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Recoverin as a Redox-Sensitive Protein

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Recoverin is a member of the neuronal calcium sensor (NCS) family of EF-hand calcium binding proteins. In a visual cycle of photoreceptor cells, recoverin regulates activity of rhodopsin kinase in a Ca^{2+}-dependent manner. Like all members of the NSC family, recoverin contains a conserved cysteine (Cys38) in nonfunctional EF-hand 1. This residue was shown to be critical for activation of target proteins in some members of the NCS family but not for interaction of recoverin with rhodopsin kinase. Spectrophotometric titration of Ca^{2+}-loaded recoverin gave 7.6 for the pK_a value of Cys38 thiol, suggesting partial deprotonation of the thiol *in vivo* conditions. An ability of recoverin to form a disulfide dimer and thiol-oxidized monomer under mild oxidizing conditions was found using SDS-PAGE in reducing and nonreducing conditions and Ellman's test. Both processes are reversible and modulated by Ca^{2+}. Although formation of the disulfide dimer takes place only for Ca^{2+}-loaded recoverin, accumulation of the oxidized monomer proceeds more effectively for apo-recoverin. The Ca^{2+} modulated susceptibility of the recoverin thiol to reversible oxidation may be of potential importance for functioning of recoverin in photoreceptor cells.

Keywords: EF-hand • NCS family • vision • recoverin • cysteine • thiol oxidation

Recoverin is a member of the neuronal calcium sensor (NCS) protein family. This family is encoded by 14 genes in the human genome and classified into five subfamilies including frequenin (NCS-1), VILIPs (visinin-like proteins), recoverins, GCAPs (guanylyl cyclase-activating proteins), and KChIPs (calsenilin, DREAM).[1,2] The NCS family has a restricted expression mainly in retinal photoreceptors or neurons and neuroendocrine cells. The physiological roles of NCS protein family include modulation of neurotransmitter release, control of cyclic nucleotide metabolism, biosynthesis of polyphosphoinositides, regulation of gene expression, phototransduction, and direct regulation of ion channels. The NCS proteins possess four EF-hand calcium binding domains, but only three or two of them bind calcium ions. Being high-affinity Ca^{2+}-binding proteins, they act as Ca^{2+} sensors rather than Ca^{2+} buffers. They undergo conformational changes on Ca^{2+}-binding, regulating target proteins. With the exception of KChIP2, KChIP3, and KChIP4, all NCS proteins are N-terminally myristoylated, allowing them to become membrane-associated.

The members of classes C and D of the NCS protein family, recoverin and GCAPs, are present in amphibia and evolutionarily subsequent species and are expressed only in the retina. Recoverin was the earliest NCS protein characterized. It specifically acts as a Ca^{2+}-dependent inhibitor of rhodopsin kinase, controlling rhodopsin inactivation process.[3−5] The NH_2-terminal glycine of recoverin molecule is covalently modified mostly by myristoleate (14:1), but 14:0, 14:2, and 12:0 acyl residues are also present.[6] The myristoyl chain exhibits the Ca^{2+}-myristoyl switch phenomenon:[7,8] being harbored at the N-terminal domain in apo-recoverin,[9] it becomes solvent exposed upon sequential binding of two Ca^{2+} ions by EF-hands 3 and 2 of the protein.[10−12] Such structural rearrangement enables recoverin to associate with ROS disk membranes in a Ca^{2+}-dependent manner.[7,8]

A characteristic feature of the NCS protein family is a sequence (D/E)CP(S/T)G within the EF-hand 1 domain. The only exception is GCAP3, containing a Tyr residue instead of the Cys.[13] The Cys and Pro residues are located between the Y and Z positions of the consensus EF-hand loop, defined in PROSITE (PDOC00018). The proline at the fourth position in the loop causes a distortion of the geometry favoring Ca^{2+}-

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binding. Some other differences of the EF-hand 1 from the canonical EF-hand loop also prevent binding of a Ca^{2+} ion to this site. Some members of the family are characterized by the presence of additional, nonconservative Cys residues. Conservatism of the thiol at position 38 of recoverin within the NCS family suggests that it plays an indispensable role in the functioning of the whole protein family.

Examination of the crystal structure of the complex between KChIP1 and the N-terminal fragment of Kv4.2 (residues 1−30) (PDB code 1S6C)[14] shows that the thiol 64, presenting in S-(dimethylarsenic)cysteine form due to modification by cacodylate during crystallization, interacts directly with the Kv4.2 helix via Trp8 side chain. At the same time, the NMR structure of the complex between recombinant nonmyristoylated recoverin with two Ca^{2+} ions bound and the N-terminal fragment of rhodopsin kinase (RK25) (PDB code 2I94)[15] reveals that the Cys38 thiol of recoverin is turned toward the RK25 helix, with the sulfur atom 4.4 Å away from the backbone oxygen atom of Ala11 in RK25. Thus, both known structures of NCS proteins complexed with fragments of their targets show that the conservative thiol is located very close to the helix of the target. This circumstance indicates that the conservative Cys residue could interact with the full-size target directly or could be involved into an active or binding site.

Using peptides derived from the GCAP-1 sequence, the EF-1 motif was mapped as one of the regions likely to be involved in the interaction with guanylate cyclase.[16] Further studies of GCAP-1 have shown that changing of Cys29 and Pro30 to a single glycine, having place in the fourth position of EF-hand of calmodulin, is sufficient to cause loss of target activation without loss of Ca^{2+}-induced conformational changes.[17] Thus, a critical role of Cys29 and Pro30 of GCAP-1 for providing the correct conformation for target activation was shown. Similarly, point mutations of Cys35 and other residues in EF-hand 1 of GCAP-2 allowed drawing the same conclusion.[18] Meanwhile, recent study of recoverin with Cys38 modified by a fluorescent dye Alexa647-maleimide revealed just a minor decrease in the efficiency of the Ca^{2+}-myristoyl switch and the recoverin potency to inhibit rhodopsin kinase,[19] which evidence an insignificant role of this thiol in functioning of recoverin. Immobilization of recoverin on a SPR sensor chip surface by a thiol-specific reagent does not disturb the Ca^{2+}-myristoyl switch[20] and does not effect Ca^{2+}-dependent binding of rhodopsin kinase to the recoverin-coated surface.[21] These somewhat contradictory observations obtained on different members of the same family indicate that further investigations of the conserved thiol and its possible role in implementation of versatile functions of NCS proteins are required.

In the present work, a pK_a value for Cys38 thiol of Ca^{2+}-loaded myristoylated recoverin was determined, implying that *in vivo* partial deprotonation of the thiol should be expected. A propensity of Cys38 for oxidation was probed under mild oxidizing conditions. A calcium-dependent formation of both a disulfide dimer and oxidized monomer was observed. The established susceptibility of recoverin thiol to reversible oxidation modulated by calcium ions may be of potential importance for functioning of recoverin in photoreceptor cells.

Experimental Section

Materials. Recombinant recoverin was produced in *Escherichia coli* and purified in reducing conditions as previously described,[22,23] with minor modifications. Recombinant nonmyristoylated and myristoylated forms of recoverin were expressed in the *E. coli* strains pET11d rec/BL21 and pET11d rec/pBB131/BL21C, respectively. Cells were lyzed in buffer B (20 mM Tris-HCl, pH 8.0, 100 mM NaCl, 1 mM DTT, 1 mM phenylmethylsulfonyl fluoride, 0.1 mM EDTA) including 0.05 mg/mL lysozyme, incubated on ice for 20 min, and centrifuged at 15 000g for 20 min at 4 °C. The supernatant was adjusted to 5 mM $CaCl_2$ and was applied to a phenyl-Sepharose column previously equilibrated with buffer A, containing 5 mM $CaCl_2$ and 2 mM β-mercaptoethanol in 20 mM Tris-HCl, pH 8.0. Recoverin was eluted with buffer B, containing 5 mM EGTA and 2 mM β-mercaptoethanol in 20 mM Tris-HCl, pH 8.0. The recoverin fraction was loaded on a Mono-Q (5/5) column that was equilibrated with 20 mM Tris-HCl, pH 8.0, 2 mM β-mercaptoethanol. Recoverin was eluted with a linear gradient of 0−500 mM NaCl, 2 mM β-mercaptoethanol in 20 mM Tris-HCl, pH 8.0. Protein was stored in the same buffer, ∼120 mM NaCl at −20 °C. The purity of recoverin samples exceeded 98% as confirmed by SDS-PAGE. The degree of myristoylation was determined by analytical HPLC on Symmetry 300 C18 5m 3.9 × 150 column and exceeded 95% for myristoylated protein, not exceeding 5% for nonmyristoylated recoverin.

Recoverin concentration was measured spectrophotometrically using a molar extinction coefficient of $\epsilon_{280nm} = 24\,075$ $M^{-1}cm^{-1}$, calculated according to the procedure of Pace.[24] The extinction coefficient of urea unfolded recoverin was estimated based upon this value, knowing the volumetric effect accompanying the urea dissolution: $\epsilon_{280nm} = 22\,627$ $M^{-1}cm^{-1}$.

Baltic cod 4.40 parvalbumin was isolated from cod skeletal muscles according to known protocol.[25] The purity of protein sample was confirmed by native and SDS-PAGE. Parvalbumin concentration was determined spectrophotometrically based upon a molar extinction coefficient of $\epsilon_{280nm} = 7115$ $M^{-1}cm^{-1}$, calculated according to the procedure of Pace.[24]

Molecular mass markers for SDS-PAGE were purchased from Sigma Chemical Co. (St. Louis, MO) and Helicon (Moscow, Russia).

HEPES, H_3BO_3, and urea were ultra-grade, from Sigma Chemical Co., Merck Biosciences and Diam (Moscow, Russia), respectively. DTT and $CaCl_2$ from Fluka, DTNB from Aldrich, GSH from AppliChem, and EGTA from Sigma Chemical Co. were of analytical grade. Other chemicals were reagent grade or higher. Distilled water with conductivity ∼3 μSm/cm was used for dialysis. All buffers and other solutions were prepared using nanopure water.

Methods. SDS-PAGE. Protein separations were performed on 15% SDS-PAGE. In experiments requiring nonreducing conditions, DTT treatment step was skipped. Silver staining was carried out using Amersham Biosciences PlusOne protein silver staining kit, according to a standard protocol.

Chromatographic Separation of Monomeric and Dimeric Forms of Recoverin. Recoverin (80 μM) was applied to a column (1.5 × 54 cm) of Sephacryl S-100 HR (Amersham Biosciences) that had been equilibrated and eluted with 10 mM H_3BO_3−KOH 150 mM KCl pH 8.5 at a flow rate of 12 mL/h. The elution was monitored with LKB-Bromma 2138 Uvicord S UV detector with a 280 nm filter.

Reduction of Sulfhydryl Group of Recoverin. Recoverin (100 μM) was unfolded by 8 M urea in presence of 1 mM EDTA at pH 7.7 (10 mM HEPES-KOH), followed by incubation with 10-fold molar excess of DTT for 4 h at 70 °C. The residuals of DTT were removed either by gel-filtration using Sephadex G-25 media or via dialysis (4 h) against 1000-fold excess of buffer at pH 6.5. The completeness of thiol reduction was confirmed

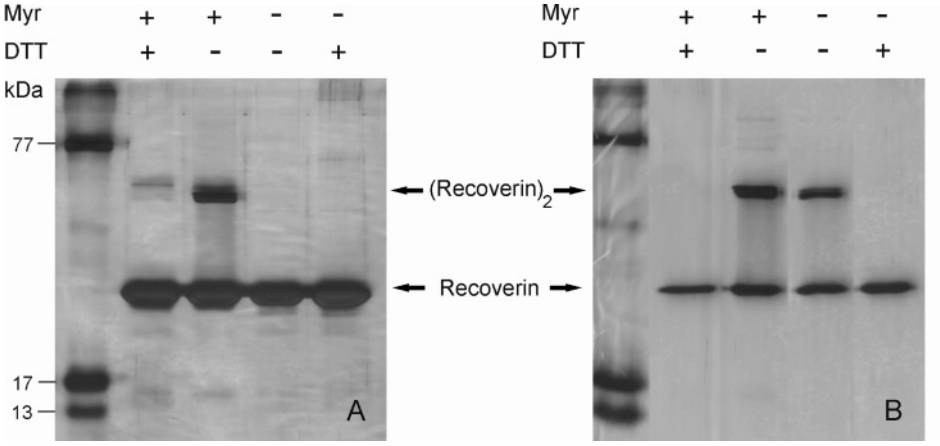

Figure 1. SDS-PAGE of myristoylated and nonmyristoylated forms of recoverin in reducing (10 mM DTT) and nonreducing conditions, followed by silver staining. (A) Recoverin isolated in reducing conditions (2 mM β-mercaptoethanol); (B) recoverin dialyzed against water and then lyophilized. The numbers in left-hand column indicate the molecular masses of markers in kilodaltons (holo-transferrin, 77 kDa; apomyoglobin, 17 kDa; ribonuclease A, 13.7 kDa).

both by SDS-PAGE in nonreducing conditions and Ellman's reagent test, which exhibited the expected 1:1 free SH-group to recoverin molar ratio. Softening of the reducing conditions resulted in marked decrease of this proportion. The reduced protein was used for experiments immediately or was kept at pH 6.5 to avoid oxidation. The correctness of recoverin folding was confirmed by intrinsic fluorescence melting experiments. The protein refolded from urea exhibits cooperative thermal denaturation transition with half-transition temperature characteristic for intact recoverin.

Ellman's Reagent Test. Quantitation of sulfhydryl groups of recoverin was performed according to the modified original procedure of Ellman.[26] Recoverin (6−16 μM) unfolded by 8 M urea in the presence of 1 mM EDTA at pH 7.7 (10 mM HEPES-KOH) was treated by 0.4 mM DTNB for 2 h at 20 °C. The kinetics of the reaction was monitored spectrophotometrically at 412 nm with a Cary 100 spectrophotometer (Varian, Inc.) in a double-beam mode versus reference solution without protein. The kinetics in most cases was described by a single exponential. Where possible, the resulting thiol-to-protein molar ratios were calculated based upon the exponentially extrapolated absorbance values. The molar extinction coefficient of 5-thio-2-nitrobenzoic acid (TNB) $\epsilon_{412nm} = 14\ 150\ M^{-1}cm^{-1}$ was used.[27]

Because urea can degrade, forming cyanates reactive to thiol groups, only newly purchased urea was used. Moreover, possible contribution of the urea contaminants was subtracted via double-beam measurement versus urea-containing reference solution.

The efficacy of the procedure used was confirmed using reduced glutathione and Baltic cod parvalbumin pI 4.40[28] samples, both containing a single thiol group.

Spectrophotometric pH Titration of Recoverin. Absorption spectra were measured with a Cary 100 spectrophotometer (Varian, Inc.). Measurements of pH-dependent changes in protein absorbance were performed by upward or downward titration of the protein solution by small aliquots of KOH or HCl, respectively. Protein absorbance at 240 nm was corrected for a minor light scattering contribution by means of extrapolation with power function of the long wavelength region of protein absorption spectrum. The absorbance was linearly corrected for concomitant dilution of the solution.

Estimation of Cys38 pK_a Value. The pK_a value of cysteine thiol group in reduced recoverin was determined by a pH-induced change in absorbance at 240 nm accompanying its ionization.[29] Over the pH range tested, the oxidized form of recoverin exhibited no absorbance change at 240 nm, indicating that the changes observed during pH titration of reduced recoverin were caused by thiol group ionization.

The reduced form of recoverin was obtained by means of treatment of urea-unfolded protein with 10-fold molar excess of DTT (pH 7.7) followed by removal of DTT via dialysis against 1000-fold excess of buffer (pH 6.5), according to the reduction procedure described above. The thiol pK_a value was estimated by nonlinear least-squares fitting of the plot of molar extinction coefficient of the reduced recoverin at 240 nm versus pH to the theoretical equation describing a single protonation mechanism:

$$\epsilon_{240nm}(pH) = \epsilon_{thiol}\cdot\alpha + \epsilon_{thiolate}\cdot(1-\alpha)$$

$$\alpha = \frac{1}{(1 + 10^{(pH - pKa)})} \quad (1)$$

Here, ϵ_{thiol} and $\epsilon_{thiolate}$ correspond to molar extinction coefficients of the protein with sulfhydryl group in thiol and thiolate forms, respectively.

Electrostatic Potential Calculation. The electrostatic potentials at sulfur atom of Cys38 residue of recoverin were calculated using the finite difference Poisson−Boltzmann method as implemented in the DelPhi module[30] of the Insight II molecular modeling environment (Insight II 2005, Accelrys Software Inc., San Diego). Electrostatics calculations were performed for myristoylated apo-recoverin (PDB code 1IKU)[9] and the myristoylated Ca^{2+}-bound protein (PDB code 1JSA).[11] The first conformer was used for calculations; hydrogen atoms were disregarded. Ionic strength of the solvent (dielectric constant 80.0, molecule radius 1.4 Å) was set to zero. The solute extent was 80%; the number of grid points was 33. Debye−Huckel-type boundary conditions were used. DelPhi's default charge and radius templates were used. The charge of thiol 38 was set to zero.

Structural Modeling of the Recoverin Disulfide Dimer. The crystal structure of a free recombinant nonmyristoylated bovine recoverin truncated at C-terminus (residues 2−190) with a Ca^{2+} ion bound to EF-hand 3 (PDB code 2HET)[31] and the NMR structure of the complex between recombinant nonmyristoylated recoverin with two Ca^{2+} ions bound and the N-terminal

fragment of rhodopsin kinase (residues 1−25) (PDB code 2I94)[15] were obtained from the Protein Data Bank (PDB).[32] The first conformer of the NMR complex structure was taken as a reference. The recoverin dimer model was built using the molecular modeling software SYBYL (Tripos Associates, Inc., St. Louis, MO). The SYBYL "Fit Atoms" function was used to perform superposition of C_α-atoms of recoverin and rhodopsin kinase fragment.

Results

The Effect of Oxidation on Dimerization of Recoverin. SDS-PAGE of myristoylated recoverin isolated in reducing conditions (2 mM β-mercaptoethanol) shows a minor fraction of dimers (Figure 1A, line 3). The excess of strong reducing agent (10 mM DTT) fully converts the dimers into their monomeric forms (Figure 1A, line 2), implying that recoverin dimer is formed by means of an intermolecular disulfide bond. At the same time, nonmyristoylated protein isolated in reducing conditions does not form the dimer (Figure 1A, line 4), indicating that non-myristoylated recoverin is less susceptible to oxidation.

SDS-PAGE of dialyzed against water and subsequently lyophilized recoverin reveals a major fraction of dimers for both myristoylated and nonmyristoylated protein (Figure 1B, lines 3 and 4). The dimer is fully converted into monomeric form after DTT treatment (Figure 1B, lines 2 and 5), confirming the involvement of an intermolecular disulfide bond. Thus, recoverin oxidation during dialysis and lyophilization processes is accompanied by effective formation of S−S-cross-linked dimer, and this process does not depend on myristoylation state of recoverin.

Separation of Monomeric and Dimeric Forms of Recoverin. Oxidized during dialysis and subsequent lyophilization, myristoylated recoverin was fractionated by gel chromatography on a Sephacryl S-100 HR column (Figure 2A). The elution profile monitored by absorbance at 280 nm exhibits two distinct symmetric peaks. SDS-PAGE of the resulting protein components in nonreducing conditions (Figure 2B) confirms that the short retention time peak corresponds to the S−S-crosslinked dimer of recoverin, whereas the second peak corresponds to the monomeric protein. Computation of the areas of the peaks 1 and 2 shows that ~35% of total recoverin is in dimeric form, giving a more reliable estimate of the dimer-to-monomer ratio than the SDS-PAGE data (Figure 1B). Notably, in some cases a minor additional short retention time peak appeared, corresponding to oligomeric form of the S−S-crosslinked dimer of recoverin, as confirmed by SDS-PAGE in nonreducing conditions (data not shown).

Quantitation of Sulfhydryl Groups of Recoverin Samples. According to the gel-filtration experiments, about 65% of recoverin oxidized during dialysis and lyophilization procedures remain in monomeric form, the oxidation state of which was assessed using thiol-specific Ellman's reagent. The results of the DTNB tests performed for myristoylated and nonmyristoylated oxidized recoverin unfolded by urea in the absence of calcium ions have shown very low SH-group content for both recoverin samples, corresponding to 16−20% of monomeric protein form. The same experiments in the absence of the denaturant gave similar results. Thus, dialysis and lyophilization of recoverin are accompanied with effective oxidation of the protein, producing both Cys−Cys dimer and oxidized monomeric forms regardless of myristoylation state of recoverin.

Spectrophotometric studies of kinetics of interaction between DTNB and urea-unfolded reduced form of recoverin at

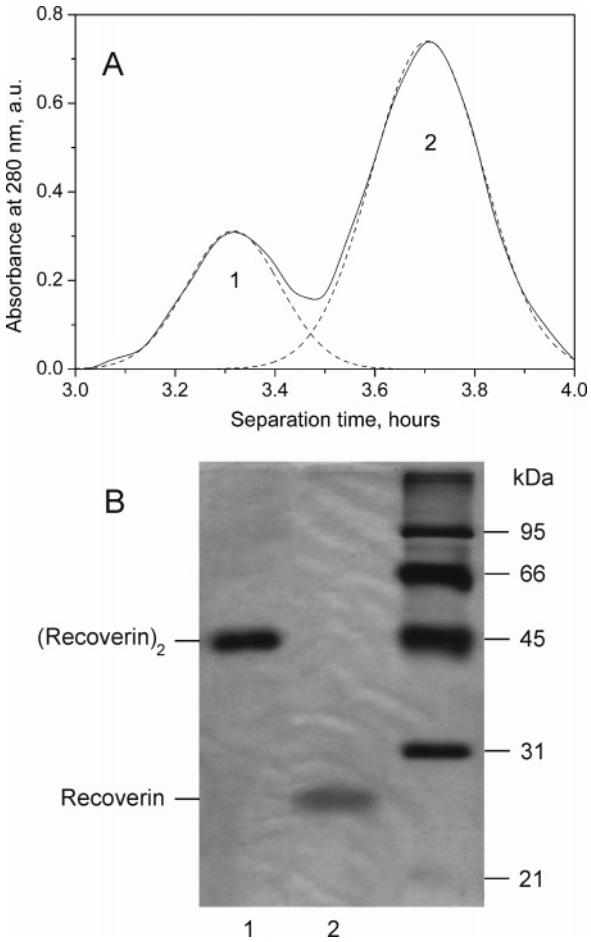

Figure 2. (A) Chromatographic fractionation of the oxidized myristoylated recoverin on a Sephacryl S-100 HR column and (B) SDS-PAGE of the resulting protein components (1 and 2) in nonreducing conditions, followed by silver staining. Protein elution was monitored by absorbance at 280 nm. The absorbance curve is deconvoluted into two components, using Gaussians (dashed lines). The numbers in right-hand column (B) indicate the molecular masses of markers in kilodaltons (cellulase, 94.6 kDa; bovine serum albumin, 66.2 kDa; ovalbumin, 45 kDa; carbonic anhydrase, 31 kDa; trypsin inhibitor, 21.5 kDa).

pH 7.7 reveal single-exponential process with about 1 h (nonmyristoylated protein; apparent rate constant 0.74 M^{-1} s^{-1}) or 2 h (myristoylated recoverin; apparent rate constant 0.34 M^{-1} s^{-1}) half-transition time. Despite the slow kinetics of the interaction, it proceeds until the full modification of Cys38 thiol is completed. An increase in pH up to 8.5 results in 14-fold acceleration of the reaction, indicating that the slow kinetics of thiol modification at pH 7.7 is due to its incomplete deprotonation.

The oxidation behavior of recoverin was compared to that for another cytosolic protein, Baltic cod parvalbumin p*I* 4.40, also containing a single Cys residue.[28] Parvalbumin subjected to analogous oxidative conditions did not prove oxidation of its thiol group, as confirmed by DTNB tests (data not shown). Thus, recoverin represents a specific case of a redox-sensitive SH-group, which may be significant for biological function of recoverin.

It should be noted that the estimates of thiol content for reduced forms of both recoverin and parvalbumin gave somewhat overestimated values, in the range 1.19−1.28, which is

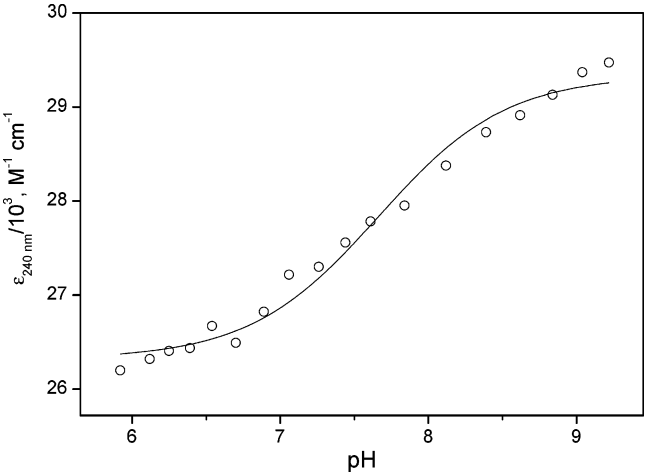

Figure 3. Spectrophotometric pH titration of reduced calcium-loaded myristoylated recoverin at 20 °C. Protein concentration was 13 μM, $CaCl_2$ concentration was 1 mM. Buffer conditions: 3 mM HEPES, 3 mM H_3BO_3. (○) Molar extinction coefficient of recoverin at 240 nm, ϵ_{240nm}. The solid curve was fitted to the experimental data according to the single protonation mechanism (eq 1).

likely to reflect a systematic error in the value of extinction coefficient of TNB used.

Thiol Group pK_a Value. The dissociated form of a simple alkyl thiol group can be identified spectrophotometrically because it displays an absorption band around 240 nm ($\epsilon \approx 4000\ M^{-1}\ cm^{-1}$), where the absorption of the protonated form is negligible.[29] Thus, estimation of the pK_a value of a single thiol group of recoverin can be carried out by pH titration with spectrophotometric monitoring the appearance of the thiolate form. The upward pH titration of reduced calcium-loaded myristoylated recoverin showed a single ionization process with a pK_a value of 7.6 (Figure 3). The downward pH titration of reduced Ca^{2+}-loaded recoverin, starting from pH 9, did not reveal appreciable absorbance changes (data not shown), suggesting effective oxidation of Cys38 at pH values far above its pK_a value. This phenomenon was confirmed by the Ellman's reagent test and seems to affect the accuracy of the determined pK_a value. Oxidized during dialysis and subsequent lyophilization myristoylated recoverin also did not show any changes in absorbance over this pH range (data not shown). The experiments on oxidized Ca^{2+}-saturated recoverin confirm that ionization of the tyrosine residues[33] does not contribute to the absorbance changes observed for the reduced protein.

The analogous experiment performed for apo-form of reduced myristoylated recoverin did not allow for getting a reliable estimate of the pK_a value of Cys38, partially due to more pronounced pH-dependent aggregation of apo-recoverin, lowering accuracy of determination of protein absorbance at 240 nm. Other obstacles in obtaining of reliable estimate of the pK_a value for apoprotein include the strong pH-dependence of calcium affinity of Ca^{2+} chelators used for removal of calcium ions from solution[34] and calcium contamination inevitably accompanying pH-measurements due to Ca^{2+} leaks from pH-electrode.

To assess the pK_a value of Cys38 of apo-recoverin, the values of electrostatic potential at the sulfur atom of the residue were calculated based upon known three-dimensional structures of Ca^{2+}-loaded[11] and apoprotein.[9] The energies of charge−charge interactions in apo- and Ca^{2+}-bound recoverin were calculated using the finite difference Poisson−Boltzmann method. The resulting energies differ by just about 0.3 kJ/mol, which is within accuracy of the calculations and corresponds to the pK_a difference between the two recoverin forms of just about 0.04.[30] Thus, the electrostatic calculations did not reveal evident reasons for the thiol pK_a value for apo-recoverin to be substantially different from that for the Ca^{2+}-loaded protein. Examination of the structure of apo-recoverin[9] revealed that the sulfur atom of Cys38 forms a hydrogen bond with the amide nitrogen atom of the backbone of Gly41 residue. At the same time, Ca^{2+}-binding induces rotation of the backbone at Gly41, resulting in drastic change in interhelical angle of EF-hand 1.[11] This rearrangement causes disruption of the Cys38-Gly41 hydrogen bond and exposure of the Cys38 side chain to the solvent. These differences in solvent accessibility and hydrogen-bonding patterns of the thiol group in apo- and Ca^{2+}-bound states of recoverin might decrease pK_a value for apoprotein.

Calcium-Dependence of Recoverin Thiol Oxidation. Because recoverin function in photoreceptor cell was shown to be calcium mediated,[3−5] it is of interest to examine the dependence of recoverin oxidation process upon Ca^{2+} ion level. The reduced myristoylated recoverin was dialyzed against degassed (by means of prolonged boiling) buffer (pH 6.5) followed by addition of either 1 mM EDTA or 1 mM $CaCl_2$ and adjustment of pH to 8.5, which is far above of pK_a value of Cys38 in recoverin. The resulting identical solutions of recoverin differing by calcium concentration were incubated for 24 h at 20 °C. The SDS-PAGE of both samples in nonreducing conditions after 2 h and 24 h of incubation reveals slow accumulation of the dimeric form of recoverin in presence of calcium, whereas apo-recoverin does not exhibit apparent changes in dimer content (Figure 4). Potentially the long term incubation accompanied with partial oxidation of protein could prevent apo-recoverin from disulfide dimerization. Nevertheless, Ellman's test shows that more than 30% of protein Cys residues remain in reduced form, which is enough for efficient formation of disulfides. The absence of the dimer accumulation for apo-recoverin confirms that calcium binding is a necessary condition for Cys−Cys dimerization of the protein.

An analogous experiment was performed without degassing of the buffer used. The reduced apo- and Ca^{2+}-loaded states of recoverin were subjected to identical oxidation conditions. After 2 h of incubation at 20 °C (pH 8.5), accumulation of the dimer was not observed, whereas estimation of sulfhydryl groups content by Ellman's test in 8 M urea demonstrated a marked Ca^{2+}-dependence: about 72% of apoprotein was oxidized versus 53% for the Ca^{2+}-loaded state. Thus, the apo-form of recoverin proved to be more vulnerable to the oxidation. This conclusion may imply that the pK_a of Cys38 is shifted toward acidic values for apo-recoverin compared to the Ca^{2+}-loaded protein.

Structural Modeling of the Recoverin Disulfide Dimer. There are two known three-dimensional structures of recoverin complexed with protein molecules. Both structures correspond to calcium-loaded recoverin, which was shown here to form a dimer more readily than apoprotein does. The complexes share common features and can be successfully utilized for modeling of the Cys−Cys dimer of recoverin.

The nontypical crystal structure of recombinant nonmyristoylated recoverin truncated at C-terminus (residues 2−190) with a Ca^{2+} ion bound to EF-hand 3 has been determined (PDB code 2HET).[31] In this case, the crystallographic cell contains four recoverin molecules (A, B, C, and D chains), positioned

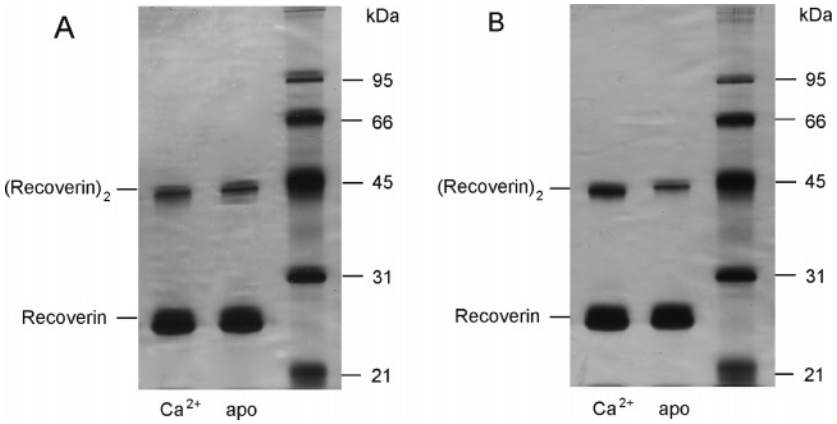

Figure 4. SDS-PAGE in nonreducing conditions of the reduced myristoylated recoverin incubated in degassed buffer at pH 8.5 for (A) 2 h or (B) 24 h (20 °C), followed by silver staining. Protein concentration during the incubation was 45 μM. Calcium concentration was controlled by the addition of 1 mM EDTA or 1 mM $CaCl_2$. Identical amounts of recoverin were applied for Ca^{2+}-loaded and apo-recoverin. The numbers in the right-hand column indicate the molecular masses of markers in kilodaltons (cellulase, 94.6 kDa; bovine serum albumin, 66.2 kDa; ovalbumin, 45 kDa; carbonic anhydrase, 31 kDa; trypsin inhibitor, 21.5 kDa).

in a head-to-tail fashion. For example, the H47 helix (residues Glu169-Asn179) of chain D is located between the H3 (residues Thr24-Cys39) and H4 (Arg46-Phe57) helices of chain A. Thus, the hydrophobic groove-helix mode of association is observed. The H4 and H47 helices run in the same direction, whereas H3 runs in the opposite direction.

The NMR structure of the complex between recombinant nonmyristoylated recoverin with two Ca^{2+} ions bound and the N-terminal fragment of rhodopsin kinase (residues 1−25, RK25) (PDB code 2I94)[15] demonstrates analogous hydrophobic groove-helix mechanism of association (PDB code 2I94).[15] RK25 in the complex forms an amphipathic α-helix (residues 4−16), interacting with the hydrophobic groove on the surface of recoverin. Likewise, the RK25 helix is located between the H3 (residues Glu25-Cys39) and H4 (residues Thr45-Phe56) helices of recoverin. In this case, however, H3 and RK25 helices run in the same direction, whereas H4 runs in the opposite direction.

Thus, both known recoverin complexes with protein molecules are formed in a similar manner, and the major difference between them is the direction of the donor helix with respect to H3 and H4 helices of recoverin. The only Cys residue of recoverin is located at the N-terminus of the H3 helix, in close proximity to the terminus of the donor helix. This circumstance allows building of recoverin homodimer in which Cys side chains are located close enough to form the disulfide bond.

Although both 2HET and 2I94 structures are suitable as the patterns for generation of the theoretical three-dimensional model of disulfide dimer of recoverin, only modeling based upon 2I94 structure will be considered in detail. The modeling based upon 2HET coordinates produces analogous structure, differing by mutual orientation of helices H3 and H4 of both recoverin molecules.

Superposition of Thr24-Leu36 (includes the major part of H3 helix (Glu25-Cys39)) fragment of an extra molecule of recoverin and Gly4-Ile16 helix of RK25 within structure 2I94 gives the root-mean-square deviation (rmsd) of 26 C_α atoms (2-fold superposition) of only 1.4 Å (Figure 5A). Moreover, Trp31 and Phe35 residues of recoverin are superimposed on Ala11 and Phe15 residues of RK25, respectively. Both Ala11 and Phe15 of RK25 are involved into hydrophobic interactions with H3 helix of recoverin through Trp31 and Phe35 residues. Thus, the resulting alignment of recoverin molecules corresponds to

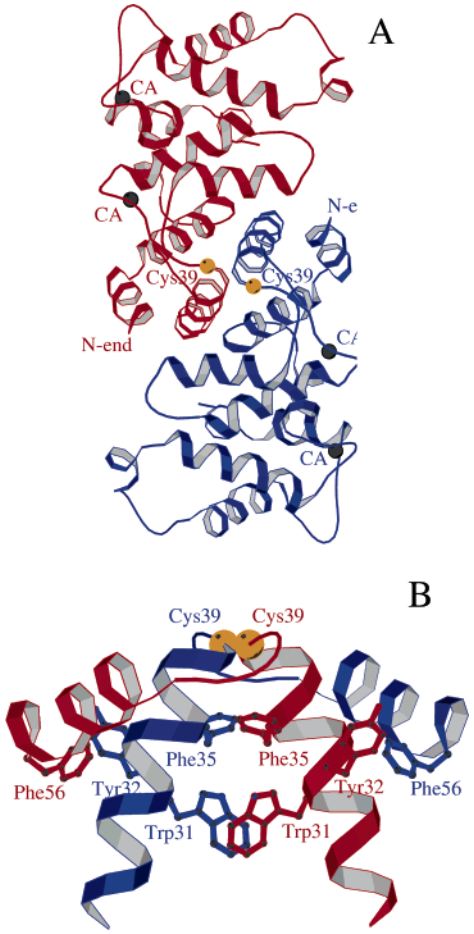

Figure 5. (A) Complete three-dimensional model of recoverin disulfide dimer and (B) hydrophobic contacts between recoverin chains A (red) and B (blue). C_α atoms of Cys residues and calcium ions are shown as yellow and black balls, respectively. In (B), side chains of the interacting residues are shown in a ball-and-stick manner. Figure was produced with MOLSCRIPT v2.1.[65]

pair wise interactions of Trp31 and Phe35 side chains (Figure 5B). Overall, four pairs of the aromatic contacts between two chains (A and B) of recoverin are formed: Trp31/A-Trp31/B, Phe35/A-Phe35/B, Tyr32/A-Phe56/B, and Phe56/A-Tyr32/B.

Phe56 residue of helix H4 is also involved into RK25-recoverin interaction. Notably, the model is free from any sterical hindrances between A and B chains, and the distance between C_α atoms of two Cys residues is 6.9 Å.

Discussion

The oxidation of recoverin isolated in reducing conditions, taking place during dialysis against water and subsequent lyophilization, is accompanied by highly effective thiol oxidation, producing both disulfide dimer (Figures 1 and 2) and oxidized monomeric forms, regardless of myristoylation state of recoverin. Artificially reduced recoverin, being exposed to alkaline pH values above pK_a value of Cys38, in mild oxidizing conditions exhibits oxidation leading to formation of mostly oxidized monomer and a minor fraction of the dimer (Figure 4). Both processes are calcium-dependent, the last being observed only for Ca^{2+}-loaded recoverin, whereas formation of oxidized monomer proceeds more efficiently for apoprotein. The Ca^{2+} dependence of recoverin oxidation can be rationalized based upon known three-dimensional structures of Ca^{2+}-loaded and apo-form of recoverin and estimations of electrostatic potential at Cys38 thiol, as described above. The fact that oxidation of Cys38 thiol is reversible, as confimed by SDS-PAGE (Figure 1) and Ellman's tests, suggests that oxidation process is accompanied with sulfenic and, less likely, sulfinic acid formation.

The formation of recoverin dimer at pH exceeding pK_a of Cys38 requires hours at protein concentration 45 μM. This is about an order of magnitude higher than physiologically relevant protein level in ROS,[35−37] which was recently shown to change in the micromolar range during the visual cycle.[38] The extremely slow kinetics of this process *in vitro* under conditions favoring disulfide formation and the reducing conditions of cytosol suggest that the Ca^{2+}-dependent spontaneous accumulation of recoverin dimer is unlikely to occur *in vivo*. At the same time, the possibility of catalytic formation/reduction of disulfide dimers of recoverin *in vivo* should not be discarded.

Previous studies on GCAP-2 and KChIP3 NCS proteins showed that they are able to undergo metal ion-dependent reversible dimerization, which seems to play a regulatory role in the manifestation of their activities.[39−41] Ca^{2+} and Mg^{2+} ions enhance the intermolecular interaction of VILIP1 and VILIP3 and lead to the formation of homo- and hetero-oligomers of VILIPs.[42] The data suggesting dimer accumulation at elevated protein concentrations for another NCS family member, NCS-1, have been reported.[43] Nevertheless, the disulfide dimer formation was not found for any of the NCS proteins, to the best of our knowledge. We believe that the Cys−Cys dimer of recoverin observed in the present work was formed due to fairly specific conditions, taking place during protein dialysis against water and further lyophilization procedure. The oxidation of the reduced Cys38 under more thoroughly controlled solvent conditions produces mostly oxidized monomer, giving just a minor contribution of the disulfide dimer. Thus, the procedures of dialysis and lyophilization of recoverin should be avoided. Otherwise, the coercive thiol reduction step is necessary.

The pronounced susceptibility of the sulfhydryl group of recoverin toward oxidizing agents seems to be mostly due to its relatively low pK_a value (7.6), promoting accumulation of highly reactive thiolate anion at physiological pH. The vast majority of thiols possess pK_a values above 8.0, which causes their protonation at cytosolic conditions. This remarkable feature of recoverin has not been reported previously for members of the NCS protein family. Nevertheless, the observed for recoverin lowered resistance of the conserved Cys residue to oxidation may be inherent to other members of the NCS family. Examination of crystal structure of the complex between KChIP1 and the N-terminal fragment of Kv4.2 (PDB code 1S6C)[14] shows that the thiol 64 is in S-(dimethylarsenic)cysteine form due to oxidation by cacodylate during crystallization.

The insufficiently studied oxidative properties of sulfhydril groups of NCS family members seem to be a result of the widespread practice of preventive addition of reducing agents during isolation and investigation of cytosolic proteins. Considering that cytosolic redox equilibrium is shifted toward reducing conditions, favoring reduction of protein sulfhydryl groups,[44] this tactic is reasonable. Nevertheless, recent studies of redox proteomics show that dozens of cytosolic proteins are able to form disulfide bonds in cardiac myocytes[45] and neuronal cells[46] exposed to various oxidative insults. Studies of the intracellular proteins of certain thermophilic bacteria show that they are rich in disulfide bonds.[47,48]

Some normal cellular functions are controlled by low concentrations of reactive oxygen and nitrogen species that are transiently generated as secondary messengers.[49−52] Cellular responses to both high and low reactive oxygen species levels involve proteins whose activities are regulated by oxidation. Several major forms of oxidative modifications can occur on amino acid residue side chains including carbonylation (for review, see ref 53), nitration of tyrosine, and oxidation of methionine to methionine sulfoxide. Protein sulfhydryls can be oxidized to intra- and interprotein disulfides, mixed disulfides (glutathionylation or cysteinylation), S-nitrosothiols, sulfenic acid, as well as more highly oxidized states such as the sulfinic and sulfonic acid forms of protein cysteines (for review, see ref 54). Many forms of thiol oxidation are reversible, which makes them attractive from the point of view of redox regulation. Over the past few years, an increasing number of thiol-containing proteins has been identified that use reactive oxygen species as mediators to quickly regulate their protein activity.[55,56] All of these proteins possess highly reactive Cys residues that are quickly and reversibly modified upon exposure to oxidative stress. In the light of these observations, basically any protein with reactive Cys residue has the potential of being redox regulated. The observed redox sensitivity of Cys38 of recoverin may indicate that this residue is also involved into some redox regulated metabolic pathways. This opportunity seems to be likely due to the fact that the retina is one of the most vascularized tissues in the body, having one of the highest oxidative metabolic rates per tissue weight, characterized by a combination of the factors favoring oxidation: high oxygen flux, light, and polyunsaturated fatty acids (for review, see ref 57).

Recent studies of recoverin with Cys38 cysteine modified by the fluorescent dye Alexa647-maleimide (molecular mass *ca* 1.3 kDa) reveal just a minor decrease of recoverin potency to inhibit rhodopsin kinase and about 2-fold increase of IC_{50} for the Ca^{2+}-dependent association of recoverin with immobilized lipids, as monitored by SPR spectroscopy.[19] Thus, oxidation of recoverin Cys38 by such a bulky reagent is accompanied with unexpectedly minor changes in functional properties of the protein. The same conclusion can be derived from SPR measurements, where recoverin immobilization on sensor chip by thiol-specific reagent does not cause profound changes in functional properties of recoverin.[20,21] Taking into account that the thiol group is located in close proximity to the rhodopsin

kinase binding site[15,58] (see Figure 5B) and to a Gly41 residue, which is the site of rotation following Ca^{2+}-binding by EF-hand 2,[11] one could expect much more prominent oxidation-induced changes in recoverin properties. At the same time, our preliminary experimental data suggest that oxidation of the recoverin thiol causes significant alteration of physicochemical properties of recoverin, which will be described in a separate paper. Nevertheless, based upon the absence of direct effect of the thiol oxidation on accepted function of recoverin, one may conclude that the Cys38, if significant for implementation of biological function of recoverin, serves to some other objectives rather than direct influence on activity of its target, as observed for GCAP-1[17] and GCAP-2.[18] Thus, most likely, a more universal function of the conserved thiol within NCS protein family exists.

The reported low level of modification of Cys38 by Alexa647 dye for E121Q (EF-hand 3) mutant of recoverin[19] is fully in accord with the present data. As shown previously, the E121Q mutant has substantially depressed calcium affinity and can be considered as a model of apo-recoverin.[12,59] As a consequence, the increased vulnerability of apo-recoverin thiol to oxidation should lower the content of free cysteine for E121Q mutant and cause proportionally inefficient labeling of Cys38 residue by Alexa647.

The Cys38 residue of recoverin could serve as a local antioxidant, intended for inactivation of harmful reactive oxygen and nitrogen species produced during photoreception. Meanwhile, in the view of millimolar concentration of the reduced form of glutathione in cytosol and presence of additional powerful antioxidants, like melatonin, stimulating antioxidant enzymes (for review, see ref 60), this function of recoverin thiol does not seem to be significant.

Among different reactive oxygen and nitrogen species existing in photoreceptor cells, nitric oxide (NO) is of special importance. It is generated in inner[61] and outer segments[62] of photoreceptors by NO synthases in a calcium-dependent manner and stimulates a soluble guanylyl cyclase, enhancing the synthesis of cyclic GMP, which in turn influences ion channel activity. This NO-cGMP cascade is well recognized as a signaling mechanism also in brain and other tissues. In retina, neuronal nitric oxide synthase-like immunoreactivity was reported for multiple cells, including rods, bipolar and ganglion cells, horizontal and amacrine cells, and Muller cells.[63] As was shown recently, recoverin undergoes light-driven translocation from rod outer segments toward the rod synaptic terminals.[38] In both principal segments of photoreceptor cell recoverin is exposed to nitric oxide, able to nitrosylate protein thiols in presence of molecular oxygen. According to one of the mechanisms of nitrosylation of protein thiol groups,[64] a target protein is a catalyst of its own nitrosylation due to oxidation of NO to highly reactive and unstable N_2O_3 form inside hydrophobic phase of the protein.[64] This mechanism exploits protein hydrophobic pockets to target NO to critical cysteines, explaining the selectivity of S-nitrosylation in regulation of protein function. The Cys38 thiol of recoverin is in close proximity to the hydrophobic pocket intended either for sequestration of myristoyl group in apo-recoverin[9] or for association with rhodopsin kinase.[15] This hydrophobic cavity could serve as catalytic media for oxidation of NO radical, resulting in nitrosylation of Cys38.

Apparently, further studies are required to ascertain possible targets of recoverin thiol group within photoreceptor cells, which could possibly enrich our understanding of the role of oxygen- and nitrogen-reactive species in processes occurring in retinal photoreceptors.

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