

Conserved Rhodopsin Intradiscal Structural Motifs Mediate Stabilization: Effects of Zinc[†]

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ABSTRACT: Retinitis pigmentosa (RP), a neurodegenerative disorder, can arise from single point mutations in rhodopsin, leading to a cascade of protein instability, misfolding, aggregation, rod cell death, retinal degeneration, and ultimately blindness. Divalent cations, such as zinc and copper, have allosteric effects on misfolded aggregates of comparable neurodegenerative disorders including Alzheimer disease, prion diseases, and ALS. We report that two structurally conserved low-affinity zinc coordination motifs, located among a cluster of RP mutations in the intradiscal loop region, mediate dose-dependent rhodopsin destabilization. Disruption of native interactions involving histidines 100 and 195, through site-directed mutagenesis or exogenous zinc coordination, results in significant loss of receptor stability. Furthermore, chelation with EDTA stabilizes the structure of both wild-type rhodopsin and the most prevalent rhodopsin RP mutation, P²³H. These interactions suggest that homeostatic regulation of trace metal concentrations in the rod outer segment of the retina may be important both physiologically and for an important cluster of RP mutations. Furthermore, with a growing awareness of allosteric zinc binding domains on a diverse range of GPCRs, such principles may apply to many other receptors and their associated diseases.

Protein misfolding and aggregation are shared etiology among many neurodegenerative disorders, including Alzheimer disease, Parkinson disease, familial amyotrophic lateral sclerosis, and transmissible spongiform encephalopathies. Over 100 distinct inherited mutations have been identified in rhodopsin, predominantly resulting in misfolding, aggregation, and cell death, followed by clinical manifestation of the retinal neurodegenerative disorder retinitis pigmentosa (RP)¹ (1). Increasing evidence has demonstrated that biologically important and ubiquitous metals, zinc (Zn²⁺) and copper (Cu²⁺), directly bind to the β -amyloid (Alzheimer disease) and prion (transmissible spongiform encephalopathies) proteins, linking the gain of non-native, low-affinity metal binding to progression of these disorders. Moreover, Zn²⁺ promotes aggregation of the highly fibrillogenic prion peptide, PrP106–126 (2) and of β -amyloid (3–5). Such findings have led to promising clinical investigation of metal protein attenuating compounds, based on clioquinol, as potential therapeutics to mitigate β -amyloid neurotoxicity (6–8).

Numerous studies have identified Zn²⁺ as an allosteric modulator of several GPCRs (9–16), including localization

of Zn²⁺ coordination sites to extracellular loops of melanocortin (13, 14), chemokine (15), olfactory (16), and tachykinin (12) receptors. These receptors are located within the central nervous system (CNS), where zinc is predominantly stored in synaptic vesicles as a modulator of neuronal excitability (17), and can be found with acute concentrations exceeding 300 μ M (18, 19), suggesting that Zn²⁺ coordination may represent a conserved mechanism regulating GPCRs in the CNS. In a recent study, we identified an essential high-affinity Zn²⁺ coordination site in the transmembrane domain of rhodopsin (20). Interestingly, we also identified a low-affinity zinc-associated component that significantly destabilized rhodopsin, an effect specific to zinc and copper (21). Molecular modeling of the rhodopsin crystal structure, in conjunction with data from our previous study (20), indicates His¹⁰⁰ and His¹⁹⁵ in the intradiscal domain of rhodopsin as primary candidates for this Zn²⁺-mediated effect.

The intradiscal domain of rhodopsin, corresponding to the extracellular domain of related GPCRs, is composed of the N-terminal region (residues 1–33), loop II–III (101–105), loop IV–V (173–198), and loop VI–VII (277–285). Mutations in this region prevent rhodopsin from folding properly *in vitro*, a property which precipitates aggregation in the endoplasmic reticulum (ER) (22, 23) and subsequent degradation by retinal pigment epithelial cells *in vivo*. The N-terminal tail plays an important role in rhodopsin folding and trafficking (24, 25), while loop IV–V (173–198) contains Cys¹⁸⁷, which forms a highly conserved disulfide bond with Cys¹¹⁰ (26, 27). This disulfide linkage is essential for proper folding, stability, and function of rhodopsin (26, 28–30). Interestingly, a cluster of RP mutations occurs in this region, many of

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¹ Abbreviations: A₅₀₀, absorbance at 500 nm; ANOVA, analysis of variance; ED₅₀, half-maximal effective dose; EDTA, ethylenediaminetetraacetic acid; GPCR, G-protein-coupled receptor; His, histidine; ICP-MS, inductively coupled plasma mass spectrometry; Lys, lysine; Phe, phenylalanine; pK_a, acid dissociation constant; Pro, proline; RP, retinitis pigmentosa; SDS, sodium dodecyl sulfate; SEM, standard error of the mean; SFM, scanning force microscopy; Tyr, tyrosine; UV–vis, ultraviolet–visible; WT, wild type; Zn, zinc.

which promote formation of alternate disulfide bonding (29). RP mutations Cys¹¹⁰Ala, Cys¹¹⁰Phe, and Cys¹¹⁰Tyr all form an alternate Cys¹⁸⁵–Cys¹⁸⁷ disulfide bond, lacking 11-*cis*-retinal binding (27, 29). Additionally, formation of an ion pair between Arg¹⁷⁷ and Asp¹⁹⁰ located at the ends of the second extracellular loop is critical for folding and stability of dark state rhodopsin (31).

In this study, our goal was to identify and characterize the low-affinity metal coordination sites mediating destabilization and determine potential influence of this phenomenon on normal and pathological RP rhodopsin. We report that two structural networks in the intradiscal domain surrounding His¹⁰⁰ and His¹⁹⁵ are susceptible to pathophysiological changes in trace metal concentrations. Trace metal chelation assisted in stabilizing the most common form of RP mutations, Pro²³His, demonstrating such modality as a possible avenue for therapy in select RP mutations.

EXPERIMENTAL PROCEDURES

Site-Directed Mutagenesis and Preparation of Rhodopsin Protein Samples. Amino acid substitutions were introduced into the synthetic rhodopsin gene through PCR mutagenesis, as previously described (32). For single mutants, a synthetic wild-type rhodopsin gene was used as the template (32). For the His¹⁰⁰Phe/His¹⁹⁵Phe double mutant, the His¹⁹⁵Phe mutant was synthesized using PCR mutagenesis, followed by mutagenesis of His¹⁰⁰Phe using His¹⁹⁵Phe as the template. COS-1 cells were transfected with pMT4 vectors carrying the opsin genes. Cells were harvested, opsins regenerated with 11-*cis*-retinal, and rhodopsin proteins purified by immunoaffinity chromatography as previously described (32). Rhodopsin concentration was determined through UV–vis spectroscopy (500 nm absorbance) (32).

UV–Visible (UV–Vis) Absorption Spectroscopy. UV–vis absorption spectra were recorded on a Perkin–Elmer λ -40 UV–vis spectrophotometer at 2°C, 10, or 25 °C (32). Rhodopsin thermal stability was determined by monitoring the decay of the 500 nm absorbance at a constant temperature of 50 °C, unless otherwise noted. Protein samples were allowed to equilibrate (approximately 2 min) to 50 °C prior to acquiring absorption spectra. Absorption spectra were obtained at 5 min intervals until the 500 nm absorbance decreased to background levels. Initial values of the 500 nm absorbance were normalized between experiments to account for baseline shifts and differences in sample protein concentrations (approximately 1.3 μ M).

Computer Modeling. The rhodopsin crystal structures (PDB ID 1L9H (33) and 1U19 (34)) were visualized with Swiss PDB Viewer (GlaxoSmithKline, Geneva, Switzerland) (35), with some images generated using WebLab Viewer Pro 3.2 (Molecular Simulations, Inc., San Diego, CA). In some models, as described, particular residues were altered from their crystallized structure to propose alternate interactions.

Data Analysis. Values for all experiments are expressed as mean \pm SEM. All means and SEM were calculated from at least three distinct experiments using separate protein preparations. Exponential decay rate constants (k) and half-lives were derived through nonlinear regression using GraphPad Prism software. A 95% confidence interval was used for all curve-fitting procedures using GraphPad Prism. Where applicable, statistical significance of the data was evaluated

using an ANOVA and/or unpaired t test (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$).

RESULTS

Wild-type rhodopsin becomes less thermostable in the presence of exogenous zinc (Figure 1A). Chelative treatment with 1 mM EDTA stabilized the wild-type rhodopsin receptor (Figure 1A). In a recent study, using inductively coupled plasma mass spectrometry (ICP-MS), we quantified 1.33 ± 0.27 Zn²⁺ ions per molecule of rhodopsin under 10 μ M treatment conditions (20). This suggested the presence of other lower affinity sites, in addition to the single high-affinity transmembrane site identified (20). Moreover, despite the use of approximately 100 mM zinc in the crystallization procedure, the presence of additional sites is supported by rhodopsin's crystal structure (33, 36, 37). To confirm that other physiologically relevant sites are present, we performed a zinc dose response (thermostability) and calculated the zinc log(EC₅₀) for the alternative site(s) to be 1.76 ± 0.08 (log[Zn²⁺], μ M), corresponding to an EC₅₀ of 57.1 μ M (Figure 1B), confirming both specificity and the low-affinity nature for these additional zinc binding sites. This is consistent with previous findings and supports the identification of greater than one zinc per rhodopsin molecule detected using ICP-MS (20). A pH profile for thermostability confirmed a pK_a of ~ 6.7 , supporting the possible involvement of His in zinc coordination (Figure 1C). Our goals were then to precisely identify the site(s), determine mechanism for their actions, and determine whether they may be relevant to retinitis pigmentosa rhodopsin mutations.

Analysis of the Rhodopsin Crystal Structure Indicates His¹⁰⁰ and His¹⁹⁵ as Potential Sites of Zn²⁺ Coordination. From the four sites depicting Zn²⁺ coordination in the crystal structure (33), combined with our previous data (20), the sites containing histidine residues His¹⁰⁰ and His¹⁹⁵ provide the only suitable candidates for Zn²⁺ coordination. The lack of a histidine, cysteine, or aspartic acid residue at one of the potential Zn²⁺ binding sites, predicted to be formed by Glu²⁰¹ and Gln²⁷⁹, suggests this site to be artifactual (Figure 2A). Based on our pH profile analysis of receptor stability and zinc destabilization, histidines are critical to the zinc response. The two remaining sites (Figure 2B,C) each contain a histidine residue (His¹⁰⁰ and His¹⁹⁵, respectively). In the rhodopsin crystal structure, one Zn²⁺ ion is located at the dimeric interface between the two rhodopsin monomers, coordinated by Tyr⁹⁶ and His¹⁰⁰ (from one monomer "a"), Lys³¹¹, and a backbone amine group from the peptide bond between Gly³²⁹ and Asp³³⁰ (from the second monomer "b"; Figure 2B). As tyrosine and lysine are inadequate for zinc coordination, attention focused on the corresponding histidine (His¹⁰⁰) in the intradiscal domain of rhodopsin. Additionally, His¹⁰⁰ is in proximity of other polar residues, allowing for alternate interactions conceivably masked by the presence of zinc. As such, both His¹⁰⁰ and His¹⁹⁵ were suitable candidates for further analysis.

We based our evaluation of the intradiscal hydrogen-bonding network on the 1L9H and 1U19 rhodopsin structures. Of the other available rhodopsin crystal structures (PDB ID 1F88, 1HZX, 1GZM, 3C9L, and 3C9M), evaluations of 1HZX and 3C9L were included in our initial assessment and deemed suitably consistent to continue with

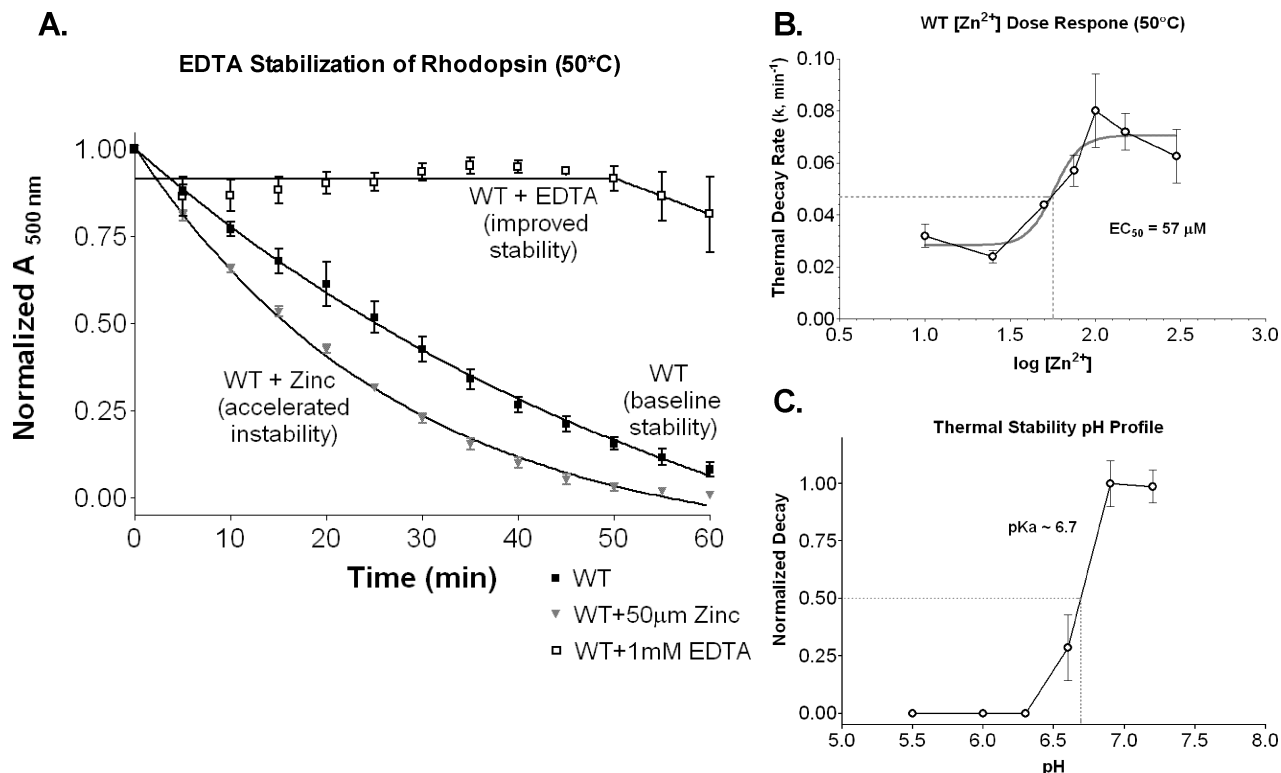


FIGURE 1: (A) Thermal decay (at 50 °C) time course for wild type (WT), wild type plus 50 μM ZnCl_2 , and wild type treated with 1 mM EDTA. (B) Dose–response relationship of zinc concentrations ($\log[\text{Zn}^{2+}]$, μM) to receptor thermal decay rate (k). Nonlinear regression determined a $\log(\text{EC}_{50})$ of 1.76 ± 0.08 , corresponding to an EC_{50} of 57.1 μM ZnCl_2 . (C) Thermal decay of wild-type rhodopsin adopts a pH profile with an apparent pK_a of 6.7, consistent with histidine involvement.

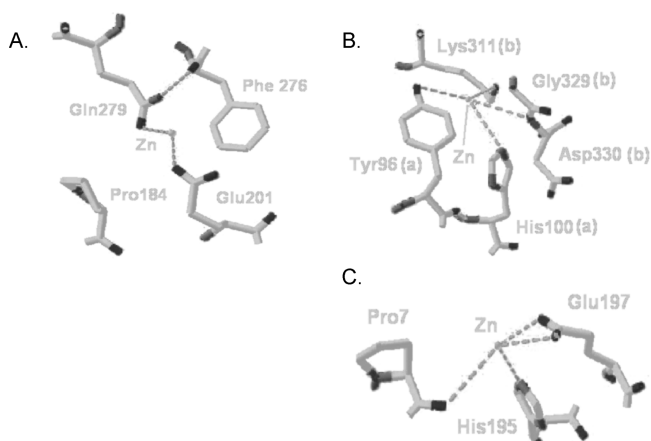


FIGURE 2: Three positions (nontransmembrane) illustrating potential zinc coordination in the rhodopsin crystal structure. (A) The lack of residues commonly implicated in zinc coordination at the Gln²⁷⁹ site suggests this position to be artifactual. (B) The interfacial zinc coordination site centered at His¹⁰⁰ bridges two inverted monomers (polypeptide chains a and b) in the crystal structure. (C) Zinc coordinated by His¹⁹⁵ connects the intradiscal loop to the N-terminal chain through Pro⁷.

our current working model. Our focus on the Okada rhodopsin structures, despite the availability of additional structures, was based on the other published structures having removed divalent cations in postprocessing, leaving manually inserted water molecules in their place. The substantial resolution of the structures by Okada et al. strengthened and supported our decision.

His¹⁰⁰ Mediates the Destabilization Conferred by Trace Metals. Of the 102 sequenced vertebrate type I opsin proteins (GPCRdb (38)), His¹⁰⁰ is highly conserved, present in 79%

of the sequences (Figure 3A), followed by asparagines (19%). Histidine and asparagine each possess a protonatable nitrogen at the δ position. Interestingly, the remaining 2% of sequences contain nitrogen at the ϵ position, suggesting complete conservation of this functional group among vertebrate type I opsins. We initially performed site-directed mutagenesis of His¹⁰⁰ to a similarly sized phenylalanine, lacking the polarity to coordinate Zn^{2+} or to form hydrogen bonds. There was no significant difference in the ability of His¹⁰⁰Phe rhodopsin to bind 11-*cis*-retinal at 25 °C, as the A_{280}/A_{500} ratio and absorbance maxima of 2.0 are similar to wild type (WT) (Supporting Information Figure 2). When we treated wild-type rhodopsin with 50 μM ZnCl_2 (EC_{50} concentration), rhodopsin decayed at an increased rate of $0.044 \pm 0.002 \text{ min}^{-1}$ (WT = $0.026 \pm 0.002 \text{ min}^{-1}$, a ratio of 1.69, $p < 0.001$; Figure 3C). The k rate constant of rhodopsin decay also increased from $0.026 \pm 0.002 \text{ min}^{-1}$ for wild-type rhodopsin to $0.057 \pm 0.005 \text{ min}^{-1}$ for His¹⁰⁰Phe (H100F) (a ratio of 2.19, $p < 0.001$). Additionally, comparing the H¹⁰⁰-related decay curves with a two-way ANOVA confirms their significance ($p < 0.001$). These data indicate that disruption of His¹⁰⁰-mediated interactions, through site-directed mutagenesis, results in rhodopsin destabilization similar to Zn^{2+} treatment. Treating His¹⁰⁰Phe with 50 μM ZnCl_2 led to further destabilization, decaying at an accelerated rate of $0.084 \pm 0.009 \text{ min}^{-1}$, compared to the initial rate of $0.057 \pm 0.005 \text{ min}^{-1}$ for His¹⁰⁰Phe alone (a ratio of 1.47, $p < 0.05$). The similar level of destabilization between His¹⁰⁰Phe and wild type treated with zinc supports the notion of His¹⁰⁰ involvement; however, additional loss of receptor thermostability with postmutational addition of zinc suggests

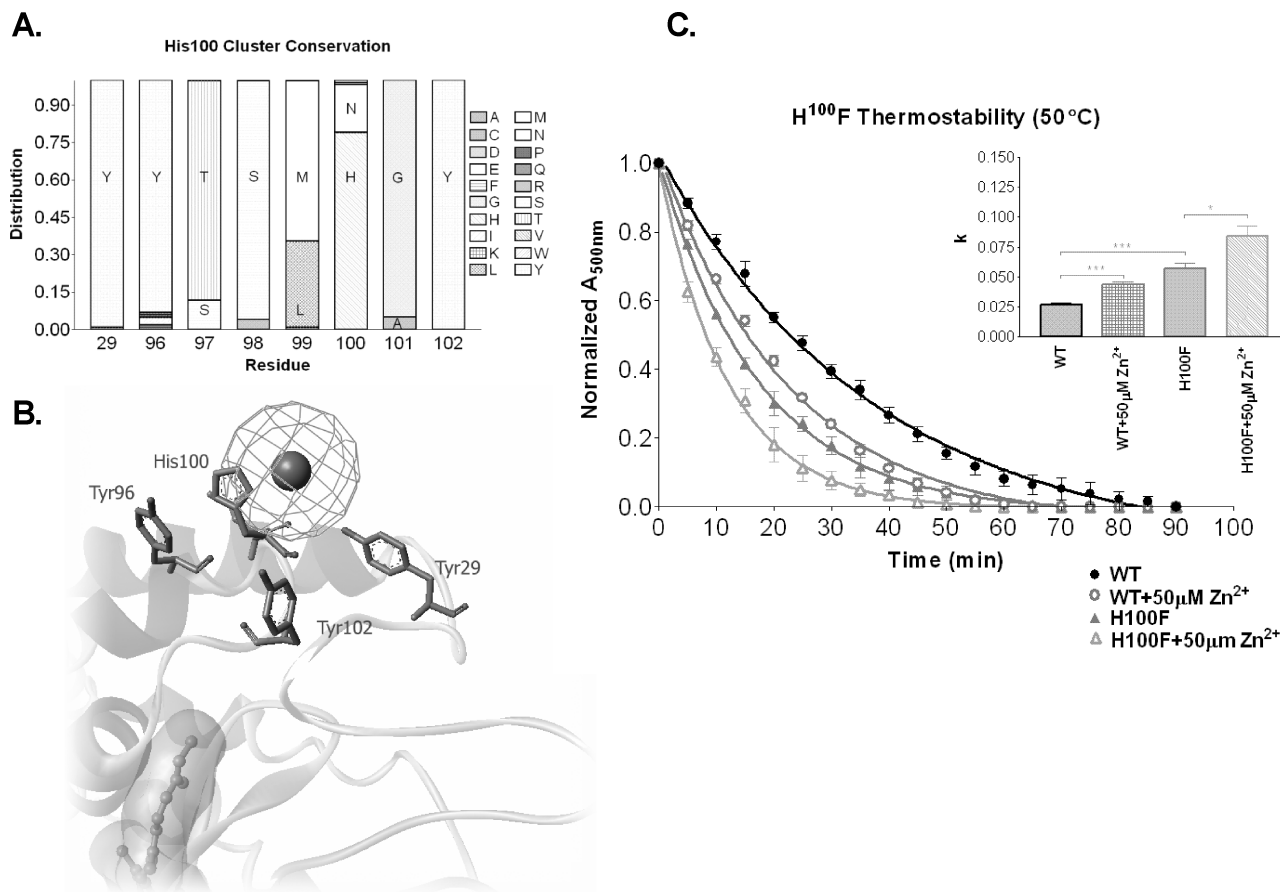


FIGURE 3: (A) Histogram showing conservation of amino acids at specified residue positions comparing 102 sequenced vertebrate type I opsin proteins (GPCRdb (38)). (B) The conserved tyrosine residues surround the His¹⁰⁰ residue in close enough proximity for potential interactions in the presence of zinc (sphere). (C) The thermal decay rate of wild-type rhodopsin in the presence and absence of 50 μM Zn²⁺. Thermal decay of H¹⁰⁰F is also shown in the presence and absence of 50 μM Zn²⁺. A bar graph showing the rate constants (*k*) for each of the constructs and treatments. (*, *p* < 0.05; ***, *p* < 0.001). Two-way ANOVA analysis confirmed multipoint significance at *p* < 0.0001.

that either an additional or alternative site is directly involved in the observed zinc-mediated destabilization.

Crystal Structure Analysis Indicates the Presence of a Highly Conserved Hydrogen-Bonding Network Coordinated by His¹⁰⁰. Disrupting the receptor structure with zinc or mutagenesis, at position 100, impairs receptor stability, suggesting important native interactions with proximal amino acids. We identified Tyr⁹⁶ (100% conserved in transmembrane domain II) and Tyr¹⁰² (93% conserved in extracellular loop II) as likely candidates (Figure 3B). A Tyr-His-Tyr triad appears in 79% of the vertebrate opsins and in each opsin containing a His¹⁰⁰ residue, with tyrosine 96 having 93% conservation and tyrosine 102 completely conserved (Figure 3A). Extending this evaluation beyond simple sequence conservation to the relative position of functional groups supports the hypothesis of regional structural preservation. Threonine has 88% conservation at position 97, with the remaining sequences possessing serine at this site, resulting in 100% conservation of the γ-hydroxyl functional group. Interestingly, position 101 maintains 100% conservation of small flexible Gly or Ala residues.

His¹⁹⁵ Additionally Mediates Trace Metal Regulated Thermostability. Further receptor destabilization with addition of zinc to His¹⁰⁰Phe suggests that another site also contributes to the effect. An alternative extracellular histidine (His¹⁹⁵) was similarly characterized using site-directed mutagenesis and thermal stability assessment. In contrast to His¹⁰⁰, His¹⁹⁵

is unique to bovine rhodopsin (the source of the crystal structure), with a lysine residue at this position in human rhodopsin. Lysine is 42% conserved at position 195 across type I vertebrate opsins, second only to alanine (44%) (Figure 4A). His¹⁹⁵Phe and His¹⁹⁵Lys showed no significant difference in the ability to bind 11-*cis*-retinal at 25 °C, with an absorbance ratio similar to wild type (WT). Both His¹⁹⁵Phe and His¹⁹⁵Lys mutations destabilized rhodopsin less dramatically than mutation of His¹⁰⁰. Interestingly, while His¹⁹⁵Lys was less stable than WT, this mutation also reduced the destabilizing impact of zinc treatment. The *k* rate constant of rhodopsin decay increased from the wild type ($0.026 \pm 0.002 \text{ min}^{-1}$) to $0.037 \pm 0.005 \text{ min}^{-1}$ for His¹⁹⁵Lys and $0.069 \pm 0.005 \text{ min}^{-1}$ (a ratio of 2.65, *p* < 0.0001) for His¹⁹⁵Phe (Figure 4C). As before, comparing the collection of decay curves using a two-way ANOVA confirms their significance (*p* < 0.001) and suggests that, as with His¹⁰⁰, disruption of His¹⁹⁵ also mediates the destabilizing effects observed with ZnCl₂ treatment. To evaluate involvement of His¹⁹⁵ in metal coordination, we treated these two rhodopsin mutants (His¹⁹⁵Phe and His¹⁹⁵Lys) with ZnCl₂. Both mutants demonstrated elevated destabilization with Zn²⁺ treatment, though this effect was modest for His¹⁹⁵Lys. The His¹⁹⁵Lys mutant decayed at a rate of $0.037 \pm 0.005 \text{ min}^{-1}$, with an increased rate of $0.042 \pm 0.003 \text{ min}^{-1}$ upon addition of 50 μM ZnCl₂. The His¹⁹⁵Phe mutant decayed at a rate of $0.069 \pm 0.005 \text{ min}^{-1}$, compared to an accelerated rate of 0.087 ± 0.005

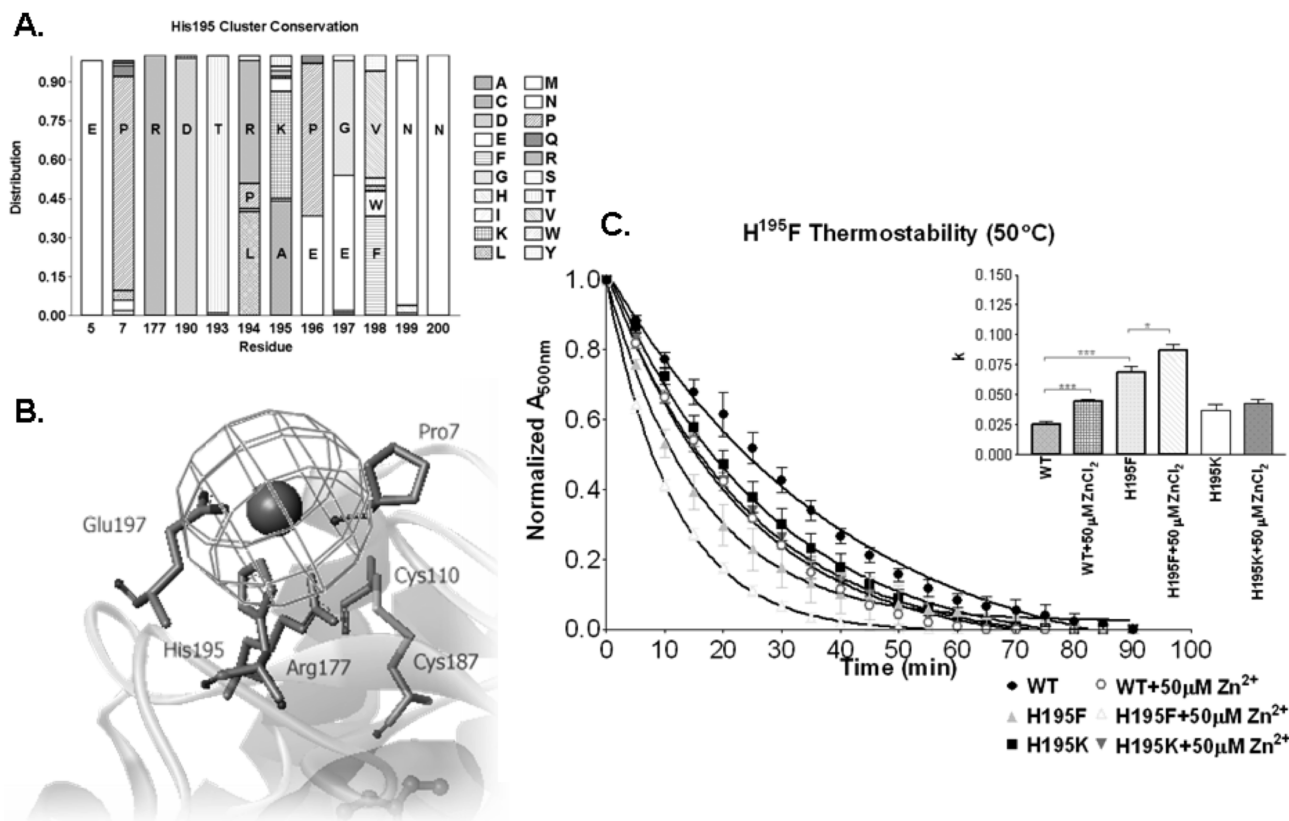


FIGURE 4: (A) Histogram showing conservation of amino acids at specified residue positions comparing 102 sequenced vertebrate type I opsin proteins (GPCRdb (38)). Sequence divergence follows a motif of $[R^{194}/K^{195}]xE$, manifest predominantly as LKPEV and RAEGF for land and aquatic vertebrates, respectively. (B) Charged residues, corresponding to the $[R^{194}/K^{195}]xE$ motif, cluster around His¹⁹⁵ and the associated zinc in the crystal structure. (C) The thermal decay rate of wild-type rhodopsin in the presence and absence of 50 μ M Zn²⁺. Thermal decay of H¹⁹⁵F and H¹⁹⁵K is also shown in the presence and absence of 50 μ M Zn²⁺. The bar graph shows rate constants (k) for each construct and treatment. (*, $p < 0.05$; ***, $p < 0.001$). Two-way ANOVA analysis confirmed multipoint significance at $p < 0.0001$.

min⁻¹ (a ratio of 1.26, $p < 0.05$) when treated with 50 μ M ZnCl₂. Not surprisingly, His¹⁹⁵Lys remains a great deal more stable than His¹⁹⁵Phe, as it retains similar polarity to the native bovine rhodopsin histidine, again highlighting important intradiscal interactions involving the residue at this position. Interestingly, the observed differences between His¹⁹⁵ and His¹⁹⁵Lys suggest that while Lys¹⁹⁵ is less stable than His¹⁹⁵, it may provide some degree of protection from additional structural instability induced by zinc association.

Opsin Sequence Analysis Supports the Presence of a Conserved Interaction between Residues 195 and 197. Sequence analysis indicates the His¹⁹⁵-Glu¹⁹⁷ pair to be unique to bovine rhodopsin; however, a Lys¹⁹⁵-Glu¹⁹⁷ pair is 32% conserved across the vertebrate opsins including human rhodopsin (Figure 4A). Beginning at residue 194, a motif of LKPEV accounts for 31% of all type I vertebrate opsins with RAEGF accounting for 35%. Taken together, a motif of $(R^{194}/K^{195})xE$ is 82% conserved. Phylogenetic distribution of these structural motifs, among type I vertebrate opsins, reveals segregation between land (LKPEV dominant) and aquatic (RAEGF dominant) vertebrates. Modeling illustrates that the LKPEV motif maintains a pocket capable of accommodating zinc coordination, whereas the RAEGF motif may not (Figure 4B). Bovine rhodopsin is unique at this position, containing a PHEET motif. Furthermore, our study suggests that disrupting this interaction, through site-directed mutagenesis or zinc coordination, alters the structure of this region resulting in dramatic protein instability. This

instability may occur through subsequent disruption of the proposed ion pair between Arg¹⁷⁷ and Asp¹⁹⁰ (Figure 4A,B) located at the ends of the second extracellular loop which is critical for folding and stability of dark state rhodopsin (31).

The His¹⁰⁰Phe/His¹⁹⁵Phe Double Mutant Is Unaffected by Further Zinc Treatment. To evaluate whether these histidines are sufficient to mediate the observed Zn²⁺-induced instability, we constructed the His¹⁰⁰Phe/His¹⁹⁵Phe double mutant rhodopsin protein. The His¹⁰⁰Phe/His¹⁹⁵Phe mutant displayed reduced 11-*cis*-retinal binding at 25 °C. Thermal stability of the His¹⁰⁰Phe/His¹⁹⁵Phe double mutant (Figure 5), with an accelerated decay rate of 0.277 ± 0.027 min⁻¹, demonstrated dramatic impact on protein stability relative to wild-type rhodopsin (0.026 ± 0.002 min⁻¹) (a ratio of 10.65, $p < 0.0001$). The resulting instability appears to be synergistic, as each individual mutation accounts for less than half the observed decay rate (His¹⁰⁰Phe at 0.057 ± 0.005 min⁻¹ and His¹⁹⁵Phe at 0.069 ± 0.005 min⁻¹). There was no statistically significant difference between the His¹⁰⁰Phe/His¹⁹⁵Phe double mutant treated with 50 μ M ZnCl₂ that decayed at a rate of 0.225 ± 0.023 min⁻¹ and the untreated double mutant, which decayed at a rate of 0.277 ± 0.027 min⁻¹ ($p > 0.05$; ns; Figure 5A), supporting the identification of the critical zinc coordination sites. Further studies were performed at 37 °C to assess whether there were any significant differences at a lower temperature where rhodopsin would be more stable (Figure 5B). As expected, the double mutant receptor demonstrated increased stability at 37 °C (0.144 ± 0.017

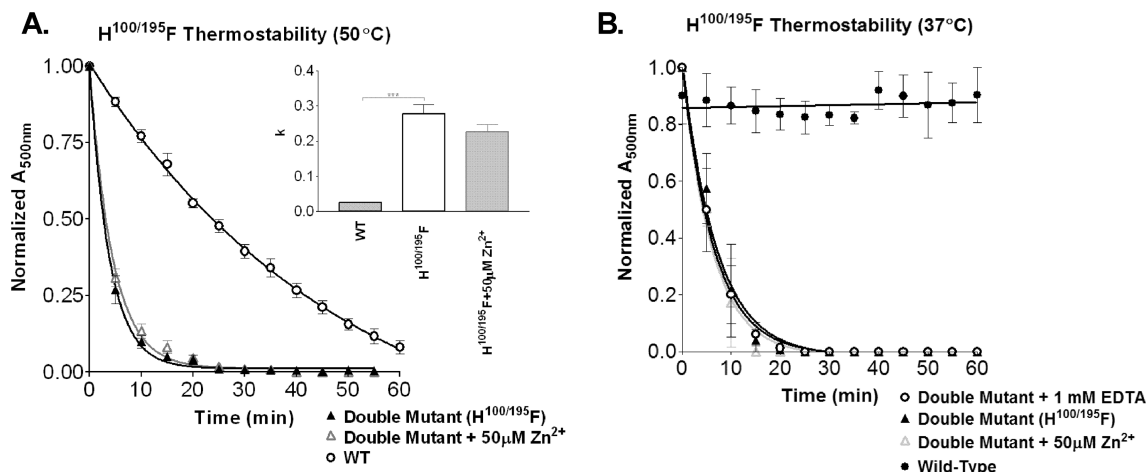


FIGURE 5: (A) Simultaneous removal of both His¹⁰⁰ and His¹⁹⁵ results in a dramatically unstable photoreceptor ($0.277 \pm 0.027 \text{ min}^{-1}$). Additional treatment of the double mutant with 50 μM zinc has no further impact on thermal stability ($0.225 \pm 0.023 \text{ min}^{-1}$). Shown in the bar graphs are the respective rate constants (k). (***, $p < 0.001$). (B) The double mutant receptor ($0.144 \pm 0.017 \text{ min}^{-1}$) evaluated at a lower temperature (37 °C) remained consistent with the higher temperature, showing no significant differences in thermal stability with the addition of either 50 $\mu M Zn^{2+}$ ($0.165 \pm 0.015 \text{ min}^{-1}$) or 1 mM EDTA ($0.155 \pm 0.016 \text{ min}^{-1}$).

min^{-1}) compared to 50 °C. Consistent with the higher temperature experiments, the addition of 50 $\mu M Zn^{2+}$ ($0.165 \pm 0.015 \text{ min}^{-1}$) or 1 mM EDTA ($0.155 \pm 0.016 \text{ min}^{-1}$) made no further impact on the double mutant. These results support both His¹⁰⁰ and His¹⁹⁵ as critical rhodopsin stabilizing sites which are exquisitely sensitive to destabilization by zinc coordination.

Chelation of Trace Zn^{2+} Can Stabilize Retinitis Pigmentosa Mutation Pro²³His. As exogenous zinc demonstrated a physiological role in modulating rhodopsin structural stability, we hypothesized that it may additionally play a role for structurally impaired RP mutants. Wild-type rhodopsin treated with EDTA showed significant thermal stabilization (Figure 1A). The Pro²³His rhodopsin mutation (most common RP mutations in North America) results in a structure significantly less stable than WT rhodopsin, with a decay rate of $0.450 \pm 0.032 \text{ min}^{-1}$. Pretreatment of Pro²³His rhodopsin with 1 mM EDTA significantly increases receptor thermostability ($0.173 \pm 0.021 \text{ min}^{-1}$) at high temperature (a ratio of 2.60, $p < 0.05$; Figure 6A). Although this corresponds to greater than a 2-fold increase in receptor half-life, the rapidity of receptor unfolding at this high temperature results in a low resolution of decay rates for highly unstable forms of rhodopsin such as Pro²³His. To increase resolution of this observation, a lower temperature (37 °C) demonstrates enhanced stability, with a decay rate of 0.122 ± 0.012 for Pro²³His increasing to 0.027 ± 0.007 with 1 mM EDTA treatment (a ratio of 4.52, $p < 0.05$; Figure 6B). This difference corresponds to more than a 4-fold increase in receptor half-life. We hypothesize that, as with wild type, EDTA chelation stabilizes the Pro²³His through prevention of zinc coordination at the native His¹⁰⁰, His¹⁹⁵Phe sites. We cannot exclude the formation of a third zinc binding site involving Pro²³His, though we surmise that zinc can similarly influence intradiscal RP mutations and may therefore play a role in disease progression and therapy.

DISCUSSION

Allosteric effects of trace metals on G-protein-coupled receptors have demonstrated implications in both health and disease (9–16, 39, 40). Trace metals such as zinc can

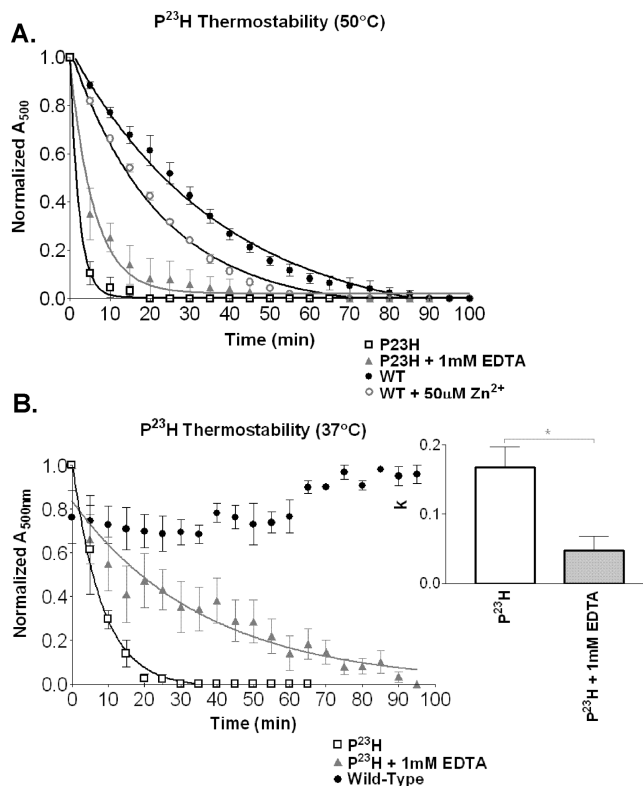


FIGURE 6: (A) Thermal decay (50 °C) for the most prevalent rhodopsin RP mutant, Pro²³His, in the absence and presence of EDTA. Results are compared to wild-type protein in the presence and absence of Zn^{2+} . Wild type plus EDTA is shown in Figure 1B. (B) Evaluating the unstable mutants at a lower temperature (37 °C) improves resolution between the decay curves for Pro²³His and the same mutant treated with EDTA. The bar graph illustrates improvement in decay rate (k) with EDTA (*, $p < 0.05$). Two-way ANOVA analysis confirmed multipoint significance at $p < 0.0001$.

accumulate to high concentrations within organs and subcellular compartments (41), influencing many neurodegenerative diseases (2–4, 41, 42). The rhodopsin crystal structure provides a unique opportunity to elucidate precise mechanisms of allosteric action. This ultimately can serve to establish important principles for other GPCRs.

Zinc Binding Has Dual and Opposing Roles. Rhodopsin displays differential responses to zinc: Transmembrane coordination helps to stabilize the receptor (20), and intradiscal coordination appears to destabilize purified rhodopsin (current study). Dual zinc association (His¹⁰⁰ and His¹⁹⁵) within the intradiscal region mediates disruption of receptor thermostability through low-affinity histidine coordination. Significant conservation surrounds the His¹⁰⁰ zinc-coordinating region, in contrast to His¹⁹⁵, which is unique to bovine rhodopsin. Simultaneous replacement of these groups with phenylalanine results in severe structural impairment and eliminates further metal-induced destabilization. Interestingly, human Lys¹⁹⁵ appears to assist structural integrity in this region, reducing wild-type rhodopsin susceptibility to metal interference in comparison to bovine rhodopsin.

With sensitivity to zinc in the physiological range (as observed from our dose response) and evidence of a gradient free zinc distribution within the rod cell itself (high ER concentrations, low in outer segment and very high in the RPE) (43), we speculate that the observed destabilization may serve both physiological and pathological roles. The high zinc concentrations around the ER may be involved in rhodopsin folding (or dimerization as proposed by Park et al. (44)), particularly with the assistance of chaperonins. The absence of available free zinc across the outer segment (as with our EDTA experiments) imposes maximal stability and thus optimal rhodopsin function. Finally, high zinc concentrations observed in the RPE may assist in the destabilizing, unfolding, and proteolytic degradation of rhodopsin. In the absence of chaperonins or proteolytic cleavage, instability arising from zinc coordination may promote oligomerization and/or aggregation. Formation of stable oligomers is supported by previous scanning force microscopy (SFM) data suggesting that zinc coordination promotes self-association of the photoreceptor (44).

Implications for Retinitis Pigmentosa. Our findings with the most prevalent rhodopsin RP mutation, Pro²³His, support a potential pathological involvement of zinc coordination in rhodopsin. In the context of retinitis pigmentosa, the impairment of receptor integrity observed with trace metal coordination would accelerate the progression of disease. Specific RP mutations, particularly those among the intradiscal region, may amplify the impact of metal-induced destabilization. The instability of Pro²³His may be related to potential aberrant Zn binding sites, or influence the known sites, in addition to effects on the native disulfide bond. Unfortunately, misfolding and aggregation currently prevent accurate assessments of these possibilities. Although chelative zinc removal does not completely restore the P²³H photoreceptor to wild-type stability levels, half-life increases of 3-fold to 5-fold may be suitable to slow the progression of retinal degeneration. The impact of structural impairment in RP progression, coupled with metal-induced misfolding, permits speculation regarding the possibility for chelative agents to inhibit disease progression. Metal protein attenuating compounds, such as PBT2 (45), are being developed and evaluated for utility in chelating metals from protein aggregates. Though currently focused on Alzheimer's plaques, if analogues can distribute, or be delivered, to photoreceptors, they may be useful in a subset of RP mutants. The influence of metal coordination on rhodopsin structural integrity extends insight into native

receptor structure, enhancing general understanding of GPCRs, and may point toward potential therapeutics.

SUPPORTING INFORMATION AVAILABLE

Additional data regarding mutagenesis, modeling, and spectrophotometry. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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