+}-binding motifs among which only two, EF2 and EF3, are capable of Ca^{2+} binding. The N-terminal myristoyl group of apo-recoverin is buried in the hydrophobic pocket of the protein, while the association of two Ca^{2+} ions results in a transfer of the myristoyl group to water environment, which allows an attachment of the protein to photoreceptor membranes ["Ca^{2+}-myristoyl switch" (Zozulya and Stryer 1992)], and provides an exposed hydrophobic crevice for target recognition (Valentine et al. 2003). A set of biochemical and electrophysiological studies, confirmed by the data of genetically modified animal models (Makino et al. 2004; Chen et al. 2010), evidences that recoverin serves as a modulator of the lifetime of the photoexcited state of rhodopsin via the Ca^{2+}-dependent interaction with rhodopsin kinase.

An inherent feature of the NCS protein family is a conservative Cys residue in the inactive EF1 loop, which is located at the third position of the consensus EF-loop. Being fully extrinsic to the EF-hand motifs, this cysteine performs obscure function. The known structures of NCS proteins complexed with fragments of their targets show that the conservative thiol is located in vicinity of the target helix [described in (Permyakov et al. 2007)]. Few studies aimed at elucidating the physiological role of this residue disclose a contradictory picture: while the conservative Cys was shown to be important for target activation by GCAPs 1 and 2, modifications of this residue in recoverin did not cause distinct changes in its ability to inhibit rhodopsin kinase [reviewed in (Permyakov et al. 2007)]. This fact indicates that the function of the conservative Cys residue in the NCS proteins remains to be established.

The previously reported vulnerability of the conserved Cys39 of recoverin to oxidation under mild oxidizing conditions in vitro (Permyakov et al. 2007) is of potential physiological importance, considering an emerging role of sulfenic and sulfinic acids derivatives as thiol redox switches in signaling (Poole et al. 2004; Jacob et al. 2004; Forman et al. 2010). The significance of conservative cysteines for redox signaling was shown for close relatives of NCS proteins, the multifunctional family of S100 proteins [reviewed in (Lim et al. 2009)]. Meanwhile, the information on impact of the oxidative modification of the Cys39 thiol group upon the generally accepted function of recoverin is lacking. To address this question, a C39D recoverin mutant, mimicking oxidative conversion of Cys39 into sulfenic, sulfinic or sulfonic acids, was obtained and its structural and functional properties were studied. The oxidation state of Cys39 is shown to be critical for effective functioning of the Ca^{2+}-myristoyl switch in recoverin. This fact indicates that the Cys38 residue may serve as redox sensor in vision. Furthermore, the necessity to maintain the thiol group of recoverin in the reduced state for its proper functioning enabled us to put forward an original interpretation of the available data on cellular localization and the light-dependent translocation of recoverin in rod photoreceptors.

Materials and methods

Materials

Recoverin concentration was evaluated using molar extinction coefficient $\varepsilon_{280\ nm}$ of 24,075 M^{-1} cm^{-1} (Permyakov et al. 2007). All buffer components were ultra grade. All solutions were prepared using nano-pure water. Plastics or quartz ware was used instead of glassware. DTT solutions were prepared immediately before usage to avoid oxidation of the reagent. The buffers to be used with DTT were preliminarily degassed.

Protein isolation and physico-chemical methods

DNA construct for the expression of C39D recoverin mutant was generated using full-length recoverin cDNA in a pET-11d plasmid as a template and oligonucleotides 5′-GAAGGAGGACCCCAGTGGCCGGATCA-3′ and 5′-ATCCGGCCACTGGGGTCCTCCTTCAG-3′ as direct and reverse primers for PCR, respectively (Alekseev et al. 1998). PCR fragments were inserted to pET-11d plasmid between the *Nco*I and *Bam*HI restriction sites. The integrity of the nucleotide sequences obtained was confirmed by sequencing using Sanger's method. The myristoylated wild type and C39D recoverin forms were expressed in *E. coli* BL-21 (DE3) cells which contained pBB131 vector co-expressing *N*-myristoyl transferase. The purification of recombinant proteins from cell lysates was performed as previously reported by Senin et al. (2002a, b). The solutions of purified recoverin were stored in aliquots at −18°C. The content of myristoylated forms in protein preparations was determined by analytical reversed-phase HPLC using C18 "Symmetry" column (3.9 × 150 mm). The elution profile of the C39D mutant was similar to that of wild type recoverin indicating the same degree of myristoylation (>95%) for both recoverin forms.

Purification of the protein from Ca^{2+} was performed using the Sephadex G-25 gel-filtration method by Blum et al. (1977) with slight modifications. 1 mM EDTA was

complemented by 1 mM DTT to ensure reduction of −SH groups in recoverin. The quantitation of sulfhydryl groups of recoverin was performed according to the modified Ellman's procedure (Permyakov et al. 2007). Urea was avoided. The Ellman's test evidenced that at least 87% of −SH groups of WT recoverin were reduced.

Circular dichroism measurements were carried out with a JASCO J-810 spectropolarimeter (JASCO Inc., Japan), equipped with a Peltier-controlled cell holder. Cuvettes with pathlength of 1.00 mm were used for far-UV region. Bandwidth was 2 nm, averaging time 2 s and accumulation 3. The contribution of buffer (pH 8.2, 10 mM H_3BO_3–KOH, 20 μM DTT) was subtracted from experimental spectra. Estimations of the secondary structure contents were made using the CDPro software package (Sreerama et al. 2000). The experimental data were treated by CDSSTR and CONTIN algorithms, using SDP48 and SMP56 reference protein sets; the resulting estimates were averaged.

Fluorescence measurements were performed on a Cary Eclipse spectrofluorimeter (Varian Inc.), equipped with a Peltier-controlled cell holder. Protein fluorescence was excited at 280 nm. All spectra were corrected for the spectral sensitivity of the instrument and lognormally fitted using LogNormal software (IBI RAS, Pushchino). The positions of the fluorescence spectrum maxima (λ_{max}) were obtained from these fits.

Spectrofluorimetric thermal stability measurements were performed as described in Permyakov et al. (2008). The mid-transition temperatures, $T_{1/2}$, were calculated from temperature dependence of λ_{max} using Boltzmann sigmoid as implemented in OriginPro 8.0 (OriginLab Corporation) software.

Estimation of equilibrium Ca^{2+} association constants of recoverin was performed as described in Permyakov et al. (2008) using the sequential Ca^{2+}-binding scheme:

$$\begin{aligned} &\text{Protein} + Ca^{2+} \overset{K_1}{\leftrightarrow} \text{Protein} \cdot Ca^{2+} \\ &\text{Protein} \cdot Ca^{2+} + Ca^{2+} \overset{K_2}{\leftrightarrow} Ca^{2+} \cdot \text{Protein} \cdot Ca^{2+}, \end{aligned} \quad (1)$$

where K_1 and K_2 are equilibrium Ca^{2+}-binding constants for the two active EF-hand sites of the protein.

Electrostatics calculations were performed as described in Permyakov et al. (2008) for NMR structures of myristoylated recoverin (PDB codes 1iku and 1jsa, chain 1) using Discovery Studio Software (Accelrys Software Inc.). The total energy of charge–charge interactions was estimated via summing up the electrostatic energies of all charges and division by a factor of 2.

The binding of recoverin to photoreceptor membranes was performed using equilibrium centrifugation assay as previously described (Zozulya and Stryer 1992; Senin et al. 2002a, b). Samples of recoverin (30 μM) were mixed with bleached urea-washed photoreceptor membranes containing 0–100 μM rhodopsin in 20 mM Tris–HCl (pH 8.0), 150 mM NaCl, 20 mM $MgCl_2$, 1 mM DTT, 1 mM dibromo-BAPTA, and 0–2 mM $CaCl_2$ in total volume of 50 μl. The probes were incubated at 37°C for 20 min and centrifuged (15 min, 14,000 rpm). The supernatants were discarded and the pellets were dissolved in 50 μl of sample buffer [125 mM Tris–HCl, pH 6.8, 4% (w/v) SDS, 20% (v/v) glycerol, 10% (v/v) β-mercaptoethanol, 0.004% (w/v) bromphenol blue] and analyzed by SDS-PAGE. The amounts of recoverin forms bound to photoreceptor membranes were evaluated by densitometric scanning of the corresponding bands in polyacrylamide gel.

Results

Structural properties of C39D recoverin mutant

The measurement of far-UV CD spectra of C39D and WT recoverin forms was performed for both apo- and Ca^{2+}-loaded states (Fig. 1). Although CD estimate of α-helices content for apo-form of WT recoverin is in agreement with the literature NMR data (Table 1), the same estimate for Ca^{2+}-loaded WT protein shows 10% difference from the NMR derived value. The latter fact is likely due to higher protein concentrations used in NMR experiments, which should promote protein aggregation in the case of Ca^{2+}-loaded myristoylated recoverin (Kataoka et al. 1993). The CD estimates evidence just a minor Ca^{2+}-induced decrease in the content of both α-helices and β-sheets. Opposite to WT recoverin, Ca^{2+} association induces a minor increase (ca. 2%) in α-helical content of the C39D mutant (Table 1). Furthermore, apo-form of the C39D demonstrates substantially lower (ca. 5%) content of α-helices and somewhat higher (ca. 2%) content of β-sheets.

Thermal stability of C39D recoverin

The disturbance of secondary structure of C39D recoverin is in accord with the altered thermal stability of the mutant, measured by intrinsic fluorescence technique (Fig. 2). The position of protein fluorescence spectrum maximum (λ_{max}) is related to polarity and mobility of the polar environment of emitting tryptophans in recoverin and reflects accessibility of the chromophores to solvent molecules. At elevated temperatures, tryptophan fluorescence spectrum of the protein is red shifted (Fig. 2), which indicates a transfer of the emitting Trp residues to water environment caused by the thermally induced protein unfolding. The mid-transition temperatures ($T_{1/2}$) of C39D and WT recoverin, calculated from temperature dependences of λ_{max} value,

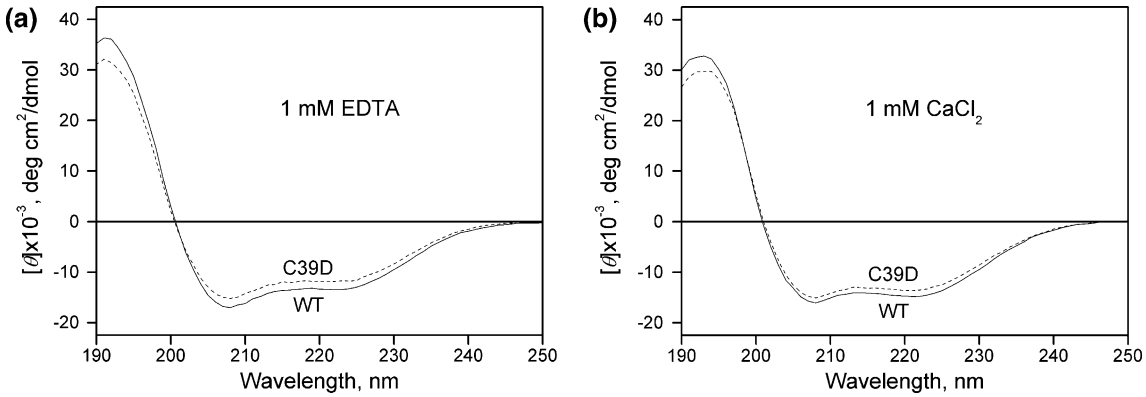

Fig. 1 Far-UV CD spectra of the metal-free (**a**) and Ca^{2+}-loaded (**b**) forms of C39D (*dashed curve*) and WT (*solid curve*) recoverin at 20°C (pH 8.2, 10 mM H_3BO_3–KOH, 20 μM DTT). Protein concentration was 3.1–3.5 μM. Calcium depletion/saturation was achieved by addition of either 1 mM EDTA or 1 mM $CaCl_2$ to the Ca^{2+}-free protein (Blum et al. 1977), respectively

Table 1 The results of estimation of the secondary structure contents (in %) for apo- and Ca^{2+}-loaded forms of C39D and WT recoverin from CD data in Fig. 1, using the CDPro software package (Sreerama et al. 2000), in comparison with the values derived from NMR structures (PDB entries 1iku and 1jsa) using DSSP program (Kabsch and Sander 1983)

Protein	α	β	*T*	*U*	α, NMR	β, NMR
Apo-form						
C39D	49.2	11.0	15.0	24.7	n.a.	n.a.
WT	54.6	9.2	13.6	22.5	55.7	4.0
Ca^{2+}-loaded protein						
C39D	50.9	10.1	14.0	24.7	n.a.	n.a.
WT	54.2	8.6	15.0	21.7	43.8	3.0

T and *U* correspond to turns and unordered structures, respectively

n.a. data not available

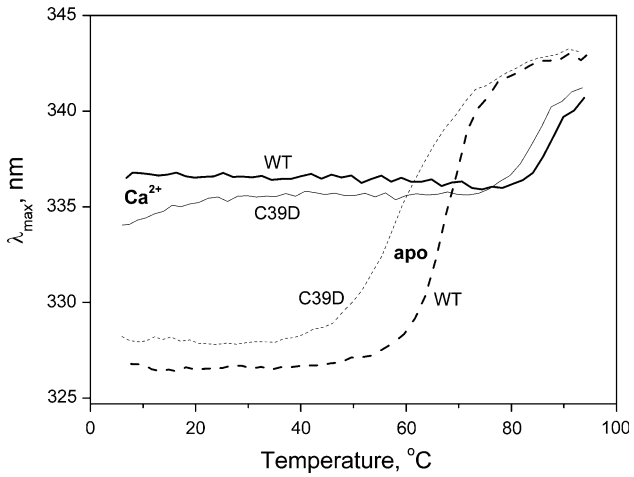

Fig. 2 Thermal denaturation of apo- (*dashed curves*) and Ca^{2+}-loaded (*solid curves*) C39D (*normal curves*) and WT (*thick curves*) recoverin monitored by intrinsic protein fluorescence technique (pH 8.05, 10 mM HEPES–KOH, 1 mM DTT). Protein concentration was 4–8 μM. Calcium depletion/saturation was achieved by the addition to the Ca^{2+}-free protein (Blum et al. 1977) of either 1 mM EDTA or 1 mM $CaCl_2$, respectively

are presented in Table 2. The thermal stability of C39D mutant is 7 and 3°C lowered with respect to that of the WT protein, for apo- and Ca^{2+}-loaded states, respectively. The lowered thermal stability of the apo-state of C39D mutant correlates with 1.5 nm red shift in λ_{max} at low temperatures, which evidences higher accessibility of emitting Trp residues to water molecules, consistent with a destabilized protein structure.

The destabilization of both apo- and Ca^{2+}-loaded states of C39D recoverin can be rationalized on the basis of electrostatic calculations. The FDPB calculations of the total energies of charge–charge interactions in apo- and Ca^{2+}-bound forms of C39D and WT recoverin forms show that the C39D substitution is accompanied by an unfavorable enthalpy increase of 5.5–6 kT (13.5–15 kJ/mol) [equivalent to the decrease in mid-transition temperature for ca. 13°C, as estimated from the microcalorimetry data presented in Permyakov et al. (2003)]. The residues with the highest destabilizing contribution in apo-protein include D(C)39 and D82, and the highest stabilizing contributions give K84 and R43. Thus, the electrostatic changes mostly affect a close environment of the C39 residue, being confined to the EF-loops 1 and 2 (residues K37-E48 and D74-E85, respectively). Analogous estimates for the Ca^{2+}-bound recoverin show even more local effects of the C39D substitution on nearby charges, limited to EF1-loop: D(C)39, E38 and E48.

Calcium affinity of the C39D mutant

Our previous study of calcium affinities of recoverin mutants with disabled EF-hands 2 or 3 (EF2 and EF3) has shown that the binding of Ca^{2+} to myristoylated recoverin can be regarded as a sequential process (Scheme 1) with EF3 being filled first (Permyakov et al. 2000). Furthermore,

Table 2 The thermal stabilities and calcium affinities of C39D and WT recoverin

Protein	$T_{1/2}$, °C 1 mM EDTA	$T_{1/2}$, °C 1 mM $CaCl_2$	K_1, 1/*M*	K_2, 1/*M*
C39D	60.5	84.3	$(1.5 \pm 0.6) \times 10^6$	$(0.34 \pm 0.13) \times 10^6$
WT	67.8	87.5	$(0.7 \pm 0.3) \times 10^6$	$(0.23 \pm 0.04) \times 10^6$

Mid-transition temperatures ($T_{1/2}$) were estimated from data presented in Fig. 2 using Boltzmann sigmoid

The equilibrium Ca^{2+} association constants of sites 1 and 2 of recoverin at 20°C, K_1 and K_2, were estimated from spectrofluorimetric measurements within the model of sequential filling of Ca^{2+}-binding sites of the protein (Scheme 1) (Permyakov et al. 2000)

Buffer conditions: pH 8.1, 10 mM HEPES–KOH, 1 mM DTT; protein concentration was 4–10 μM

analysis of the process of Ca^{2+} association using fluorescent phase plots (Burstein 1977) indicates population of at least one intermediate recoverin state in the course of the Ca^{2+} titration, supporting the sequential Ca^{2+}-binding scheme (Scheme 1). Analogous analysis of the Ca^{2+} titration of C39D mutant reveals qualitatively similar behavior (data not shown), thereby confirming that the Ca^{2+}-binding mechanism was not affected by this mutation. The results of spectrofluorimetric calcium affinity measurements of C39D and WT recoverin forms at 20°C within the model of sequential filling of Ca^{2+}-binding sites of recoverin are shown in Table 2. The C39D substitution results in minor (1.5–2-fold) increase in the calcium affinities of both active EF-hands of recoverin, although these effects are close to the accuracy limit of the method used in the calcium affinity measurements.

The C39D mutation induced increase in calcium affinity of the nearby EF-hand 2 is fairly expected, since introduction of the negatively charged Asp residue should decrease electrostatic potential at the point of Ca^{2+} coordination (energetic gain about 0.8 kT, from FDPB calculations). Meanwhile, the overall electrostatic energy gain in the process of Ca^{2+} binding is less favorable in the C39D mutant by ca. 0.6 kT. The change in the Ca^{2+}-binding constant corresponding to 0.7 kT change in the free energy of metal binding corresponds to a coefficient of 2.0. The latter is close to the accuracy limit of the experimental determination of the protein Ca^{2+} affinity. Thus, the purely electrostatic effect of the C39D substitution in recoverin on its Ca^{2+} affinity is expected to be undetectable.

$$\text{Protein} + Ca^{2+} \overset{K_1}{\leftrightarrow} \text{Protein} \cdot Ca^{2+}$$

$$\text{Protein} \cdot Ca^{2+} + Ca^{2+} \overset{K_2}{\leftrightarrow} Ca^{2+} \cdot \text{Protein} \cdot Ca^{2+},$$

Scheme 1 The scheme of successive filling of EF-hands of recoverin

Effect of C39D substitution on affinity of recoverin to photoreceptor membranes

The relationship between recoverin Ca^{2+} binding and membrane association can be considered within the following simplest scheme (P, C and M denote apo-protein, calcium and recoverin-binding membrane site, respectively) (Zozulya and Stryer 1992) (Scheme 2).

where $K_{C,P}$ and $K_{C,MP}$ are equilibrium effective Ca^{2+} association constants for free and membrane-bound protein forms, respectively; $K_{M,P}$ and $K_{M,PC2}$—equilibrium effective membrane-binding constants for apo- and Ca^{2+}-bound protein states, respectively. The following relationship can be easily derived:

$$K_{C,MP}/K_{C,P} = K_{M,PC2}/K_{M,P} \tag{2}$$

Because Ca^{2+} binding causes an association of recoverin with membranes (Zozulya and Stryer 1992), the latter ratio significantly exceeds 1. Hence, the membrane association should increase calcium affinity of the protein ($K_{C,MP} \gg K_{C,P}$). Therefore, under conditions of saturating free Ca^{2+} concentrations the fraction of membrane-bound recoverin can be calculated from:

$$\begin{aligned} R &= ([MP] + [MPC_2])/([P] + [PC_2] + [MP] + [MPC_2]) \\ &\approx [MPC_2]/([PC_2] + [MPC_2]) = [M]/\left(1/K_{M,PC2} + [M]\right) \end{aligned} \tag{3}$$

The fitting of the data on recoverin binding to photoreceptor membranes at saturating free Ca^{2+} concentration of 2 mM (Fig. 3a) using Eq. (3) gives the following effective $K_{M,PC2}$ values for C39D and WT recoverin forms:

$$\begin{array}{ccc} & \overset{K_{C,P}}{} & \\ P + 2C & \leftrightarrow & PC_2 \\ + & & + \\ M & & M \\ K_{M,P} \updownarrow & & \updownarrow K_{M,PC2} \\ MP + 2C & \leftrightarrow & MPC_2 \\ & \underset{K_{C,MP}}{} & \end{array}$$

Scheme 2 The simplest scheme of chemical equilibria between apo-recoverin and its calcium- and membrane-bound forms

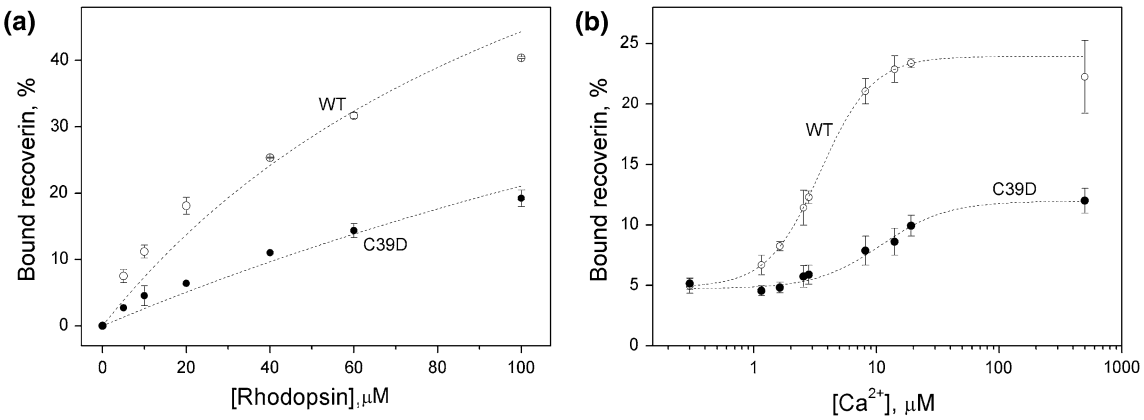

Fig. 3 The quantitative determination of C39D and WT recoverin forms bound to photoreceptor membranes as a function of rhodopsin (**a**) and Ca^{2+} concentration (**b**) using an equilibrium centrifugation assay. Recoverin concentration was 30 μM. Data points are from 2 to 5 different evaluations. **a** Free Ca^{2+} concentration was 2 mM. The *curves* are theoretical (Eq. 3). **b** Concentration of rhodopsin was 50 μM. $EC_{50} = 3.5$ μM Ca^{2+} and $n = 2.0$ (Hill coefficient) for WT recoverin; $EC_{50} = 11$ μM Ca^{2+} and $n = 1.6$ for C39D mutant

2.7×10^3 and 8×10^3 M^{-1}, respectively. Thus, the C39D substitution results in a threefold decrease in photoreceptor membrane affinity of the Ca^{2+}-bound recoverin. Similar ratios of $K_{M,PC2}$ values for WT and C39D proteins (1.9–2.1) can be obtained from the last points of the experimental curves in Fig. 3a and from the data on recoverin binding to membranes in Fig. 3b.

The analysis of the Ca^{2+}-dependent membrane association of recoverin gives the following relationship for the mid-transition Ca^{2+} concentration (neglecting $K_{M,P}$ value):

$$[C]_{1/2}^2 \approx 1/\left[K_{C,P} \cdot \left(1+K_{M,PC2} \cdot [M]\right)\right] \quad (4)$$

Neglecting the difference in Ca^{2+} affinities of membrane-free C39D and WT recoverin forms (Table 2), the threefold lower $K_{M,PC2}$ value for C39D mutant may cause according to the Eq. (4) 1.7-fold increase in the $[C]_{1/2}$ value. The experimentally observed effect is qualitatively identical, but about 80% higher (Fig. 3b). Similar effect has been observed in surface plasmon resonance measurements of the Ca^{2+}-dependent binding of recoverin to immobilized lipids for recoverin form with C39 residue labeled by negatively charged Alexa647 dye (Gensch et al. 2007).

The lowered membrane affinity of the Ca^{2+}-loaded C39D recoverin can be readily rationalized using literature NMR data which show that *K*5, *K*11, *K*22, *K*37, *R*43, and *K*84 of myristoylated Ca^{2+}-bound recoverin form close contacts with membrane (Valentine et al. 2003). Because membrane-bound recoverin was shown to retain the same overall three-dimensional structure that it has in solution, the position of C39 can be judged upon 1jsa PDB entry: C39 is located in close proximity to these basic residues, lying nearly in the same plane with all of them, except for *K*5 and *K*11. Hence, the negative charge of D39 side chain in the C39D mutant should be located in the immediate vicinity of the negatively charged surface of membrane. This fact expectedly should result in electrostatic repulsion of the carboxylate of D39 residue, making the association of the C39D mutant with membrane less favorable.

Discussion

The substitution of Cys39 of recoverin by Asp, considered as a model of its oxidative conversion into a negatively charged group, is accompanied with essential changes in the protein properties. The Ca^{2+}-depleted form of C39D mutant demonstrates significantly lower (ca. 5%) α-helical content (Table 1). The secondary structure changes correlate well with the protein thermal stability changes showing 7 and 3°C decrease in mid-transition temperatures for apo- and Ca^{2+}-bound protein states, respectively (Table 2). The destabilization of the C39D mutant can be reasonably explained based on the electrostatic calculations. The structural consequences of the C39D mutation do not cause measurable calcium affinity changes (Table 2), but result in a drastic change in affinity of recoverin to photoreceptor membranes (Fig. 3). The latter effect seems to be a consequence of the proximity of the residue 39 to the negatively charged membrane in the membrane-bound protein. Notably, the observed suppression of recoverin ability to membrane binding is unexpectedly strong in comparison with the effects of much more serious modifications of the protein, like truncation of the 12 C-terminal residues (Weiergraber et al. 2006), or inactivation of its Ca^{2+}-binding loops (Senin et al. 2002a, b).

Because the key features of the effect of the C39D substitution on physico-chemical properties of recoverin can be adequately described on the basis of electrostatic

calculations, similar effects are expected from oxidative conversion of the Cys39 sulfhydryl group into sulfenic, sulfinic or sulfonic acid, all bearing negative charge at neutral pH. Hence, the oxidation of highly susceptible to that in vitro conservative thiol of recoverin (Permyakov et al. 2007) may greatly reduce the efficiency of functioning of the Ca^{2+}-myristoyl switch in recoverin, due to the lowered membrane affinity of the protein. This feature of Cys39 may impart to recoverin sensitivity to redox conditions of rod photoreceptors. Because spectrofluorimetric measurements of Zn^{2+}-binding constants for apoforms of C39D and WT recoverin at 20°C did not reveal noticeable difference between them (data not shown), Cys39 is not involved into coordination of Zn^{2+}. Hence, Zn^{2+} binding is unable to directly affect oxidation of Cys39 residue. If redox sensory function is conserved within NCS proteins, they may be involved in multiple physiological and pathological processes related to cellular redox state changes, because reactive oxygen species (ROS) in the nervous system were shown to have important effects on central neural mechanisms involved in blood pressure regulation, volume homeostasis, autonomic function and implicated in the neuronal dysregulation associated with some forms of hypertension and heart failure (reviewed in Zimmerman and Davisson 2004; Schipper 2004). In the visual system, ROS initiate and accompany a number of disorders including cataract (Spector 1995; Truscott 2005), glaucoma (for review, see Saccà et al. 2007), as well as retinal inflammatory diseases, such as age-related macular degeneration (for review, see Beatty et al. 2000; Khandhadia and Lotery 2010), diabetic retinopathy (reviewed in Giacco and Brownlee 2010) and others.

The exposure of retina to photons in the presence of a near-arterial level of oxygen is known to favor intensive production of ROS (Organisciak and Vaughan 2010; Noell et al. 1966; Fliesler and Anderson 1983; Anderson et al. 1994; Boulton et al. 2001). In the same time, the redox potential regulation in outer segments of rod and cone photoreceptors is rather specific due to the absence of reduced glutathione, thioredoxin and thioredoxin reductase (reviewed by Winkler 2008). The harmful effect of ROS is compensated via continuous phagocytosis and degradation of retinal photoreceptor outer segment material by the retinal pigment epithelium (Young 1967; Miceli et al. 1994). The only Cys residue of recoverin, being prone to oxidation (Permyakov et al. 2007), is likely to be subjected to oxidation under oxidizing conditions of rod outer segments. The lowered membrane affinity of the Cys39-oxidized protein is expected to cause permanent activation of rhodopsin kinase, eventually resulting in an increase in efficiency of rhodopsin inactivation, similarly to the effect observed in the case of a knockout of recoverin gene (Makino et al. 2004). The intense regeneration of rhodopsin, in turn, is expected to cause accelerated production of ROS (reviewed by Organisciak and Vaughan 2010). Thus, the oxidation-induced modification of recoverin is likely to give rise to a positive loopback, further favoring production of ROS. Therefore, the oxidation of Cys39 residue of recoverin should be considered as a highly undesirable process.

Because the vast majority (>88%) of recoverin is located in rod inner segment (Strissel et al. 2005), which is characterized by more reducing conditions (Winkler 2008), such localization may rescue recoverin from oxidizing conditions of rod outer segment. The light-dependent exchange of recoverin between rod inner and outer segments (Strissel et al. 2005) may serve as a mechanism ensuring effective reduction in the protein oxidized in rod outer segment. Moreover, the fact that concentration of recoverin in rod outer segment is minimal in the light (below 2%) may be considered as a way of avoidance of excessive oxidation of recoverin during the most intense production of ROS. Thus, the necessity to maintain the thiol group of recoverin in the reduced state for its proper functioning is able to rationalize both the localization of recoverin in rod photoreceptors and the light-dependent intracellular translocation of recoverin in rods.

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