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Recoverin Is a Zinc-Binding Protein

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Recoverin is an N-myristoylated 23 kDa calcium-binding protein from retina, which modulates the Ca^{2+}-sensitive deactivation of rhodopsin via Ca^{2+}-dependent inhibition of rhodopsin kinase. It was shown by intrinsic and bis-ANS probe fluorescence, circular dichroism, and differential scanning calorimetry that myristoylated recombinant recoverin interacts specifically with zinc ions. Similar to the calcium binding, the binding of zinc to Ca^{2+}-loaded recoverin additionally increases its α-helical content, hydrophobic surface area, and environmental mobility/polarity of its tryptophan residues. In contrast to the calcium binding, the binding of zinc decreases thermal stability of the Ca^{2+}-loaded protein. Zn^{2+}-titration of recoverin, traced by bis-ANS fluorescence, reveals binding of a single Zn^{2+} ion per protein molecule. It was shown that the double-mutant E85Q/E121Q with inactivated Ca^{2+}-binding EF-hands 2 and 3 (Alekseev, A. M.; Shulga-Morskoy, S. V.; Zinchenko, D. V.; Shulga-Morskaya, S. A.; Suchkov, D. V.; Vaganova, S. A.; Senin, I. I.; Zargarov, A. A.; Lipkin, V. M.; Akhtar, M.; Philippov, P. P. *FEBS Lett.* **1998**, *440*, 116−118), which can be considered as an analogue of the apo-protein, binds Zn^{2+} ion as well. Apparent zinc equilibrium binding constants evaluated from spectrofluorimetric Zn^{2+}-titrations of the protein are 1.4×10^5 M^{-1} (dissociation constant 7.1 μM) for Ca^{2+}-loaded wild-type recoverin and 3.3×10^4 M^{-1} (dissociation constant 30 μM) for the E85Q/E121Q mutant (analogue of apo-recoverin). Study of the binding of wild-type recoverin to ROS membranes showed a zinc-dependent increase of its affinity for the membranes, without regard to calcium content, suggesting further solvation of a protein myristoyl group upon Zn^{2+} binding. Possible implications of these findings to the functioning of recoverin are discussed.

Keywords: recoverin • zinc-binding sites • site-directed mutagenesis • thermal stability • structure

The significance of zinc ions in various biological processes is well documented. In some cases, zinc is important as a structure-supporting agent, stabilizing protein domains such as zinc fingers, RING fingers, and B-boxes. Zinc finger proteins are known to take part in DNA recognition, RNA packaging, transcriptional activation, regulation of apoptosis, protein folding and assembly, and lipid binding (for a review, see ref 1), while RING fingers and B-boxes mediate protein−protein interactions.[2,3] Zinc may also be directly involved in the catalytic activity of enzymes (see, for example, ref 4). Sometimes, zinc ion is neither strictly structural nor catalytic, as in aminoacyl-tRNA synthetases, where zinc is involved in amino acid discrimination.[5,6]

The presence of high amounts of zinc in retina has been known for decades, implying an essential role of the cation in this tissue.[7] Nevertheless, the first studies elucidating the role of zinc in the phototransduction process were made quite recently.[8] It was shown that the localization of histochemically reactive zinc in rat photoreceptors varies during light and dark adaptation. We propose that this effect may be attributed to the light-stimulated translocation of zinc from the photoreceptor perikarya to its inner and outer segments followed by binding to specific molecules in the outer segment. This idea is supported by in vitro studies demonstrating the ability of rhodopsin[9] and cyclic guanosine monophosphate (cGMP)

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phosphodiesterase[10,11] to bind zinc ions. In both cases, zinc binding affected the functional properties of the target (see also refs 12 and 13), suggesting a regulatory role of zinc ions in photoreceptor cells. Therefore, knowledge of other regulatory proteins, which are able to bind zinc in photoreceptor outer segments, could enrich our understanding of zinc-mediated regulation in vision. In the present work, we investigated zinc-binding properties of recoverin, a key regulatory protein in rod outer segments.

Recoverin is an N-myristoylated 23 kDa calcium-binding protein that participates in visual signal transduction.[14−16] The protein modulates the Ca^{2+}-sensitive deactivation of rhodopsin via Ca^{2+}-dependent inhibition of rhodopsin kinase.[17,18] The myristoylation of the amino terminus of recoverin imposes cooperativity on its inhibitory efficiency with respect to rhodopsin kinase.[19] It is also essential for the binding of recoverin to membranes via the Ca^{2+}-dependent solvation of its myristoyl group.[20,21] The X-ray crystal structure of recoverin[22] shows that the protein molecule is composed of two domains, each containing two EF-hand type motifs (helix-Ca^{2+}-binding loop-helix). Potential Ca^{2+}-binding sites are distributed relatively uniformly within the recoverin sequence and include regions 36−48 (EF1), 73−85 (EF2), 109−121 (EF3), and 159−170 (EF4). Interestingly, of the four potential Ca^{2+}-binding sites, only two EF-hands (the second and the third) are capable of binding Ca^{2+}, while the remaining two sites (the first and the fourth) possess structural features that prevent them from binding calcium.[22] Our previous study showed that the binding of calcium ions to recoverin is a sequential process with the EF3-hand being filled first.[23]

In the present work, we demonstrate that recoverin exhibits specific zinc binding at a site distinct from the calcium-binding sites.

Materials and Methods

Materials. Recombinant myristoylated wild-type recoverin and its mutants with substitutions in the second (E85Q), third (E121Q), and fourth (G160D, K161E, K162N, D165G, and K166Q) EF-hands were produced in *E. coli* and purified as previously described.[19,23,24] Protein concentration was measured spectrophotometrically using a molar extinction coefficient of $\epsilon_{280nm} = 24\,075\ M^{-1}\ cm^{-1}$, calculated according to ref 25. The fluorescent probe bis-ANS (4,4′-bis[1-(phenylamino)-8-naphthalenesulfonic acid]) was purchased from Molecular Probes (Eugene, OR). The dye concentration was estimated from optical absorption using $\epsilon_{385nm} = 16\,790\ M^{-1}\ cm^{-1}$.[26]

Methods. Circular dichroism measurements were carried out with an AVIV 60DS spectropolarimeter (Lakewood, NJ) equipped with a temperature-controlled cell holder. Typical scans were measured at the rate of 5 nm/min, time constant 8 s, with cell path lengths of 0.1 and 10.0 mm for far- and near-UV CD measurements, respectively. The protein concentration was 0.75 mg/mL (33 μM) in 10 mM HEPES−KOH buffer, pH 8.0. The CD spectrum of the buffer was subtracted from the protein spectra. The molar ellipticity of sample, [θ], was calculated as the CD signal × MW (Da)/[recoverin concentration (mg/mL) × number of its residues × cell path length (mm)].

Fluorescence measurements were carried out on a computerized laboratory-built spectrofluorimeter described previously.[27] Excitation wavelength was 280.4 or 365.2 nm for protein and bis-ANS fluorescence, respectively. Protein concentrations were 3 μM or lower in 10 mM HEPES−KOH buffer, pH 8.0. All spectra were corrected for spectral sensitivity of the instrument and fitted to log-normal curves.[28] The positions of fluorescence spectrum maxima, λ_{max}, were obtained from the fits.

The apparent zinc-binding constants of recoverin, K_a, were evaluated from a fit of either the protein fluorescence or the bis-ANS probe fluorescence titration data to the simplest one-site binding scheme. The optimal binding scheme was chosen on the "simplest best fit" basis taking into consideration fluorescence phase plots:[29]

$$\text{protein} + Zn^{2+} \overset{K_a}{\Longleftrightarrow} \text{protein}\cdot Zn^{2+} \quad (1)$$

The fit was achieved by variation of the K_a value, using nonlinear regression analysis.[30] The quality of the fit was judged by the randomness of residual distribution resulting in accuracy of the zinc-binding constants about $\pm^1/_4$ order of their magnitudes.

Scanning calorimetry measurements were carried out on a DASM-4M differential scanning microcalorimeter (Institute for Biological Instrumentation of the Russian Academy of Sciences, Pushchino, Russia) at a 0.9 K/min heating rate with 2.2 mg/mL protein concentration in 20 mM H_3BO_3−KOH buffer, pH 8.2. Excess pressure of 3 atm was maintained to prevent solution boiling at temperatures above 100 °C and possible degassing of the solution on heating. Protein-specific heat capacity was calculated as described in ref 31. Partial molar volume of the protein was calculated according to ref 32. Temperature dependencies of protein specific heat capacity (C_p) were fitted to a simple two-state scheme, assuming that the difference between heat capacities of denatured and native protein states (V) is independent of temperature:

$$C_p = C_{p,n} + \{V + (P + VT)^2/\{[1 + \exp(s)]RT^2\}\}\exp(s)/[1 + \exp(s)], \quad (2)$$

$$s = \{V \ln(T/T_0) - P[(1/T) - (1/T_0)]\}/R$$

Here, $C_{p,n}$ is a specific heat capacity of native protein state, linearly extrapolated into the transition region. Fitting parameters V, T_0 (mid-transition temperature), and P were estimated using the Marquardt algorithm.[30] The enthalpies of protein denaturation were calculated as ($P + VT_0$).

Preparation of ROS was performed from fresh bovine retinae as previously described.*18* Urea-washed ROS membranes were prepared by homogenization of ROS in 5 M urea in 20 mM Tris−HCl, pH 7.5, in the dark. Then ROS were incubated on ice for 5 min and centrifuged at 100000*g* for 40 min at 4 °C. The pellet was resuspended, washed three times with buffer A (20 mM Tris−HCl, pH 7.5, 2 mM $MgCl_2$, 0.1 mM EDTA, 1 mM DTT, 1 mM PMSF), and stored at −80 °C.

Quantitative determination of recoverin bound to ROS membranes was performed using equilibrium centrifugation according to ref 24 Briefly, 30 μM recoverin was mixed with bleached urea-washed ROS membranes (50 μM rhodopsin) and incubated at 37 °C upon mixing (1000 rpm) for 15 min in 20 mM HEPES (pH 7.5), 150 mM NaCl, 20 mM $MgCl_2$, 1 mM DTT, or 2 mM EGTA, 2 mM $CaCl_2$, 2 mM $ZnCl_2$, or 2 mM $CaCl_2$ and 2 mM $ZnCl_2$ (total volume 100 μL). Membranes were separated by centrifugation (15 min, 14 000 rpm), and the supernatant was removed. The pellet was analyzed using SDS−PAGE electrophoresis.

Results and Discussion

Fluorescent Analysis of Zinc Binding to Native Recoverin. To study the Zn^{2+}-binding ability of myristoylated recombinant

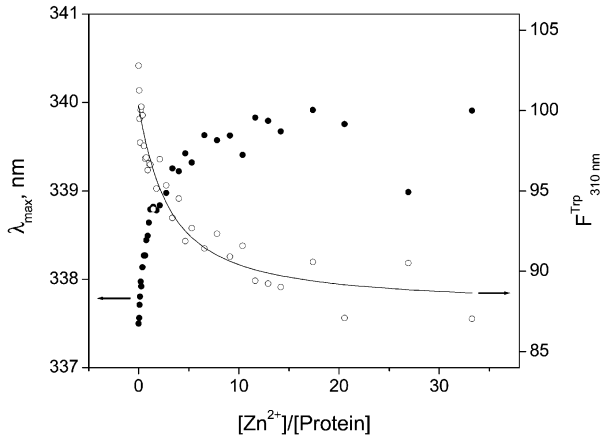

Figure 1. Spectrofluorimetric $ZnCl_2$ titration of recombinant recoverin of wild type (3.0 μM) in the presence of $CaCl_2$ (5.4 μM) at 20 °C (pH 8.0; 10 mM HEPES−KOH), monitored by intrinsic tryptophan fluorescence excited at 280.4 nm. Closed circles: fluorescence spectrum maximum position, λ_{max}, nm. Open circles: tryptophan fluorescence intensity at 310 nm, F^{Trp}_{310}, arbitrary units. Points are experimental; curve is theoretically fitted to the experimental points according to a single site binding scheme (eq 1).

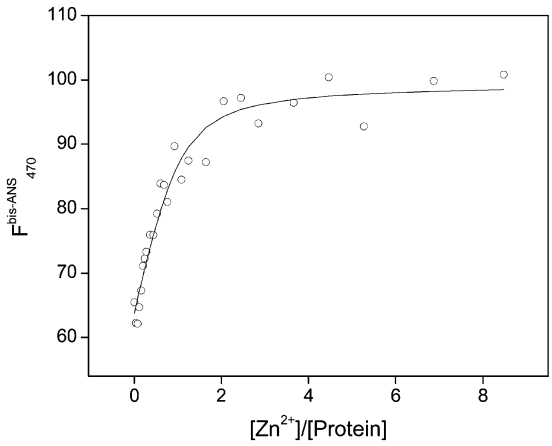

Figure 2. Spectrofluorimetric $ZnCl_2$ titration of recombinant wild-type recoverin (2.4 μM) in the presence of $CaCl_2$ (5.4 μM) at 20 °C (pH 8.0; 10 mM HEPES−KOH), monitored by bis-ANS probe (0.058 μM) fluorescence at 470 nm, $F^{bis-ANS}_{470}$, arbitrary units. Points are experimental; curve is theoretically fitted to the experimental points according to the single site binding scheme (eq 1). Fluorescence was excited at 365.0 nm.

WT recoverin, its calcium-loaded form was subjected to spectrofluorimetric Zn^{2+}-titration (Figure 1). In our previous study,[2] it has been shown that the thermal unfolding of recoverin occurs at temperatures above 50 °C at any calcium content. Besides, we found no spectral changes for recoverin in the pH range from 7.5 to 8.5. For this reason, the titration was carried out at 20 °C and pH 8.0, far from thermal and pH transitions of the protein. Zinc-induced spectral changes include 2.5 nm red shift of the maximum of tryptophan fluorescence spectrum, indicative of an increase in mobility/polarity of the environment surrounding tryptophan residues (for example, see ref 33) and decreases in fluorescence intensity at 310 nm and relative fluorescence quantum yield (area under the spectrum, not shown). Thus, the binding of zinc ion(s) to recoverin is accompanied by spectral changes similar to those observed for the binding of calcium ions to this protein, except the difference in fluorescence quantum yield behavior.[23,24] It should be noted that the calcium-induced red shift of the tryptophan fluorescence spectra is highly unusual, since normally the binding of calcium ions stabilizes the rigid structure of a protein.[35] Analysis of the 3D structures of apo- and calcium-loaded states of recoverin[36,37] shows that the effect may be due to an ion-induced increase in the degree of solvation of all tryptophan residues of recoverin (Trp31, Trp104, and Trp156).

The titration data presented in Figure 1 were also used to determine the apparent zinc-binding constant of recoverin according to the simplest one-site binding scheme (eq 1). The best fit was achieved when the apparent equilibrium binding constant, K_a, equaled 1.4×10^5 M^{-1} (dissociation constant, K_d = 7.1 μM). This value is close to the zinc-binding constant of cGMP phosphodiesterase (PDE) ($K_a = 2 \times 10^6$ M^{-1}, K_d = 0.5 μM),[10] another protein that plays a central role in vertebrate vision. Similarly, for the γ-subunit of cGMP PDE, K_a was shown to be of 1×10^6 M^{-1} (K_d = 1 μM).[40] Thus, the relatively low value of the zinc-binding constant of recoverin shows that the zinc-binding site in this protein may be composed of carboxylic groups of aspartic/glutamic acids and carbonyl groups. It is worth mentioning that it is generally accepted that calcium- and zinc-binding sites in proteins are completely different in structure (see, for example, refs 38 and 39), which prevents these ions from competition with each other for the same binding sites. Calcium binding sites are composed of oxygen atoms of carboxyl and carbonyl groups, while strong zinc-binding sites usually include histidines and cysteines (for example, see refs 1−4). While zinc-binding sites with lower affinities can be composed of carboxyl/carbonyl oxygen atoms, their geometry is different from those of the calcium binding sites.

Since zinc was shown to affect the functional properties of PDE,[10,13] the visual signal transduction cycle might be modulated by micromolar concentrations of free zinc ions. While information about free zinc concentration in photoreceptor outer segments is very restricted, data on the total zinc content in dark-adapted rat retina indicate that photoreceptor outer segments possess about 0.5 mM of zinc.[41] Presumably, the majority of this zinc is tightly bound to various cell components, as in brain.[42]

Zinc binding to WT recoverin was also monitored by emission of a fluorescent hydrophobic probe, bis-ANS. Zinc was added to the myristoylated calcium-loaded protein in the presence of a low concentration of bis-ANS (40-fold molar excess of the protein) to reduce possible perturbation of equilibrium induced by the presence of the dye. Figure 2 demonstrates that the binding of zinc to recoverin resulted in an increase of bis-ANS fluorescence intensity and quantum yield (data not shown), indicative of an increase in the hydrophobic surface area of the protein. It should be noted that the characteristic bend of the Zn^{2+}-titration curve in Figure 2, corresponding to saturation of the protein by zinc ions, takes place around Zn-to-protein ratio 1, which is evidence of the binding of a *single zinc ion per protein molecule*. The zinc-binding constant evaluated from these data is 2.2×10^6 M^{-1} (K_d = 0.45 μM), which exceeds the value obtained from the intrinsic fluorescence data (Figure 1). This discrepancy may be due to effects of equilibrium between dye-bound forms of zinc-free and zinc-loaded protein. In this case, the data suggest an increase in affinity of the bis-ANS-bound form of recoverin for zinc ions in comparison with the dye-free form.

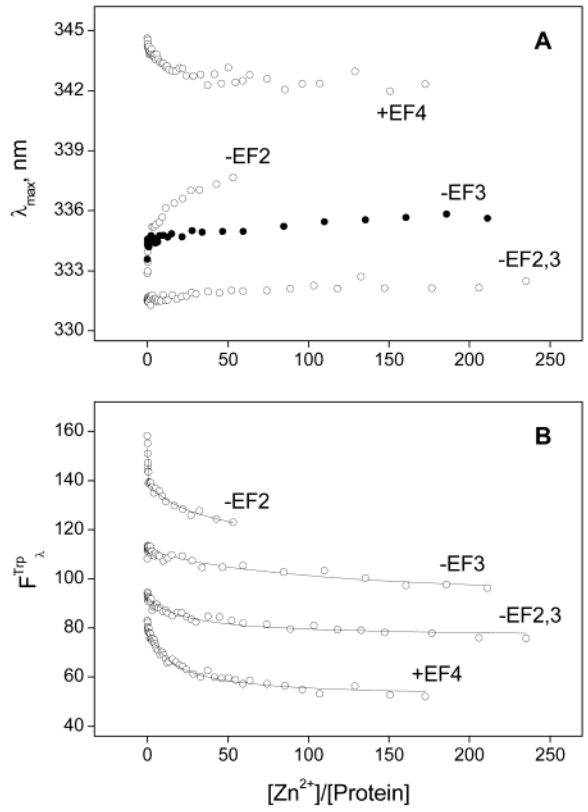

Figure 3. Spectrofluorimetric $ZnCl_2$ titration of −EF2 (1.86 μM), −EF3 (1.54 μM), −EF2,3 (1.33 μM), and +EF4 (0.89 μM) mutants of recoverin in the presence of $CaCl_2$ (5.4 μM) at 20 °C (pH 8.0; 10 mM HEPES−KOH). A: changes monitored by shift in tryptophan fluorescence spectrum maximum position, λ_{max}. B: changes monitored by modulation of fluorescence intensity at fixed wavelength, F^{Trp}_{λ}, arbitrary units. Points are experimental; curves are theoretically fitted according to the single site binding scheme (eq 1). Fluorescence was excited at 280.4 nm.

Fluorescent Analysis of Zinc Binding to Mutant Recoverin. It is usually very difficult to study the binding of zinc ions to apo-forms of calcium-binding protein because of possible artifacts due to contaminating Ca^{2+}, which is inevitably contained in the apo-protein or zinc preparations. Fortunately, this problem can be solved by the use of recoverin mutant −EF2,3, with inactivated second (substitution E85Q) and third (E121Q) calcium-binding sites.[24] Previously, we have shown[23] that these mutations did not significantly change the native structure of the protein, but eliminated strong calcium binding. For this reason, the −EF2,3 mutant can be used as an analogue of wild-type apo-recoverin.

Figure 3 presents the data on Zn^{2+}-titration of the −EF2,3 mutant of recoverin. The zinc titration data clearly showed that the −EF2,3 mutant exhibited spectral changes similar to those observed for wild-type recoverin (see Figure 1). The estimated apparent zinc binding constant was somewhat lower, $K_a = 3.3 \times 10^4$ M^{-1} ($K_d = 30$ μM), compared to WT recoverin ($K_a = 1.4 \times 10^5$ M^{-1}). Hence, calcium binding seemed to result in a minor increase of recoverin affinity for zinc ions. Similar situation was observed for either −EF2 ($K_a = 3.2 \times 10^4$ M^{-1}, $K_d = 31$ μM) or −EF3 ($K_a = 6.3 \times 10^3$ M^{-1}, $K_d = 158$ μM) mutants of recoverin, with inactivated second or third site, respectively (see Figure 3). Since the inactivation of the Ca^{2+}-binding sites of recoverin did not result in disappearance of the protein affinity for zinc, one may additionally conclude that recoverin possesses a Zn^{2+}-binding site distinct from the calcium sites.

We have shown previously[23] that the structural properties of the +EF4 mutant of recoverin containing reconstructed fourth EF-hand[24] are substantially different from properties of any other mutant studied. In this work, we found that it also bound zinc ions (Figure 3) with $K_a = 8.4 \times 10^4$ M^{-1} ($K_d = 11.9$ μM), i.e., with affinity close to that of WT protein. This binding occurred despite a severe destabilization of protein tertiary structure as a consequence of the amino acid substitutions.[23] As a result of the structure distortion and in contrast to all other forms of recoverin, the +EF4 mutant exhibited a 2.5 nm *blue* shift of tryptophan fluorescence spectrum maximum upon the binding of zinc, indicating that the environment of some tryptophan residues becomes less polar or/and less mobile (indicative of their immersion into protein interior). Since the calcium binding to recoverin, resulting in structural changes mostly in the N-terminal domain of the protein,[37] induces an increase in the protein affinity for zinc, and the structural perturbations in the C-terminal domain in +EF4 recoverin mutant do not cause any noticeable changes in protein affinity for zinc ions, the Zn^{2+}-binding site of recoverin may be located in the N-terminal domain of the protein. Although groups involved in Zn^{2+} coordination in proteins include the carboxylate groups of Asp and Glu, peptide carbonyl groups, His imidazoles and Cys thiolates (typical for structural high affinity Zn^{2+}-sites), in the case of low affinity site of recoverin it is reasonable to restrict this set to carboxyl/carbonyl groups. Examination of the NMR structural data for Ca^{2+}-bound and apo-form of recoverin[36,37] in the region of N-terminal domain reveals several clusters of grouped carboxylates: (1) Glu11, Glu14, and Glu15 (helical region); (2) Glu24, Glu25, and Glu26 (interface between helical and loop regions); (3) Glu58 and Asp60 (loop region). Purely helical region is unlikely to be suitable for effective Zn^{2+}-binding, while two other groups of residues along with nearby peptide carbonyl groups can be of potential interest for further search of recoverin zinc site. Both regions are two residues away from the residues (Leu28 and Phe56) directly involved in retention of protein myristoyl moiety to hydrophobic pocket of apo-recoverin[36,37] and potentially can affect the position of myristoyl residue upon zinc binding.

Circular Dichroism Analysis of Zinc Binding to Recoverin. Figure 4 represents the results of analysis of zinc-induced structural changes in far- and near-UV CD spectra of recombinant WT recoverin. Similar to the binding of Ca^{2+} to the apo-recoverin,[23] the binding of Zn^{2+} to calcium-saturated myristoylated WT recoverin decreased the ellipticity in the near-UV region (reflecting a decrease in the asymmetry of aromatic residues environment likely due to an increase in their accessibility to the solvent) and slightly increased ellipticity in the far-UV region (consistent with a minor increase in α-helical structure of the protein molecule). Unlike calcium binding, the zinc-induced increase in the mobility of environment surrounding aromatic residues is not limited to tryptophans, but occurred also in the environment of tyrosines and phenylalanines, as it is clearly seen from the near-UV CD spectra (Figure 4A). These data, along with the red shift of tryptophan fluorescence (Figure 1), support an increase in Trp exposure to solvent upon zinc binding to recoverin.

The −EF2,3 and +EF4 mutants of recoverin show similar zinc-induced changes in their near- and far-UV CD spectra

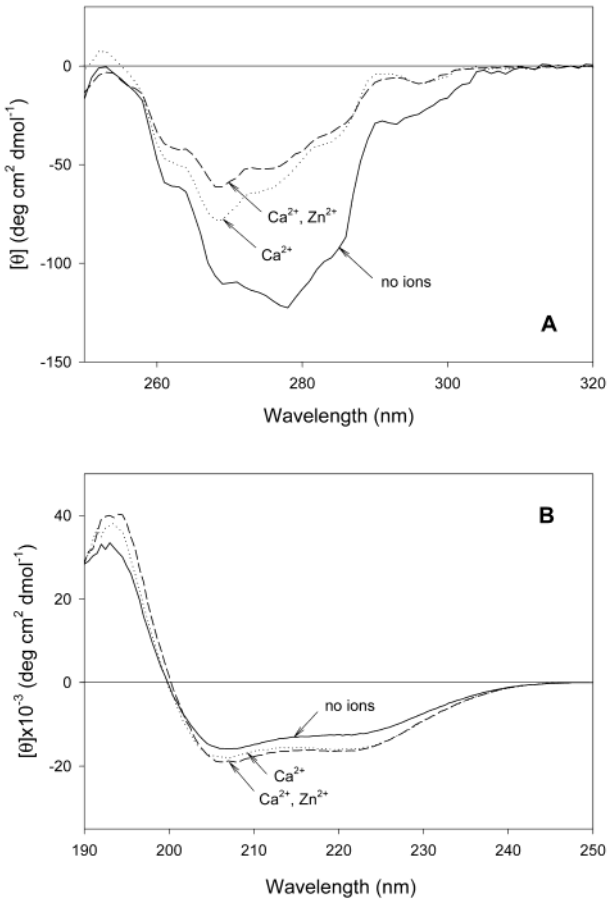

Figure 4. Effect of zinc binding on near (A) and far UV (B) circular dichroism spectra of recombinant wild-type recoverin, 20 °C (pH 8.0; 10 mM HEPES−KOH). Spectra were collected under different metal content: no ions added (solid lines), 1 mM Ca^{2+} added (dotted lines), and in the presence of both 1 mM Ca^{2+} and 0.5 mM Zn^{2+} (dashed lines). The protein concentration was 33 μM.

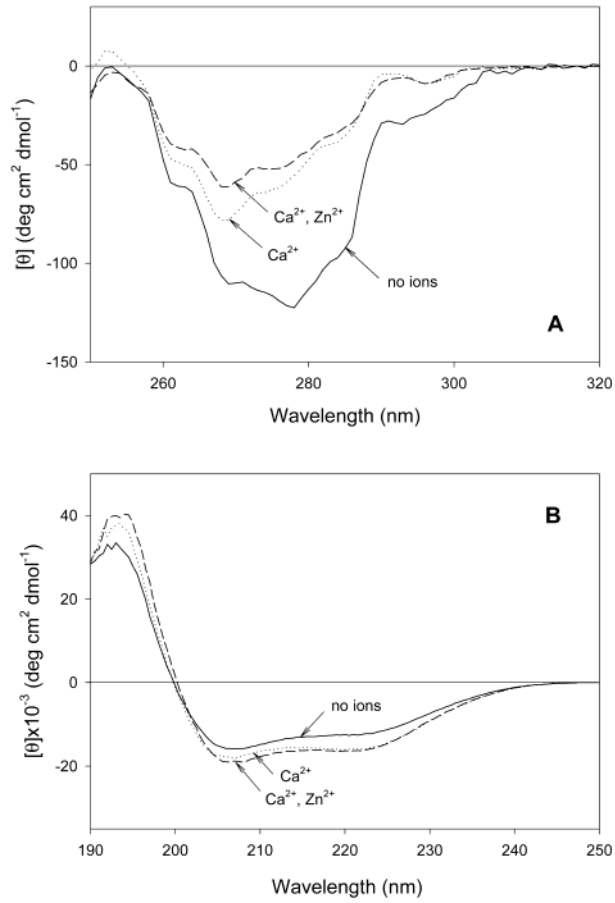

Figure 5. Effect of zinc binding on near (A) and far UV (B) circular dichroism spectra of −EF2,3 and +EF4 mutants of recoverin, 20 °C (pH 8.0; 10 mM HEPES−KOH). Spectra were collected in excess of Ca^{2+} (1 mM) or in the presence of both Ca^{2+} (1 mM) and Zn^{2+} (0.5 mM). The protein concentration was 33 μM.

(Figure 5) as wild-type recoverin, although zinc binding to +EF4 mutant did not affect protein secondary structure (Figure 5B).

Thermal Stability Studies. previously, we have shown that the binding of calcium ions to recoverin slightly increases its thermal stability.[23] In contrast to this, the binding of zinc decreased the thermal stability of Ca^{2+}-loaded WT recoverin (Figure 6): the thermal transition in the presence of zinc ions occurred at lower temperatures (by about 9 °C). Both heat sorption curves presented are satisfactorily approximated by the simple two-state transition model (eq 2), although some deviations from theoretical curve are clearly seen for the melting of recoverin in the absence of zinc. This effect may be a result of aggregation processes, which effect follows from the characteristic downward bend of the calorimetric curve at temperatures above the thermal transition. Thermodynamic parameters of thermal denaturation of recoverin estimated from calorimetric data are listed in Table 1. Thermodynamically, the fact of structure destabilization upon Zn^{2+}-binding means that Zn^{2+} ions have higher affinity for the thermally unfolded state of the protein than for the native state however this suggestion is hard to check because of serious experimental difficulties of Zn^{2+}-titration and protein aggregation at high temperatures (above 90 °C). It should be noted that the heat capacity of the Zn^{2+},Ca^{2+}-loaded protein in the pre-denaturation temperature range is higher than that for the calcium-loaded form of recoverin. This indicates an increase in solvation of hydrophobic residues upon the zinc binding, which correlates well with our spectroscopic data.

The zinc-induced exposure of hydrophobic residues could imply that zinc binding to recoverin causes an exposure of the myristoyl group to the solvent, accompanied with destruction and solvation of a hydrophobic pocket that retains the myristoyl moiety inside the apo-protein.[37]

Recoverin Binding to ROS Membranes. The exposure of the myristoyl group of recoverin to the solvent can be judged upon measurement of its propensity for binding to membranes. Figure 7 depicts results of quantitative study of effects of the zinc binding on the interaction of WT-recoverin with ROS membranes. It is clearly seen that the zinc binding to the calcium loaded protein makes its interaction with membranes even more prominent than in the presence of only calcium. Addition of zinc to calcium-saturated recoverin additionally increases the fraction of membrane-bound protein. These observations confirm the suggestion about liberation of the recoverin myristoyl group upon zinc binding.

Thus, similar to the Ca^{2+} binding, the binding of zinc ion(s) to Ca^{2+}-loaded recoverin increases α-helical content of the protein, its hydrophobic surface area, and the mobility/polarity of environment of its tryptophan residues and increases its affinity for ROS membranes. At the same time, Zn^{2+} binding to recoverin demonstrate some unique features. For example,

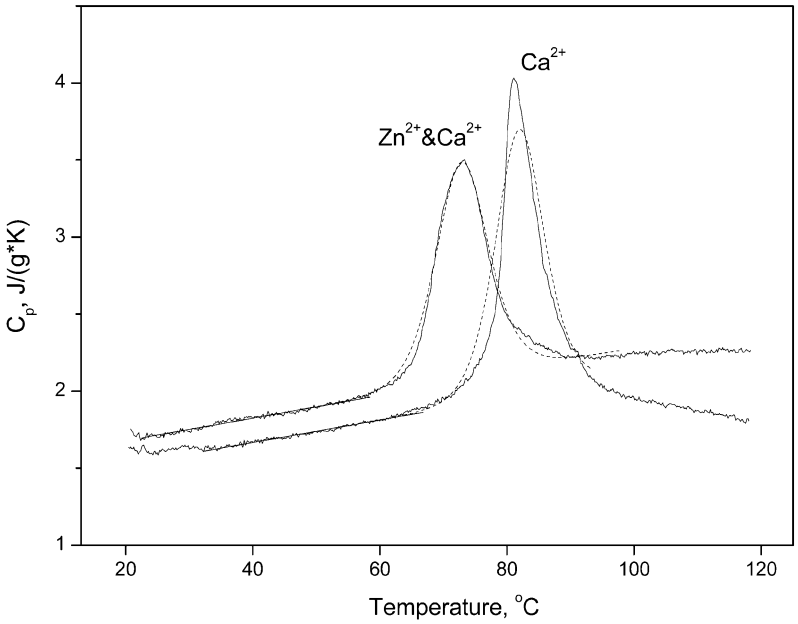

Figure 6. Scanning microcalorimetry curves for zinc-free and zinc-loaded (0.25 mM) Ca^{2+}-saturated (1 mM) recombinant wild-type recoverin (pH 8.2, 20 mM H_3BO_3−KOH). The protein concentration was 90 μM. Heating rate: 0.9 K/min. Solid lines: experimental data. Dotted lines: theoretical curves fitted to the experimental points according to the simple two-state scheme (eq 2).

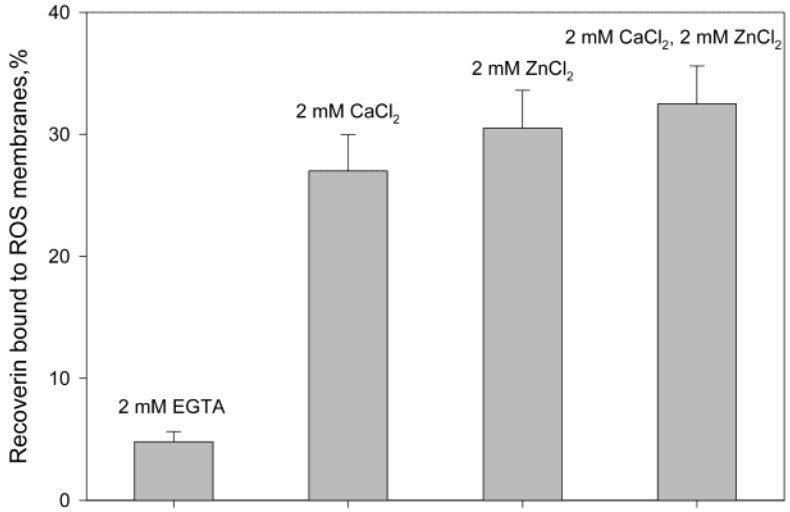

Figure 7. Ion-dependent binding of WT-recoverin to ROS membranes. Quantitative determination of WT-recoverin bound to ROS membranes after centrifugation in the presence of 2 mM EGTA, 2 mM $CaCl_2$, 2 mM $ZnCl_2$, or 2 mM $CaCl_2$ and 2 mM $ZnCl_2$ (pH 7.5). Data points were calculated on the basis of three separate evaluations.

Table 1. Thermodynamic Parameters of Thermal Denaturation of Zinc-Free and Zinc-Loaded (0.25 mM) Ca^{2+}-Saturated (1 mM) Recombinant Wild-Type Recoverin (pH 8.2, 20 mM H_3BO_3-KOH)[a]

recoverin state	$t_{1/2}$ (°C)	ΔH (kJ/mol)	ΔS (kJ/(mol·K))
Ca^{2+}-loaded	82.1	412	1.16
Zn^{2+},Ca^{2+}-loaded	73.0	363	1.05

[a] The protein concentration was 90 μM. The parameters are estimated from differential scanning calorimetry data according to the simple two-state scheme (eq 2). Key: $t_{1/2}$, mid-transition temperature; ΔH and ΔS, enthalpy and entropy of protein denaturation at mid-transition temperature.

it decreases thermal stability of the protein. The zinc-induced destabilization of protein structure is characteristic for some other calcium binding proteins, and a similar effect was observed for α-lactalbumin and parvalbumin.[43,44] Moreover, these proteins bind Zn^{2+} with similar affinities (1−10 μM) as recoverin and seem to have Zn^{2+} binding sites consisting of carboxylates of glutamic/aspartic acids.[45] Proteins, whose Zn^{2+}-binding sites are mostly composed of histidine and cysteine residues, possess much higher affinities for zinc, which usually stabilizes protein structure in this case (for example, see refs 1 and 2).

The fact that recoverin possesses relatively high affinity for zinc ions along with other regulatory vision proteins, such as rhodopsin[9] and cGMP phosphodiesterase,[10] allows us to suggest that zinc could be involved in regulation of visual signal transduction. The idea is supported by recent studies demonstrating the light-stimulated translocation of zinc from the photoreceptors perikarya to its inner and (possibly) outer segments.[41] If the zinc-binding constants for the vision proteins

mentioned lie in the range from 1 to 10 μM, one may conclude that zinc regulation of vision could be mediated by micromolar concentrations of free Zn^{2+}. More extensive studies of zinc allocation within and outside rod segments under varying light conditions along with investigation on the effects of zinc on the functional properties of its putative targets could give us a more thorough understanding of a regulatory role of zinc ions in vision processes.

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Abbreviations used: WT, wild type; −EF2, −EF3, and −EF2,3, myristoylated recoverin mutants with inactivated second (E85Q), third (E121Q), and both second and third EF-hands, respectively; +EF4, myristoylated recoverin mutant with reconstructed fourth EF-hand (G160D, K161E, K162N, D165G, and K166Q); ROS, rod outer segments; bis-ANS, 4,4′-bis[1-(phenylamino)-8-naphthalenesulfonic acid]; HEPES, *N*-[2-hydroxyethyl]piperazine-*N*′-[2-ethanesulfonic acid].

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