Critical Role of Transmembrane Segment Zinc Binding in the Structure and Function of Rhodopsin*

Zinc deficiency and retinitis pigmentosa are both important factors resulting in retinal dysfunction and night blindness. In this study, we address the critical biochemical and structural relevance of zinc ions in rhodopsin and examine whether zinc deficiency can lead to rhodopsin dysfunction. We report the identification of a high-affinity zinc coordination site within the transmembrane domain of rhodopsin, coordinated by the side chains of two highly conserved residues, Glu122 in transmembrane helix III and His211 in transmembrane helix V. We also demonstrate that this zinc coordination is critical for rhodopsin folding, 11-cis-retinal binding, and the stability of the chromophore-receptor interaction, defects of which are observed in retinitis pigmentosa. Furthermore, a cluster of retinitis pigmentosa mutations is localized within and around this zinc binding site. Based on these studies, we believe that improvement in zinc binding to rhodopsin at this site within the transmembrane domain may be a pharmacological approach for the treatment of select retinitis pigmentosa mutations. Transmembrane coordination of zinc may also be an important common principle across G-protein-coupled receptors.

Several neurodegenerative disorders, Alzheimer disease, Parkinson disease, familial amyotrophic lateral sclerosis, and the transmissible spongiform encephalopathies, share a common pathogenesis involving the misfolding and aggregation of specific proteins. Mounting evidence has demonstrated the direct binding of zinc (Zn\textsuperscript{2+}) to the β-amyloid (Alzheimer disease), α-synuclein (Parkinson disease), superoxide dismutase (amyotrophic lateral sclerosis), and prion (transmissible spongiform encephalopathies) proteins, linking either the gain or loss of Zn\textsuperscript{2+} to disease. Mounting evidence has demonstrated the direct binding of zinc (Zn\textsuperscript{2+}) to the β-amyloid (Alzheimer disease), α-synuclein (Parkinson disease), superoxide dismutase (amyotrophic lateral sclerosis), and prion (transmissible spongiform encephalopathies) proteins, linking either the gain or loss of Zn\textsuperscript{2+} to disease.

Published, JBC Papers in Press, June 12, 2004, DOI 10.1074/jbc.M403821200

* This work was supported in part by grants from the Department of Pharmacology & Toxicology and Medicine (Section of Cardiology), Dartmouth Medical School, Hanover, New Hampshire 03755

‡ To whom correspondence should be addressed: Dept. of Pharmacology & Toxicology, 7650 Remsen, Dartmouth Medical School, Hanover, NH 03755. Tel.: 603-650-1813; Fax: 603-650-1129; E-mail: John.Hwa@Dartmouth.edu.

This paper is available online at http://www.jbc.org
oresent detection of Zn$^{2+}$ and site-directed mutagenesis, we confirm that Zn$^{2+}$ is bound to rhodopsin and that the coordination site formed by residues Glu$^{195}$ and His$^{311}$ is a high-affinity zinc coordination site. In contrast to a low-affinity, solvent-accessible Zn$^{2+}$ coordination site that destabilizes rhodopsin, the coordination of Zn$^{2+}$ within the transmembrane domain is critical for dark state rhodopsin stability, 11-cis-retinal binding, and may play a vital role in transition to the active metarhodopsin II state.

EXPERIMENTAL PROCEDURES

Preparation of Rhodopsin Protein Samples—Amino acid substitutions were introduced into the synthetic rhodopsin gene through PCR mutagenesis, as described previously (25). COS-1 cells were transiently transfected with pMT4 vectors carrying the opsin genes. Cells were harvested and opsins regenerated with 11-cis-retinal and rhodopsin proteins purified by immunoaffinity chromatography. In experiments where rhodopsin was treated with EDTA (Sigma), CaCl$_2$ (Sigma), CoCl$_2$ (Sigma), or ZnCl$_2$ (Fisher Scientific) or examined under various pH values, the respective phosphate-buffered saline buffer, with those changes, was prepared and utilized throughout the protein purification process. ROS (rod outer segments) from bovine retinas was a kind gift from Dr. Philip Reeves (M.I.T.). The presence of rhodopsin protein, both from COS-1 cells and from ROS, was determined through UV-visible spectroscopy (500 nm absorbance) and Western blot analysis (25).

UV-visible Absorption Spectroscopy—UV-visible absorption spectra were recorded on a PerkinElmer Life Sciences A-40 UV-visible spectrophotometer at 2 or 25 °C (25). Rhodopsin thermal stability was determined by monitoring the decay of the 500 nm absorbance at a constant temperature of 50 °C. Protein samples were allowed to equilibrate (2–5 min) to 50 °C prior to acquiring absorption spectra. Absorption spectra were obtained at 5-min intervals, until complete disappearance of the 500 nm absorbance. Initial values of the 500 nm absorbance were normalized between experiments to account for base-line shifts and protein concentration.

Fluorescent Zn$^{2+}$ Detection—Purified rhodopsin protein or rhodopsin from ROS was incubated with a 1 μM concentration of the fluorescent Zn$^{2+}$ indicator, FluoZin$^{TM}$-3 (Molecular Probes). Samples were excited in a CytoFluo II spectrophotometer (Applied Biosystems) at a wavelength of 485 nm (bandwidth of 20 nm), and the emission was measured at a wavelength of 508 nm (bandwidth of 20 nm). This was within the active range of FluoZin-3 (optimal excitation at 494 nm and emission at 515 nm). A standard curve of Zn$^{2+}$ binding to FluoZin-3 was determined by the addition of 10-fold differing concentrations of Zn$^{2+}$ to FluoZin-3 (six Zn$^{2+}$ concentrations from 1.0 × 10$^{-6}$ M to 1.0 × 10$^{-5}$ M; n = 6). To exclude fluorescence due to binding of alternate cations, we measured fluorescence of FluoZin-3 upon the addition of Ca$^{2+}$ (CaCl$_2$). To determine background Zn$^{2+}$, the same concentration of FluoZin-3 was incubated with each solution in which rhodopsin was solubilized and purified. Rhodopsin was incubated with FluoZin-3 and assayed for fluorescence in either the dark or activated state. Three fluorescence scans were obtained and averaged for each cycle. For photoactivation, protein samples were illuminated for 30 s with a 150-watt fiber optic light. These samples were then returned to the dark for the remainder of the experiment.

ICP-MS—Purified rhodopsin protein was dissolved in a HNO$_3$ solution and digested in an MARS-5 microwave (300 watts for 10 min). ICP-MS analyses were performed on an Agilent 7500s ICP-MS equipped with an Agilent High Solids nebulizer. The solution was diluted 4- and 10-fold with deionized water and analyzed for zinc. Three isotopes of zinc ($^{65}$Zn, $^{67}$Zn, and $^{68}$Zn) were averaged for measurements and calculations. To check for contamination of the digestion procedure and sample manipulation, a blank solution was prepared and carried through each set of analyses. Gallium was used as internal standard for the determination of zinc.

Computer Modeling—The rhodopsin crystal structure (PDB ID: 1L9H) (24) was visualized with the Swiss PDB Viewer computer program (GlaxoSmithKline, Geneva, Switzerland) (26). Putative H-bonds were depicted in green. Zn$^{2+}$ ions were depicted as gray spheres.

Data Analysis—Values for all experiments are expressed as mean ± S.E. All means and S.E. were calculated from at least three separate experiments using separate protein preparations. K rate constants, half-lives, and FluoZin-3 Zn$^{2+}$ binding rates were acquired with non-linear regression using GraphPad Prism® software. A 95% confidence interval was used for all curve-fitting procedures using GraphPad Prism® software. A 95% confidence interval was used for all curve-fitting procedures using GraphPad Prism® software.

RESULTS

Through examination of the rhodopsin crystal structure (PDB ID: 1L9H) (24), we identified four putative Zn$^{2+}$ coordination sites in each monomer of rhodopsin (Fig. 1A, Zn$^{2+}$, Zn$^{2+}$, Zn$^{2+}$, and Zn$^{2+}$). Interestingly, while many metal binding sites occur in solvent-exposed regions, there was a predicted Zn$^{2+}$ coordination site within the TM 11-cis-retinal binding pocket of rhodopsin, 8 Å from the β-ionone ring of 11-cis-retinal (Fig. 1B). Furthermore, we had previously identified a large clustering of retinitis pigmentosa mutations in this same region (9). As the rhodopsin crystallization procedure required supraphysiological concentrations of Zn$^{2+}$ (~100 mM), it was crucial to determine which Zn$^{2+}$ coordination sites were physiologically and biochemically relevant and to determine the role played by the coordination of such Zn$^{2+}$ ions. Determination of Zn$^{2+}$ binding (ICP-MS and FluoZin-3) was followed by Zn$^{2+}$ treatment (pre- and postprotein purification), biochemical removal of the solvent exposed Zn$^{2+}$ binding sites (pH and EDTA), and mutagenic removal of the Zn$^{2+}$ from the transmembrane site. These assays in combination with the rhodopsin crystal structure confirmed a structurally important, buried, high-affinity Zn$^{2+}$ coordination site with the rhodopsin transmembrane domain and one or more low-affinity, solvent-exposed extracellular Zn$^{2+}$ coordination sites.

Rhodopsin Binds One High-affinity and One Low-affinity Zn$^{2+}$

Using ICP-MS and fluorescent chelation of Zn$^{2+}$, we confirmed that each rhodopsin molecule possessed at least one
Zn$^{2+}$ binding site. ICP-MS detected a total binding of $1.33 \pm 0.27$ Zn$^{2+}$ ions per molecule of rhodopsin (Fig. 2A). We hypothesized complete occupancy of a high-affinity site (TM) and partial occupancy of low-affinity sites (extracellular). The Zn$^{2+}$-dependent fluorophore, FluoZin-3, was used to quantitate low-affinity Zn$^{2+}$ coordination in rhodopsin. FluoZin-3, a Zn$^{2+}$ chelator, removes Zn$^{2+}$ that are loosely bound to rhodopsin or free in solution. In Fig. 2B, we show a standard curve of Zn$^{2+}$ binding to FluoZin-3. 10-Fold differences in Zn$^{2+}$ concentrations were initially used (0.1 nM to 1 mM), followed by more detailed assessment of the linear portion of the curve to correlate fluorescence (508 nm) to Zn$^{2+}$ concentration (inset, Fig. 2B). The calculated linear regression was determined to be $Y$ (A.U.) = 428.7 $X$ (log M) + 3150. This binding was shown to be specific for Zn$^{2+}$, as incubation of FluoZin-3 with Ca$^{2+}$ showed no significant fluorescence at equivalent concentrations of metal (Fig. 2B).

Wild type rhodopsin was incubated with FluoZin-3 and assayed for fluorescence in the dark or upon photoactivation. In the absence of added Zn$^{2+}$, FluoZin-3 chelated $0.21 \pm 0.05$ Zn$^{2+}$ in the dark state and $0.57 \pm 0.06$ (p < 0.001) Zn$^{2+}$ per rhodopsin in the photoactivated state (Fig. 2A). These data supported our hypothesis that one solvent exposed Zn$^{2+}$ coordination site was partially bound and solvent exposed (easily chelatable by FluoZin-3), while a second, higher affinity Zn$^{2+}$ coordination site (hypothesized to be the TM site) required photoactivation for chelation. We next examined Zn$^{2+}$ affinity in a His$^{211} \rightarrow$ Cys mutant, which was predicted to reduce the affinity of Zn$^{2+}$ at the Zn$^{2+}$ site natively coordinated by His$^{211}$ and Glu$^{122}$. FluoZin-3 chelated $0.40 \pm 0.03$ Zn$^{2+}$ in the dark state, and the total zinc (1.37 $\pm 0.12$ Zn$^{2+}$ (p < 0.001)) was readily detected in the photoactivated state (Fig. 2A). Data from the wild type rhodopsin proteins supported that the Zn$^{2+}$ coordination site within the transmembrane domain was a physiologically relevant and high-affinity site; however, it became an exposed, lower affinity site upon photoactivation of rhodopsin.
The His211 → Cys mutation reduced the affinity for Zn2+ within the transmembrane domain, allowing rapid total chelation upon photoactivation.

To validate the physiological relevance of our findings, rhodopsin from ROS was analyzed using FluorOzin-3. FluorOzin-3 chelated 0.18 ± 0.01 Zn2+ in the dark state and 0.20 ± 0.01 Zn2+ per rhodopsin in the photoactivated state. While these data were not found to be statistically significant, a small light-sensitive trend similar to that observed with rhodopsin purified from COS-1 cells was consistently observed with rhodopsin from ROS. Nevertheless, these studies were able to confirm that the low-affinity portion of Zn2+ bound to rhodopsin was less than half a Zn2+ per rhodopsin molecule in both COS-1-expressed and ROS rhodopsin. As a result of the much higher stability of rhodopsin in ROS versus the dodecyl maltoside system used for rhodopsin expressed in COS-1 cells, the Zn2+ ion in the TM domain may be less accessible and detectable by FluorOzin-3 upon light activation.

Treatment of Rhodopsin with ZnCl2 Reveals One Stabilizing and One Destabilizing Zn2+ Coordination Site

Previous studies have used high concentrations of Zn2+ after rhodopsin purification thus accessing only the solvent accessible site. The TM domain is buried in a highly hydrophobic region and only exposed either during protein folding or upon photoactivation, as observed with FluorOzin-3 chelatation. To further support our hypothesis that the Zn2+3 site and coordination of Zn2+ within the transmembrane domain is physiologically relevant, we both pretreated cells immediately following transfection (to promote access to the Zn2+3 coordination site during protein folding and processing) in addition to the conventionally used posttreatment procedure (after rhodopsin purification). Control samples were left untreated. UV-visible absorption spectra, obtained from the three rhodopsin populations (untreated, pretreated (50 μM ZnCl2 added to COS-1 cells), or posttreated (50 μM ZnCl2 added to purified protein)) show identical formation of a 500 nm peak (not shown). Upon photoactivation, all three populations also show identical formation of a 380 nm peak (not shown). Immunoblotting of rhodopsin showed that, at concentrations as high as 50 μM ZnCl2 in cell culture, no change in the expression or glycosylation of rhodopsin was detected with 50 μM ZnCl2, pretreatment with 50 μM CuCl2, or posttreatment of purified protein, allowed Zn2+ coordination at the stabilizing site. As we initially hypothesized, only the highly hydrophobic Zn2+3 coordination site (Fig. 1A) would be inaccessible to ZnCl2 posttreatment of purified rhodopsin. Altogether, our data suggested that the Zn2+3 coordination site within the transmembrane domain was a higher affinity, protein-stabilizing site, while one of the solvent-exposed (Fig. 1A, Zn2+1, Zn2+2, and Zn2+4) coordination sites was a lower affinity, protein-destabilizing site. To further support our hypothesis we used biochemical and mutagenic techniques to remove these coordination sites.

Removal of Zn2+ from a Solvent-exposed Zn2+ Coordination Site Stabilizes the Rhodopsin Dark State

To support the presence of a low-affinity, solvent-accessible, destabilizing site, we treated purified rhodopsin with EDTA, a strong Zn2+ chelator. We detected no fluorescence of FluorOzin-3 in rhodopsin samples treated with 1 mM EDTA (pH 7.4), suggesting that EDTA chelated all Zn2+ that were solvent-accessible (not shown). Removal of Zn2+ from the solvent-accessible Zn2+ coordination site stabilized rhodopsin at 50 °C, as treatment with EDTA increased the thermal stability t50 of rhodopsin to greater than 60 min (Fig. 3B and Table I). While untreated rhodopsin began to immediately decay at 50 °C, EDTA-treated rhodopsin did not decay for the first 45 min. The protein then began to degrade at a rate constant of 0.025 ± 0.006 min−1, similar to untreated rhodopsin (Fig. 3B and Table I). As EDTA chelates other divalent cations we also examined the use of pH.

The solvent-exposed Zn2+ coordination sites, Zn2+1 and Zn2+4 each has a histidine residue as a critical coordinating ligand (Zn2+2 does not). Since protonated histidine has a much lower affinity for Zn2+, we altered solvent pH levels near the pK0 of histidine (pH 6.5) to determine whether coordination of Zn2+ occurred at one of these two sites. When we purified rhodopsin at pH 5.5 and 6.0 (equilibrium shifted toward protonated histidine), rhodopsin exhibited no detectable decay in the dark state for a period of at least 120 min (Fig. 3C and Table I). Purification of rhodopsin at pH 7.4, 8.0, and 9.0 (equilibrium shifted toward unprotonated histidine) destabilized rhodopsin in a pH-dependent manner, increasing the decay rates to 0.024 ± 0.001 min−1 (p < 0.001; Fig. 3A and Table I). Posttreatment of rhodopsin protein with 10 μM ZnCl2 increased the decay rate to 0.027 ± 0.002 min−1, although t test analysis confirmed these data as not statistically significant. Posttreatment of rhodopsin protein with 50 μM ZnCl2 markedly and significantly increased the decay rate to 0.052 ± 0.006 min−1 (p < 0.01; Fig. 3A and Table I).

To evaluate the selectivity of the predicted Zn2+ coordination site in the rhodopsin transmembrane domain, COS-1 cells were transiently transfected with wild type rhodopsin, pretreated with 10 μM CaCl2, CoCl2, CuCl2, or ZnCl2, and 11-cis-retinal binding and rhodopsin thermal stability evaluated. Similar to ZnCl2, pretreatment with CaCl2, CoCl2, CuCl2 did not alter the 11-cis-retinal binding affinity of rhodopsin (data not shown). While ZnCl2 pretreatment and coordination of Zn2+ in the TM domain improved rhodopsin thermal stability (Table I and Fig. 3B), none of the additional metals showed such an effect. These studies confirm that the metal coordination site in the TM domain of rhodopsin is selective for Zn2+. Interestingly, 10 μM CuCl2 pretreatment led to significant destabilization. This may be related to extracellular solvent-exposed binding.

These data suggested the presence of a stabilizing Zn2+, selective coordination site whose occupancy could be detected with a 10 μM ZnCl2 pretreatment and a destabilizing Zn2+ coordination site whose occupancy could be detected with a 50 μM ZnCl2 posttreatment. These findings also indicated that only ZnCl2 pretreatment, and not posttreatment of purified protein, allowed Zn2+ coordination at the stabilizing site. As we initially hypothesized, only the highly hydrophobic Zn2+3 coordination site (Fig. 1A) would be inaccessible to ZnCl2 posttreatment of purified rhodopsin. Altogether, our data suggested that the Zn2+3 coordination site within the transmembrane domain was a higher affinity, protein-stabilizing site, while one of the solvent-exposed (Fig. 1A, Zn2+1, Zn2+2, and Zn2+4) coordination sites was a lower affinity, protein-destabilizing site. To further support our hypothesis we used biochemical and mutagenic techniques to remove these coordination sites.
Mutation of the Zn$^{2+}$/H11001 Coordination Site Transmembrane Domain Residues His211, Glu122, and Trp126 Alters Spectral Properties, 11-cis-Retinal Binding, and Rhodopsin Thermal Stability

Mutagenesis studies were performed on the highly conserved residues that were predicted to form and stabilize the putative Zn$^{2+}$/H11001 coordination site within the transmembrane domain. Characterization of such mutations, utilizing a variety of experimental techniques, would provide critical information on the contribution of each residue to Zn$^{2+}$/H11001 coordination.

His211—A crystal structure-based model (Fig. 1B) suggested that His211 was the primary Zn$^{2+}$/H11001 coordinating residue. More interestingly, FluoZin-3 data of the His211$^{3}$Cys mutant indicated the important role of this residue to Zn$^{2+}$/H11001 affinity. Site-directed mutagenesis to His211$^{3}$Cys (a residue still able to coordinate Zn$^{2+}$/H11001) and His211$^{3}$Phe (a residue unable to coordinate Zn$^{2+}$/H11001) was performed. His211$^{3}$Cys and His211$^{3}$Phe mutants bind 11-cis-retinal with an absorbance ratio lower than wild type, at both 2 and 25 °C (Fig. 4A and Table II). Fig. 4A also shows a shift in the $\lambda_{\text{max}}$ from the wild type 500 to 493 nm, for both His211$^{3}$Cys and His211$^{3}$Phe. Such data suggest the destabilization and altered structure of the 11-cis-retinal binding pocket. Interestingly, the decrease in 11-cis-retinal binding was much more evident at the higher temperature (25 °C), indicating a potential role of this site in thermal stability of the native protein. Mutation to His211$^{3}$Cys and His211$^{3}$Phe also increased the thermal decay rate constant from the wild type 0.024 ± 0.001 min$^{-1}$ to 0.157 ± 0.009 min$^{-1}$ ($p < 0.01$; Fig. 4B and Table I) and 0.201 ± 0.010 min$^{-1}$ ($p < 0.01$; data not shown), respectively.

We hypothesized that each mutant had lowered the affinity for Zn$^{2+}$/H11001 and that ZnCl$_2$ pretreatment of COS-1 cells, express-
Rhodopsin Transmembrane Segment Zinc Binding

Statistical significance for treatments with zinc, EDTA, and altered pH is compared with non-treated sample of same rhodopsin population (i.e. wild type versus wild type + 1 mM EDTA). Statistical significance (for mutants at pH 7.4) is compared with wild-type rhodopsin at pH 7.4 (i.e. wild type versus Glu122 → Cys). ND, not detectable. ns, not statistically significant.

| Sample                     | $k$ rate | $t_{1/2}$ |
|----------------------------|----------|-----------|
|                            | min$^{-1}$ | min       |
| WT rhodopsin (pH 7.4)      | 0.024 ± 0.001 | 27.9 ± 0.7 |
| WT (10 μM zinc pretreatment) | 0.021 ± 0.001 ($p < 0.05$) | 33.7 ± 1.5 ($p < 0.01$) |
| WT (50 μM zinc pretreatment) | 0.030 ± 0.001 ($p < 0.001$) | 23.6 ± 0.5 ($p < 0.01$) |
| WT (10 μM zinc posttreatment) | 0.027 ± 0.002 ($p > 0.05$ (ns)) | 25.9 ± 1.9 ($p > 0.05$ (ns)) |
| WT (50 μM zinc posttreatment) | 0.052 ± 0.006 ($p < 0.01$) | 14.2 ± 2.0 ($p < 0.001$) |
| WT (1 mM EDTA$^a$)         | ND ($p < 0.001$) | ND ($p < 0.001$) |
| WT (1 mM EDTA$^b$)         | 0.025 ± 0.006 ($p > 0.05$ (ns)) | 28.5 ± 5.8 ($p > 0.05$ (ns)) |
| WT (pH 5.5)                | ND ($p < 0.001$) | ND ($p < 0.001$) |
| WT (pH 8.0)                | 0.040 ± 0.001 ($p < 0.001$) | 17.4 ± 0.4 ($p < 0.001$) |
| WT (pH 9.0)                | 0.049 ± 0.002 ($p < 0.001$) | 13.7 ± 0.5 ($p < 0.001$) |
| Glu122 → Cys (Cys122, pH 7.4) | 0.041 ± 0.002 ($p < 0.01$) | 17.0 ± 0.8 ($p < 0.001$) |
| Cys122 (10 μM zinc pretreatment) | 0.029 ± 0.003 ($p < 0.05$) | 24.2 ± 2.0 ($p < 0.01$) |
| Cys122 (50 μM zinc pretreatment) | 0.049 ± 0.006 ($p > 0.05$ (ns)) | 14.6 ± 1.9 ($p > 0.05$ (ns)) |
| Cys122 (50 μM zinc posttreatment) | 0.046 ± 0.005 ($p > 0.05$ (ns)) | 15.3 ± 1.6 ($p > 0.05$ (ns)) |
| His211 → Cys (Cys121, pH 7.4) | 0.157 ± 0.009 ($p < 0.01$) | 4.6 ± 0.5 ($p < 0.001$) |
| Cys121 (10 μM zinc pretreatment) | 0.109 ± 0.017 ($p > 0.05$ (ns)) | 6.5 ± 1.0 ($p > 0.05$ (ns)) |
| Cys121 (50 μM zinc posttreatment) | 0.144 ± 0.005 ($p > 0.05$ (ns)) | 4.8 ± 0.2 ($p > 0.05$ (ns)) |
| Cys121 (50 μM zinc posttreatment) | 0.146 ± 0.017 ($p > 0.05$ (ns)) | 4.8 ± 0.6 ($p > 0.05$ (ns)) |

$^a$ For first 45 min, EDTA-treated WT rhodopsin did not decay.
$^b$ Beginning with the 45-min time point, EDTA-treated WT began to decay at this rate.

Modeling of the Zn$^{2+}$ Coordination Site in the Rhodopsin Transmembrane Domain

His$^{211}$ and Glu$^{122}$ are necessary for the full affinity of this Zn$^{2+}$ coordination site. The thermal stability of His$^{211}$ → Cys and Glu$^{122}$ → Cys could be rescued by pretreating with 10 μM Zn$^{2+}$ (although the results for the more severe His$^{211}$ → Cys had not reached statistical significance). Combined, these findings supported our FluoZn-3 and ICP-MS data showing a decrease in affinity for Zn$^{2+}$ with His$^{211}$ → Cys. Molecular modeling of the Zn$^{2+}$ coordination site (PDB ID: 1L9H) showed the structural basis for our results (Fig. 5). Computations performed by the Swiss PDB Viewer computer program proposed that mutation of Glu$^{122}$ → Cys would result in a Cys$^{122}$ coordination that could not interact with Zn$^{2+}$ (Fig. 5B). Such a conformation would account for the reduced Zn$^{2+}$ affinity and reduced thermal stability that we observed with this mutant. However, an alternate conformation of Cys$^{122}$ could coordinate Zn$^{2+}$ more tightly (Fig. 5C). Our data suggest that, through the addition of exogenous Zn$^{2+}$, we favored the formation of this alternate and more stable Cys$^{122}$ coordination (Fig. 5C). Improvement in Zn$^{2+}$ coordination correlated well with an increased thermal stability for this mutant. Pretreatment of Glu$^{122}$ → Leu did not show an improvement in thermal stability, as observed with the Glu$^{122}$ → Cys mutant. This was expected, since no conformation of leucine could participate in improved Zn$^{2+}$ coordination.

DISCUSSION

Zinc deficiency leads to night blindness, as do retinitis pigmentosa mutations. The role of Zn$^{2+}$ ions in the structure and...
function of rhodopsin is unclear with most studies, including the crystallization of rhodopsin utilizing supraphysiological doses. As we observed in this study, receptor destabilization through the addition of high Zn\(^{2+}\)/H11001 concentrations arises from binding to low-affinity, solvent-accessible, and perhaps non-physiologically relevant sites. More importantly, we now confirm that a Zn\(^{2+}\)/H11001 coordination site in the transmembrane domain is selective for Zn\(^{2+}\)/H11001 and plays a critical physiological role in rhodopsin stabilization in the dark. We also hypothesize that this site may also serve a role in Meta I-Meta II active state transition. Based upon these findings, caution is required in interpreting studies using engineered Zn\(^{2+}\)/H11001 coordination sites (and supraphysiological Zn\(^{2+}\) concentrations) as significant destabilizing effects may result from binding to native Zn\(^{2+}\) sites.

**Glu122 and His211 Form a Zn\(^{2+}\)/H11001 Coordination Site within the Transmembrane Domain**—Formation of the Zn\(^{2+}\)/H11001 coordination site is critical for rhodopsin dark state stability. Loss of Glu122, Trp126, or His211 lowers the affinity of rhodopsin for Zn\(^{2+}\)/H11001, resulting in a destabilized 11-cis-retinal binding pocket, altered spectral properties of the chromophore, and diminished retinal binding (Fig. 4 and Table II). His211 is obligatory to the formation of this Zn\(^{2+}\)/H11001 coordination site, as any mutation of His211 results in a severely unstable protein. Previous studies have suggested the presence of such a zinc coordination site in the transmembrane domain. Fourier transform infrared studies on rhodopsin showed that the frequency of the C=O stretch of Glu122 was very low, indicating strong hydrogen bonding in the ground state (27). The most recent 2.6-Å rhodopsin crystal
These studies support our model that Glu 122 and His 211 form a coordination site, as demonstrated by the Zn²⁺ (PDB ID: 1CA1) displayed a resemblance to the geometry as observed in the Zn²⁺ coordinated Zn in complex of rhodopsin (27, 29, 30). Thus, structural changes in the region near Glu 122 and His 211 are required for proper repositioning of Zn²⁺. Based on our studies, upon photoactivation Gly 121 is displaced displaying a Zn²⁺ interaction between 11-cis retinal and Gly 121 in rhodopsin (11, 12). An early study using atomic absorption spectroscopy for rhodopsin and rhodopsin mutant proteins concluded that the high degree of conservation. Glu 122, Trp 126, and His 211 are 100% conserved in vertebrate rhodopsins. Interestingly, His 211 is 100% conserved in vertebrate rhodopsins, and Cys 211 is 100% conserved in vertebrate cone pigments. The conserved nature of this site raises the possibility that the Zn²⁺-mediated Meta II formation may be an acquired feature that accounts for the 10-fold difference in light sensitivity between rod and cone pigments (40). The fact that Zn²⁺ deficiency results in abnormal dark adaptation and night blindness in humans and other animals (41) further supports the critical role of this site in rhodopsin structure and function.

**Rhodopsin Transmembrane Segment Zinc Binding**

| Sample | λ<sub>max</sub> | Δ<sub>399/430</sub> |
|--------|----------------|------------------|
| WT (2°C) | 500 ± 1 | 1.9 ± 0.1 |
| WT (25°C) | 500 ± 1 | 2.1 ± 0.1 |
| Glu¹²² → Cys (2°C) | 493 ± 3 | 2.2 ± 0.1 |
| Glu¹²² → Cys (25°C) | 482 ± 2 | 2.8 ± 0.1 |
| Glu¹²² → Leu (2°C) | 491 ± 1 | 2.0 ± 0.1 |
| Glu¹²² → Leu (25°C) | 492 ± 1 | 2.5 ± 0.1 |
| Trp¹²⁶ → Asp (2°C) | 502 ± 2 | 5.2 ± 0.2 |
| Trp¹²⁶ → Asp (25°C) | 484 ± 4 | 6.2 ± 0.8 |
| His¹³¹ → Cys (2°C) | 495 ± 1 | 2.1 ± 0.1 |
| His¹³¹ → Cys (25°C) | 493 ± 1 | 3.1 ± 0.3 |
| His¹³¹ → Phe (2°C) | 504 ± 3 | 2.5 ± 0.3 |
| His¹³¹ → Phe (25°C) | 493 ± 1 | 3.6 ± 0.2 |

Our data suggest that, in the ground (dark) state, the Zn²⁺ coordination site in the rhodopsin transmembrane domain is a stabilizing mechanism, retaining rhodopsin in the inactive conformation and 11-cis-retinal as an inactive agonist. Upon photoactivation, this Zn²⁺ coordination is disrupted, promoting the dissociation of TM helix III from helices IV and V and allowing the unhindered formation of photolyzed intermediates. It has been hypothesized that protonated His²¹¹ interacts electrostatically with another amino acid to drive rearrangement of TM helices (38); our studies suggest that protonated His²¹¹ loses an interaction (with Zn²⁺) to drive this helical rearrangement. Interestingly, His²¹¹ is 100% conserved in vertebrate rhodopsins, and Cys²¹¹ is 100% conserved in vertebrate cone pigments. The conserved nature of this site raises the possibility that the Zn²⁺-mediated Meta II formation may be an acquired feature that accounts for the 10-fold difference in light sensitivity between rod and cone pigments (40). The fact that Zn²⁺ deficiency results in abnormal dark adaptation and night blindness in humans and other animals (41) further supports the critical role of this site in rhodopsin structure and function.

**Night Blindness and Protein Misfolding in Retinitis Pigmentosa**—Naturally occurring mutations His²¹¹ → Pro and His²¹¹ → Arg give rise to severe opsin misfolding, leading to retinitis pigmentosa, a serious retinal degenerative disorder (reviewed in Refs. 9, 42, and 43). Interestingly one of the first manifestations of this disorder is night blindness. Moreover, a large cluster of retinitis pigmentosa mutations are located in the region near His²¹¹. We have recently shown that two of these mutations, Leu¹²⁶ → Arg and Ala¹⁶⁴ → Val, directly interfere with a Glu¹²²-Trp¹²⁶-His²¹¹ interaction (25). We now propose that the disrupted Zn²⁺ coordination site in the rhodopsin transmembrane domain directly leads to the protein misfolding and night blindness associated with these mutations. A number of studies have shown that folding of the transmembrane domain is coupled to the formation of a specific tertiary structure and the native Cys¹¹⁰-Cys¹⁸⁷ disulfide bond in the intradiscal domain (42, 44–46). Since loss of the Zn²⁺ coordination site and loss of the Cys¹¹⁰-Cys¹⁸⁷ disulfide bond promote rhodopsin unfolding, it is exciting to speculate that formation of the Zn²⁺ site could be coupled to the formation of this critical disulfide bond. Combined, our studies strongly support the importance of zinc in rhodopsin structure and function and also provide a possible molecular mechanism for night blindness and retinitis pigmentosa. Additionally, improvement in protein stability of the Glu¹²² → Cys mutant with the addition of Zn²⁺ further highlights the importance of this site and suggests a
Potential Mechanism for Neurodegeneration from Zinc Excesses and Deficiencies—Zn\(^{2+}\) promotes aggregation of the highly fibrillogenic prion peptide, PrP\(_{106–126}\) (1), and of the β-amyloid protein (2–4) into amyloidogenic aggregates, leading to cellular apoptosis and neurodegeneration. In contrast, in the mutant superoxide dismutase protein, loss of affinity for Zn\(^{2+}\) results in diminished protein activity (6), reduced protein stability (7), and formation of amyloid-like filaments (8). The mechanisms for these observations are not well understood, particularly as both the loss and gain of Zn\(^{2+}\) binding can induce neurodegeneration. We observe that both a loss of physiological Zn\(^{2+}\) binding and a gain of non-physiological Zn\(^{2+}\) binding can induce rhodopsin destabilization and misfolding through distinct mechanisms. Loss of a native high-affinity Zn\(^{2+}\) coordination site, either through Zn\(^{2+}\) deficiencies or induced by naturally occurring mutations, renders the rhodopsin protein unstable, misfolded, and eventually aggregated. Conversely, supraphysiological Zn\(^{2+}\) concentrations binding to a non-native, low-affinity Zn\(^{2+}\) coordination site in rhodopsin can also result in biochemical defects.

Selectivity of trace metals in neurodegeneration has recently gained increasing interest. Our studies confirm that the high-affinity metal coordination site is selective for Zn\(^{2+}\). Interestingly, at the solvent-accessible, low-affinity metal coordination site, recent studies suggest that high concentrations of either Zn\(^{2+}\) or Cu\(^{2+}\) can destabilize rhodopsin protein (13). This is similar to what has been observed with other proteins, where high-affinity and physiological metal coordination sites are selective, while low-affinity and non-physiological (or pathophysiological) metal coordination sites may be non-selective. High concentrations of Cu\(^{2+}\), similar to Zn\(^{2+}\), also promote aggregation of the highly fibrillogenic prion peptide, PrP\(_{106–126}\), and of the β-amyloid protein (2–4) into amyloidogenic aggregates. Our current study may help clarify the mechanisms by which a loss and/or gain of metal binding results in a wide variety of neurodegenerative diseases.

REFERENCES
1. Joljing, M. F., Huang, X., Stewart, L. R., Barnham, K. J., Curtian, C., Volitakis, I., Pergolini, M., White, A. R., Cherry, R. A., Masters, C. L., Barrow, C. J., Collins, S. J., Bush, A. I., and Cappai, R. (2001) Biochemistry 40, 8073–8084
2. Atwood, C. S., Moir, R. D., Huang, X., Scarpa, R. C., Bacarra, N. M., Romano, D. M., Hartshorn, M. A., Tanzi, R. E., and Bush, A. I. (1998) J. Biol. Chem. 273, 12817–12826
3. Huang, X., Atwood, C. S., Moir, R. D., Hartshorn, M. A., Vonsattel, J. P., Tanzi, R. E., and Bush, A. I. (1997) J. Biol. Chem. 272, 26464–26470
4. Lynch, T., Cherry, R. A., and Bush, A. I. (2000) Exp. Gerontol. 35, 445–451
5. Finefrock, A. E., Bush, A. I., and Doraiaiwamy, P. M. (2003) J. Am. Geriatr. Soc. 51, 1145–1148
6. Crow, J. P., Sampson, J. B., Zhuang, Y., Thompson, J. A., and Beckman, J. S. (1997) J. Neurochem. 69, 1936–1944
7. Rodriguez, J. A., Valentine, J. S., Eggera, D. K., Roe, J. A., Tiwari, A. Brown, R. H. Jr., and Hayward, L. J. (2002) J. Biol. Chem. 277, 15932–15937
8. Elam, J. S., Malek, K., Rodriguez, J. A., Doucette, P. A., Taylor, A. B., Hayward, L. J., Cabelli, D. E., Valentine, J. S., and Hart, P. J. (2003) J. Biol. Chem. 278, 21032–21039
9. Stejanovic, A., and Hwa, J. (2002) Receptors Channels 8, 33–50
10. Ugarte, M., and Osborne, N. N. (2001) Prog. Neurobiol. 64, 219–249
11. Shuster, T. A., Nagy, A. K., Conly, D. C., and Farber, D. B. (1992) Biochem. J. 282, 123–129
12. Tam, S. W., Wilber, K. E., and Wagner, F. W. (1978) Biochem. Biophys. Res. 

![Crystal structure-based model of the Zn\(^{2+}\) coordination site, as hypothesized in this study. Green dashed-lines represent putative hydrogen bonding.](image-url)
Rhodopsin Transmembrane Segment Zinc Binding

35941

13. del Valle, L. J., Ramon, E., Canavate, X., Dias, P., and Garriga, P. (2003) J. Biol. Chem. 278, 4719–4724
14. Schetz, J. A., and Sibley, D. R. (1997) J. Neurochem. 68, 1990–1997
15. Swaminath, G., Lee, T. W., and Kohilka, B. (2003) J. Biol. Chem. 278, 352–356
16. Swaminath, G., Steenhuis, J., Kohilka, B., and Lee, T. W. (2002) Mol. Pharmacol. 61, 65–72
17. Holst, B., and Schwartz, T. W. (2003) Ann. N. Y. Acad. Sci. 994, 1–11
18. Holst, B., Elling, C. E., and Schwartz, T. W. (2002) J. Biol. Chem. 277, 47662–47670
19. Gerlach, L. O., Jakobsen, J. S., Jensen, K. P., Rosenkilde, M. R., Skerlj, R. T., Ryde, U., Bridger, G. J., and Schwartz, T. W. (2003) Biochemistry 42, 710–717
20. Sheik, S. P., Zvyaga, T. A., Lichtarge, O., Sakmar, T. P., and Bourne, H. R. (1996) Nature 383, 347–350
21. Sheik, S. P., Vilardarga, J. P., Baranski, T. J., Lichtarge, O., Iiri, T., Meng, E. C., Nissenson, R. A., and Bourne, H. R. (1999) J. Biol. Chem. 274, 17033–17041
22. Palczewski, K., Kumasaka, T., Hori, T., Behnke, C. A., Motoshima, H., Fox, B. A., Le Trong, I., Teller, D. C., Okada, T., Stenkamp, R. E., Yamamoto, M., and Miyano, M. (2000) Science 290, 739–745
23. Teller, D. C., Okada, T., Behnke, C. A., Palczewski, K., and Stenkamp, R. E. (2001) Biochemistry 40, 7761–7772
24. Okada, T., Fujimori, Y., Sielo, M., Navarro, J., Landau, E. M., and Shichida, Y. (2002) Proc. Natl. Acad. Sci. U. S. A. 99, 5982–5987
25. Stojanovic, A., Hwang, I., Khorana, H. G., and Hwa, J. (2003) J. Biol. Chem. 278, 39020–39028
26. Guez, N., and Pecht, M. C. (1997) Electrophoresis 18, 2714–2723
27. Furutani, Y., Shichida, Y., and Kandori, H. (2003) Biochemistry 42, 9619–9625
28. Beck, M., Sakmar, T. P., and Siebert, F. (1998) Biochemistry 37, 7630–7639
29. Borhan, B., Steuto, M. L., Imai, H., Shichida, Y., and Nakanishi, K. (2000) Science 288, 2209–2212
30. Jang, G. F., Kuksa, V., Filipek, S., Bartl, F., Ritter, E., Gelh, M. H., Hofmann, K. P., and Palczewski, K. (2001) J. Biol. Chem. 276, 26148–26153
31. Han, M., Groesbeck, M., Sakmar, T. P., and Smith, S. O. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 13442–13447
32. Han, M., Groesbeck, M., Smith, S. O., and Sakmar, T. P. (1998) Biochemistry 37, 538–545
33. Han, M., Lin, S. W., Minnava, M., Smith, S. O., and Sakmar, T. P. (1996) J. Biol. Chem. 271, 32337–32342
34. Han, M., Lin, S. W., Smith, S. O., and Sakmar, T. P. (1996) J. Biol. Chem. 271, 32330–32336
35. Han, M., Lou, J., Nakanishi, K., Sakmar, T. P., and Smith, S. O. (1997) J. Biol. Chem. 272, 23081–23085
36. McCall, K. A., Huang, C., and Fierke, C. A. (2000) J. Nutr. 130, 1437S–1446S
37. Christianson, D. W., and Cox, J. D. (1999) Annu. Rev. Biochem. 68, 33–57
38. Weitz, C. J., and Nathans, J. (1992) Neuron 8, 465–472
39. Mathews, R. G., Hubbard, R., Brown, P. K., and Wald, G. (1963) J. Gen. Physiol. 47, 215–240
40. Baylor, D. A. (1987) Invest. Ophthalmol. Vis. Sci. 26, 34–49
41. Grahn, B. H., Paterson, P. G., Gottschall-Pass, K. T., and Zhang, Z. (2001) J. Am. Coll. Nutr. 20, 106–118
42. Hwa, J., Garriga, P., Liu, X., and Khorana, H. G. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 10571–10576
43. Sung, C. H., Davenport, C. M., and Nathans, J. (1991) J. Biol. Chem. 268, 26645–26649
44. Hwa, J., Klein-Seetharaman, J., and Khorana, H. G. (2001) Proc. Natl. Acad. Sci. U. S. A. 98, 4872–4876
45. Hwa, J., Reeves, P. J., Klein-Seetharaman, J., Davidson, F., and Khorana, H. G. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 1932–1935
46. Karmik, S. S., and Khorana, H. G. (1990) J. Biol. Chem. 265, 17529–17524