Bothnia dystrophy is caused by domino-like rearrangements in cellular retinaldehyde-binding protein mutant R234W

Xiaoqin He, Joel Lobsiger¹, and Achim Stocker²

Department of Chemistry and Biochemistry, University of Bern, 3012 Bern, Switzerland

Edited by Brian W. Matthews, University of Oregon, Eugene, OR, and approved September 16, 2009 (received for review July 3, 2009)

Cellular retinaldehyde-binding protein (CRALBP) is essential for mammalian vision by routing 11-*cis*-retinoids for the conversion of photobleached opsin molecules into photosensitive visual pigments. The arginine-to-tryptophan missense mutation in position 234 (R234W) in the human gene *RLBP1* encoding CRALBP compromises visual pigment regeneration and is associated with Bothnia dystrophy. Here we report the crystal structures of both wild-type human CRALBP and of its mutant R234W as binary complexes complemented with the endogenous ligand 11-*cis*-retinal, at 3.0 and 1.7 Å resolution, respectively. Our structural model of wild-type CRALBP locates R234 to a positively charged cleft at a distance of 15 Å from the hydrophobic core sequestering 11-*cis*-retinal. The R234W structural model reveals burial of W234 and loss of dianion-binding interactions within the cleft with physiological implications for membrane docking. The burial of W234 is accompanied by a cascade of side-chain flips that effect the intrusion of the side-chain of I238 into the ligand-binding cavity. As consequence of the intrusion, R234W displays 5-fold increased resistance to light-induced photoisomerization relative to wild-type CRALBP, indicating tighter binding to 11-*cis*-retinal. Overall, our results reveal an unanticipated domino-like structural transition causing Bothnia-type retinal dystrophy by the impaired release of 11-*cis*-retinal from R234W.

crystal structure | 11-*cis*-retinal | retinitis pigmentosa | *RLBP1* | vitamin A

Bothnia dystrophy is an autosomal recessive disease of the retina with high prevalence in northern Sweden (1). The disease, which affects 1 in 4500 births, is characterized by early-onset night blindness followed by macular degeneration and eventual loss of vision in adulthood (2). Bothnia dystrophy is caused by the arginine-to-tryptophan missense mutation in position 234 (R234W) in the *RLBP1* gene, which has been mapped to chromosome 15q26 (1). Several other clinical phenotypes, including autosomal recessive retinitis pigmentosa (3), retinitis punctata albescens (4), fundus albipunctatus (5), and Newfoundland rod-cone dystrophy (6), could be associated with up to 8 different amino acid substitution mutations in the same gene. However, the molecular basis of the retinal disorders that result from these *RLBP1* mutations is not well understood because of a lack of structural data.

The *RLBP1* gene encodes the 36-kDa cellular retinaldehyde-binding protein (CRALBP), a soluble retinoid carrier that first was isolated from the cytoplasm of bovine retina in complex with endogenous 11-*cis*-retinal and 11-*cis*-retinol (3, 7). CRALBP possesses high stereoselectivity toward 11-*cis*-retinoids, and it has been proposed that CRALBP protects these retinoids from premature photoisomerization and from enzymatic reverse isomerization (8, 9). In vivo and in vitro studies suggest that CRALBP is essential for the proper function of both rod and cone photoreceptors (10, 11). Interestingly, CRALBP is not present in photoreceptor cells but is expressed abundantly in the adjacent retinal pigment epithelium (RPE) and in the Müller glial cells of the retina (12, 13). In both cell types, CRALBP participates in the regeneration of active 11-*cis*-retinoids from the inactive 11-*trans* products of the rhodopsin photocycle and in the de novo synthesis of these retinoids from 11-*trans* metabolic precursors. The cycling of retinoids between

photoreceptor and adjacent pigment epithelium cells is known as the “visual cycle” (14, 15). CRALBP of the RPE cells binds 11-*cis*-retinol, which is the direct product of the isomerohydrolysis of all-*trans*-retinylesters in the visual cycle and which also is formed upon activation of stored 11-*cis*-retinylesters (16). CRALBP functions as a retinoid carrier, assisting the oxidation of 11-*cis*-retinol to 11-*cis*-retinal in the next step of the visual cycle and preventing uncontrolled storage of 11-*cis*-retinol by esterification (14). In Müller glial cells CRALBP is reported to have the opposite function—namely, assisting the esterification of 11-*cis*-retinol (17). Stable supramolecular complexes between components with retinoid-processing activity and CRALBP have been isolated from bovine retina by co-purification and co-precipitation (18, 19). Functional and structural interactions of CRALBP with the membrane-bound *cis*-retinoid-specific dehydrogenase and with the multivalent plasma membrane linker in the RPE have been demonstrated both in vitro and in vivo (20). Ligand-binding studies with CRALBP bearing mutations in the lipid-binding pocket implicate at least 10 hydrophobic protein-to-ligand interactions in retinoid binding (21, 22). The R234W mutant of CRALBP has a 2-fold increased affinity for 11-*cis*-retinal. The M226K and R151Q mutants have lost all retinoid-binding affinity (23). CRALBP contains 2 conserved domains termed “CRAL-TRIO” (pfam03765: residues 46–127; pfam00650: residues 133–314) that are characteristic for the SEC14p-like family of lipid binders and include a flexible helical gate giving access to a lipid-binding cavity (24, 25). Crystal structures of 4 homologous SEC14p-like lipid binders and a Ras-specific GTPase-activating protein (RasGap) suggest structural and functional conservation of the CRAL-TRIO fold (26–30).

Here we present the crystal structures of wild-type human CRALBP and of its disease-causing R234W mutant, both in complexes with their endogenous ligand, 11-*cis*-retinal. Extensive van der Waals interactions between the ligand-binding pocket and 11-*cis*-retinal explain the high stereoselectivity of CRALBP and how it protects the ligand against premature photoisomerization. In the R234W 11-*cis*-retinal complex, ligand binding is affected by domino-like conformation changes. Comparison of the binding-pocket conformation of wild-type and R234W CRALBP reveals enhanced packing density in the mutant resulting in a tighter binding of the ligand. A consequence of tighter binding by the R234W mutant is the reduced rate of ligand photoisomerization.

Author contributions: A.S. designed research; X.H. and J.L. performed research; X.H., J.L., and A.S. analyzed data; and A.S. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

Data deposition: The atomic coordinates and structure factors have been deposited in the Research Collaboratory for Structural Bioinformatics Database, www.rcsb.org (RCSB ID codes 3HX3 and 3HY5).

¹Present address: Institute for Molecular Biology and Biophysics, ETH Zürich, Schafmattstrasse 20, 8093 Zurich, Switzerland.

²To whom correspondence should be addressed. E-mail: achim.stocker@ibc.unibe.ch.

This article contains supporting information online at www.pnas.org/cgi/content/full/0907454106/DCSupplemental.

Tighter binding also slows down the release of 11-*cis*-retinal from the protein, explaining how and why the R234W mutation inhibits retinal metabolism in the human eye. The structures of CRALBP and of the R234W mutant provide an explanation of the molecular mechanism underlying Bothnia dystrophy.

Results and Discussion

Crystallization and Structure Determination. Apo-CRALBP did not afford diffracting crystals, probably because of aggregation-induced heterogeneity of the apoprotein. The addition of different non-ionic detergents to apo-CRALBP did not improve crystal quality. Addition of a 1.5 molar excess of 11-*cis*-retinal reduced aggregation and improved crystal quality to <4.0 Å diffraction, but the crystals were twinned. Replacement of high-entropy surface residues (lysine, glutamate) by alanine (31) and truncation of the protein from both ends did not improve crystal quality. Gel filtration chromatography to remove unbound 11-*cis*-retinal and strict selection of the fractions with the lowest polydispersity index ($<15\%$) by dynamic light scattering (DLS) yielded CRALBP that produced hexagonal crystals of space group $P6_522$ diffracting to 3 Å. New surface mutants were constructed with the goal of improving resolution further; 1 of these mutants was the disease-causing mutation R234W (23). Crystals of the selenomethionine-substituted R234W 11-*cis*-retinal complex (R234W) diffracted to 1.7 Å. Phases were determined using R234W and single-wavelength anomalous dispersion (SAD) (32). The structure of wild-type CRALBP was solved subsequently by molecular replacement using the atomic model of R234W. The data collection, refinement, and validation statistics are reported in [supporting information \(SI\) Table S1](#).

Overall Fold of Wild-Type CRALBP. The structure model of wild-type CRALBP comprises residues 23–306 of 317 residues (Fig. 1). The protein folds into the prototypical SEC14-like domain structure and is most similar to α -tocopherol transfer protein (PDB code 1OIP; overall sequence identity of 29%; ref. 26). CRALBP is composed of 2 domains, the N-terminal all-helical α domain and the C-terminal 3-layer $\alpha\beta\alpha$ sandwich domain. (The topology of the secondary structure of CRALBP is depicted in [Fig. S1](#).) The N-terminal α domain comprises helices $\alpha 1$ – $\alpha 5$. The core of the C-terminal $\alpha\beta\alpha$ domain comprises a β -sheet with 1 antiparallel and 4 parallel strands, $\beta 1$ – $\beta 5$, and 6 helices. Helices $\alpha 6$ and $\alpha 7$ are packed against the concave face, and the 4 helices $\alpha 9$ – $\alpha 12$ are packed against the convex face of the sheet. A third structural motif is formed between the aperiodic segment of the N terminus (residues 23–42) and 2 helices ($\alpha 13$, $\alpha 14$) of the C terminus of CRALBP. The retinal-binding cavity is delimited by the convex side of the β -sheet and the 4 adjacent helices. The interdomain contacts are governed by hydrophobic interactions between helix $\alpha 5$ of the N-terminal α domain and helices $\alpha 7$ and $\alpha 8$ of the C-terminal $\alpha\beta\alpha$ domain. Side-chain hydrogen bonds between Y117 of helix $\alpha 5$ and E185 of helix $\alpha 7$ and between Y124 of helix $\alpha 5$ and D225 of helix $\alpha 8$ control the orientation of the interdomain contact.

Structure of the R234W Mutant of CRALBP. The structure model of the R234W mutant comprises residues 57–306 of 317 residues. The R234W mutant crystallized in the space group C2, unlike wild-type CRALBP, which crystallized in space group $P6_522$. Because of the different crystal contacts, the N-terminal residues 1–56 are disordered in the mutant. The functionally important R234W mutation is located in the loop between helix $\alpha 10$ and β -strand $\beta 4$ (Fig. 1). In wild-type CRALBP, R234 is part of a cluster of 3 pairs of positively charged residues (R103–K104, K236–R234, and K261–R265) at the protein surface. (Electrostatic surface potentials of wild-type CRALBP are shown in [Fig. S2](#).) This cluster is not in direct contact with the retinoid ligand, but it binds a dianionic tartrate molecule that was present in the crystallization buffer (Fig. 24). In the R234W mutant, the apolar indole ring is buried by van

Fig. 1. Monomeric structure of CRALBP. (A) Ribbon diagram of wild-type CRALBP bound to 11-*cis*-retinal. The helices of the N-terminal domain are indicated in green; C-terminal helices are indicated in blue; the helical gate (helices $\alpha 11$ and $\alpha 12$) is indicated in gray; β -strands are indicated in red. The position of R234 is indicated as a yellow sphere, the 11-*cis*-retinal ligand is shown as orange sticks, and the cavity surface is shown in orange. The cavity surface was calculated with VOIDO (33). (B) View after rotation of A by 90° on the vertical axis. Images were generated with PYMOL (54).

der Waals contacts between helix $\alpha 4$ of the N-terminal α domain and helix $\alpha 7$ of the C-terminal $\alpha\beta\alpha$ domain, with contact to residues F99, A102, and L188, respectively. The conformation change at residue position 234 dislocates nearby residues R234, K104, and K261 over distances of 7.7, 5.4, and 3.4 Å, respectively, and disrupts the coordination of tartrate. The burial of W234 also induces a conformation change of the loop between $\alpha 10$ and $\beta 4$ (residues 227–238) by an average 2.3-Å rms deviation. This conformation

Fig. 2. Comparison of wild-type and R234W CRALBP. (A) Stereoview of the critically charged cleft of CRALBP superimposed on R234W. Tartrate and interacting residues of the CRALBP basic cleft are depicted as gray sticks. Dashed black lines indicate possible hydrogen bonds and short contacts; distances are given in Å. Corresponding residues of the R234W basic cleft are depicted as salmon sticks. (B) Stereoview of the domino-like structural transition in CRALBP by the R234W mutation. Side chains of wild-type CRALBP (gray) that participate in a cascade of side-chain flips in R234W (salmon) are depicted as sticks, the protein backbones are shown as a worm model. The cavity surface of the wild-type CRALBP and the 11-*cis*-retinal chromophore are shown in orange.

change triggers a series of side-chain flips including F198, F235, and I238, which are part of the retinal-binding pocket (Fig. 2B). As a consequence the side-chain C δ methyl group of I238 is rotated into the retinal-binding cavity. This rotation is the only significant structural alteration in the retinal-binding pocket of the R234W mutant. As described later, it probably is the decisive factor for the functional disorder of this mutated CRALBP.

The Retinoid-Binding Pocket. 11-*cis*-retinal is sequestered completely from bulk solvent in the binding pockets of both wild-type and R234W CRALBP (Fig. 3A). The pocket volumes were calculated using the program VOIDOO (33) and a rolling probe with a radius of 1.0 Å. The R234W volume is $5.989 \times 10^2 \text{ Å}^3$, which is 7.2% less than that of wild-type CRALBP. The volume reduction can be attributed to the I238 conformation change that is triggered by the R234W mutation, as described earlier. The side-chain conformations of the other residues lining the pocket remain unaltered between mutant and wild-type CRALBP. The I238 C δ methyl group improves the shape complementarity between the ligand and the binding pocket by protruding into a pit at the junction between the β -ionone ring and the polyene tail (Fig. 3A). The better fit between I238 and the ligand increases the local packing density and tightens the binding of 11-*cis*-retinal. Structural evidence for tighter packing is provided by the greater electron density of 11-*cis*-retinal. In the mutant CRALBP, the electron density map of all carbons from the β -ionone ring to the terminal aldehyde (C1–C15) is well defined (Fig. 3B). In wild-type CRALBP, the electron density map of the C11–C15 terminal tail is poorly defined, suggesting that this part of the ligand is slightly disordered in the less tightly packed ligand-binding pocket. The excellent resolution of the R234W structure and the more restricted conformation of the 11-*cis*-retinal ligand provide a detailed picture of the retinoid conformation and of the retinoid-to-protein interactions (Fig. 3B). The absolute conformation of the 11-*cis*-retinal is 6-*s-trans*, 11-*cis*, twisted 12-*s-cis*. The polyene chain is almost planar up to C12. The β -ionone ring

Fig. 3. Ligand binding pocket of CRALBP. (A) Cavity-overlay of wild-type CRALBP (gray) onto R234W (orange). The corresponding 11-*cis*-retinal ligands are shown as gray and orange sticks, respectively. (B) Close-up view of the ligand-binding pocket of R234W. Side chains (gray) that form van der Waals contacts with 11-*cis*-retinal (orange) or participate in hydrogen bonding in the ligand binding pocket are shown. The protein backbone is shown as a worm model. The electron density of the ligand is shown as a mesh at 1 σ in the $2F_o - F_c$ map. Dashed black lines indicate hydrogen bonds; distances are given in Å.

is twisted by -7° around the C6–C7 bond relative to the plane of the polyene chain. The tail of the polyene chain from C12 to the α,β -unsaturated aldehyde and including the methyl group at C13 also is planar. The 2 planes defined by C7–C12 and C13–C15 of the polyene are twisted by 45° around the C12–C13 bond. 11-*cis*-retinal interacts with the side chains of the binding pocket as follows: The α,β unsaturated aldehyde is stacked between the phenyl ring of F161 and the sulfur of M223. The carbonyl oxygen of the aldehyde serves as the hydrogen bond acceptor for the phenol group of Y180 and the acidic oxygen of E202 (2.8 Å and 2.6 Å hydrogen bonding distances). The β -ionone ring and the polyene chain are fixed by van der Waals interactions with the apolar side chains V224, L227, I238, V254, and L258 and I163, W166, F173, L215, L220, F240, F247, and Y251, respectively. In the R234W mutant, the I238 side chain improves the shape complementarity by a particularly close van der Waals contact with the methyl group C16 of the β -ionone ring. Recent fluorescence titration of CRALBP with different ligands adds evidence for stronger ligand binding by the R234W mutant (23). R234W has a 2-fold stronger affinity ($K_d \sim 10 \text{ nM}$) for 11-*cis*-retinal than does wild-type CRALBP. Furthermore, the same study shows that wild-type and mutant CRALBP have a 2.5-fold higher affinity for 11-*cis*-retinoids than for 9-*cis*-retinoids (23). The structural basis for this 11-*cis* versus 9-*cis* stereoselectivity is the shape complementarity between the binding pocket and the

Fig. 4. 3-D overlay of the binding pockets of wild-type CRALBP and α -TTP. Ribbon diagrams of CRALBP (salmon) and of α -TTP (gray) are shown with a numbering scheme according to the CRALBP secondary structure. 11-*cis*-retinal (salmon) and RRR- α -tocopherol (gray) are depicted as sticks; oxygen atoms are shown in red.

11-*cis*-retinal with a 45° out-of-plane twist between C12 and C13, which cannot be realized with the 9-*cis* isomer. For the same reasons, apo-CRALBP cannot combine with either 13-*cis*-retinal or all-*trans*-retinal. The 3-fold stronger affinity of CRALBP for 11-*cis*-retinal than for 11-*cis*-retinol (23, 34) may be caused by the stronger stacking interaction of F161 with pi orbitals of a carbonyl group than with sigma orbitals of a primary alcohol and by better hydrogen-bonding geometry between the E202 and Y180 donors and the planar sigma orbitals of the aldehyde than with the tetrahedral sigma orbitals of a primary alcohol. The specificity of the CRALBP retinoid-binding pocket reflects the need for stringent control of retinoids in metabolic processes, particularly the 11-*cis*-retinoids in the eye that are sensitive to light. Similarly, the closest structural homologue of CRALBP, the α -tocopherol transfer protein (α -TTP), selectively binds RRR- α -tocopherol out of 8 vitamers of the vitamin E group (26). A structural comparison of the 2 proteins reveals the similarity of the overall backbone architecture of the 2 binding pockets, with an average 1.9-Å rms deviation over 205 C α atoms (Fig. 4). Furthermore, the side chains of both ligands adopt similarly bent shapes through van der Waals interactions with apolar side chains. However, the positions and orientations of the respective ligands within their binding pockets are grossly different. Our findings suggest that both proteins accomplish shape complementarity between cognate ligand and binding pocket by individual fine tuning of shape and size of a set of amino acid side chains.

Protective Role of CRALBP Against Premature Ligand Photoisomerization. The photoisomerization rate of 11-*cis*-retinal in complex with CRALBP is only 4% of the rate observed in complex with rhodopsin, suggesting that a function of CRALBP is to protect the chromophore from premature photoisomerization (8). A difference between rhodopsin and CRALBP is that 11-*cis*-retinal is covalently bound as Schiff-base in rhodopsin but is bound non-covalently in CRALBP. Another difference is the unequal conformations of 11-*cis*-retinal in the binding pockets of CRALBP and rhodopsin (35, 36). (Chromophore conformations are depicted in Fig. S3.) The molecular basis of retinoid chemoprotection by CRALBP was not well understood because of the lack of structural data. The geometry of 11-*cis*-retinal, as seen in the x-ray structures of wild-type and mutant CRALBP, may offer the following “molecular” explanation of this difference. In ground-state bovine

Fig. 5. Time courses of the photoisomerization of 11-*cis*-retinal in the presence of ethanol, wild-type CRALBP, and R234W. The amount present at $t = 0$ is a_0 ; the amount present after illumination is a . For a first-order decay process, $a = a_0 e^{-kt}$. The first-order rate constants calculated from this experiment are $k_{\text{ethanol}} = 0.859 \times 10^{-3} \text{ s}^{-1}$; $k_{\text{CRALBP}} = 0.197 \times 10^{-3} \text{ s}^{-1}$; $k_{\text{R234W}} = 0.035 \times 10^{-3} \text{ s}^{-1}$.

rhodopsin the 11-*cis*-retinal Schiff-base adopts a 6-*s-cis*, 11-*cis*, 12-*s-trans* conformation (37), and the polyene chain is helically distorted between C10 and C13. This distortion facilitates light induced π - π^* transitions of the chromophore (38). Moreover, the *cis-trans* isomerization of the electronically excited state is favored in rhodopsin by the torsional moment provided by the intramolecular interactions between groups of the polyene chain—namely, steric repulsions between the hydrogen (C10) and the methyl group (C20) at C13 and dipole-dipole repulsion between the methyl groups (C18 and C20) at C5 and C13. This strain is released in the distorted all-*trans* conformation of bathorhodopsin that can be trapped within a few hundred femtoseconds following light absorption. In CRALBP, in contrast, the central region of 11-*cis*-retinal adopts a planar conformation that is less favorable for light-induced π - π^* transitions (38). In addition, because of the 6-*s-trans* conformation (as opposed to 6-*s-cis* in rhodopsin) the dipole-dipole repulsion between methyl groups (C18 and C20) at C5 and C13 is relieved. In conclusion, the hydrophobic pocket of CRALBP imposes conformational strain upon the 11-*cis*-retinal that disfavors photoisomerization.

Nevertheless, illumination of 11-*cis*-retinal complexed to CRALBP or R234W results in a loss of absorbance at 425 nm and an increase at 380 nm, consistent with the production of the all-*trans* geometrical isomer of retinal that is released for lack of affinity for CRALBP (39). We have determined first-order rate constants for the photoisomerization of uncomplexed 11-*cis*-retinal in solution or bound to CRALBP and R234W using the method previously described (8) (see SI Methods and Materials for details). The measurements revealed a 5-fold reduction of R234W photosensitivity ($191.5 \text{ M}^{-1} \text{ cm}^{-1}$) relative to wild-type CRALBP (Fig. 5). Interestingly, the binding of 11-*cis*-retinal to wild-type CRALBP or R234W results in an equal decrease of the extinction coefficient from 25,000 to $15,400 \text{ M}^{-1} \text{ cm}^{-1}$. This finding indicates that the observed 5-fold reduction in photosensitivity of 11-*cis*-retinal

Fig. 6. Missense mutations in CRALBP associated with retinitis pigmentosa. The side chains of residues mutated in retinitis pigmentosa are shown as green sticks on a worm model of the protein backbone; the interacting amino acid residues are shown in gray. The 11-*cis*-retinal ligand is shown as an orange stick, and the cavity surface is shown in orange. Dashed black lines indicate hydrogen bonds; distances are given in Å.

bound to R234W must be attributed to the local increase in packing density through the I238 C δ methyl group and not to a change in the extinction coefficient. Overall, the estimated free energy gain for the burial of a single tryptophan (-1.6 to -2.6 kcal/mol) (40) in R234W is relayed over a distance of 15 Å toward the central core where it results in increased packing through the I238 C δ methyl group (-0.6 to -1.0 kcal/mol) (41).

Other mutations of CRALBP Associated with Retinitis Pigmentosa.

Maw et al. (3) first reported a case of autosomal recessive retinitis pigmentosa associated with a pathological *RLBP1* gene defect, the 4763G-A nucleotide substitution. Known allelic variants of the gene encoding CRALBP now encompass missense mutations, frameshift mutations that result in truncations, and mutations affecting a canonical splice donor site that prevent translation of the protein.

Of the allelic variants identified thus far, 3 missense mutations (R151Q, R234W, and M226K) have been characterized in vitro with the recombinant proteins (23). Recombinant CRALBP R151Q was shown to be largely insoluble and to lack retinoid-binding capability; M226K was moderately soluble but also lacked retinoid-binding. As shown in Fig. 6, residue R151 is located at the end of beta-strand $\beta 1$ and is connected by a network of hydrogen bonds (2.9 Å, 3.0 Å, and 3.0 Å) to N190 and T193 of helix $\alpha 8$ and to the carbonyl oxygen of residue G155, respectively. Helix $\alpha 7$ and the adjacent helix $\alpha 10$ represent a central building block of the CRAL-TRIO fold defining 1 wall of the 11-*cis*-retinal-binding cavity. Disruption of the connecting hydrogen bonds toward helix $\alpha 8$ in R151Q may destabilize the overall fold and most probably is the cause of the association of R151Q with retinitis punctata albescens (3, 4). A similar clinical phenotype has been reported for a patient who had the related R151W mutation of *RLBP1* (42). M226 is located in the hydrophobic interface between helices $\alpha 7$ and $\alpha 10$ and thus affects the same area of the 11-*cis*-retinal binding cavity as R151Q. Substitution of an uncharged methionine residue by a positively charged lysine at position 226 is associated with the loss of retinoid-binding capability and retinitis punctata albescens (4). The M226K mutation may weaken van der Waals interactions by introducing a positive charge between helices $\alpha 7$ and $\alpha 10$. A phenotype of retinitis punctata albescens similar to M226K is found in association with R103W (4, 43). The R103W mutation maps to the critically charged cleft of wild-type CRALBP that harbors R234 as well (Fig. 2A). The CRALBP structural model indicates that

both R103 and R234 are involved in the coordination of dianionic tartrate deriving from the crystallization buffer. The comparison with the R234W model has revealed burial of R234 and disruption of the tartrate binding site. It is likely that the R103W mutation may exert its pathologic effect through a similar mechanism. Saari et al. (44) have proposed that the CRALBP basic cleft is physiologically relevant, stimulating retinoid transfer through specific binding of acidic phospholipids, particularly the dianionic glycerophospholipid phosphatidic acid. Accordingly, the interaction of acidic phospholipids with the basic cleft has been suggested to serve CRALBP for membrane docking (26, 44, 45). Structural superimposition of CRALBP on human α -TTP and on phosphatidylinositol transfer protein (SEC14p) from yeast reveal that the basic cleft motif is highly conserved (26–30). The R103W mutation of CRALBP represents the equivalent of the cleft mutation R59W in α -TTP that causes ataxia with vitamin E deficiency (26). Furthermore, K236 and R265 of the CRALBP cleft map to the pathologic cleft mutations R192H and R221W in α -TTP (46). In addition, R265 is equivalent to K239A in yeast SEC14p that is reported to abolish phosphatidylinositol transfer activity completely *in vitro* (47). These findings suggest that the cleft motif in CRALBP, α -TTP, and SEC14p has an essential role in stimulating the transfer of cognate ligands by acidic phospholipids. It is tempting to speculate that the CRALBP basic cleft may be involved in routing retinoids for the cytoplasmic leaflet of the plasma membrane because of the relatively high content of acidic phospholipid headgroups of the leaflet (48).

Concluding Remarks. Mutations in CRALBP are a possible cause of defective retinal metabolism that leads to the clinical phenotype of retinitis pigmentosa. The crystal structure of CRALBP provides insight into stereospecific binding and into the chemical protection of 11-*cis*-retinal in the human eye. The crystal structure of the pathologic R234W mutation of CRALBP reveals impaired 11-*cis*-retinal release through stabilization of the ligand complex and disruption of a conserved basic surface patch with putative loss of lipid stimulation of retinoid transfer. The results of our study suggest impaired 11-*cis*-retinal release may be a major cause of night blindness and retinal pathology in patients carrying the R234W missense mutation in the *RLBP1* gene (1, 2). This study may help elucidate the issue of visual cycle regulation in the human eye.

Materials and Methods

Cloning. Human *RLBP1* cDNA was obtained from Deutsches Ressourcenzentrum für Genomforschung GmbH and was cloned into the NdeI and XhoI sites of the pET-28a vector (Stratagene). Point mutations were introduced by using the QuikChange kit (Stratagene). A detailed description of methods used is given in *SI Materials and Methods*.

Expression and Purification. *Escherichia coli* BL21 (DE3) cells were transformed and cultured overnight with agitation at 37 °C in LB medium containing 30 µg/mL kanamycin. The cultures were grown at 20 °C to an OD₆₀₀ of 0.7 and then were induced with isopropyl-thiogalactopyranoside at a final concentration of 1 mM for 16 h. Cells were harvested by centrifugation at 5000 × g for 45 min and were resuspended in 250 mL of ice-cold lysis buffer (20 mM imidazole; 100 mM NaCl; 20 mM Tris-HCl, pH 7.4; 1% wt/vol Triton X-100). The cells were disrupted by ultrasonication for 20 min, and the lysate was centrifuged at 20,000 × g for 35 min to remove debris. Proteins were purified by nickel-nitrilotriacetic acid (Ni-NTA) affinity chromatography and subsequent size-exclusion chromatography. The CRALBP mutant R234W was expressed and purified in the same way as the wild-type protein. Selenomethionine-labeled R234W was prepared by the method of methionine biosynthesis inhibition (49) and purified in the same way as the wild type.

Illumination of 11-*cis*-Retinal. 11-*cis*-retinal and its complexes with CRALBP and R234W were illuminated according to the methods of Saari and Bredberg (8). The time-dependent photoisomerization of 11-*cis*-retinal in ethanol, in CRALBP, and in the mutant R234W was recorded by measuring the absorbance at 380 nm, which was used to calculate the amounts of 11-*cis*-retinal ($\epsilon = 24,935 \text{ M}^{-1} \text{ cm}^{-1}$ at 380 nm) and all-*trans*-retinal ($\epsilon = 42,880 \text{ M}^{-1} \text{ cm}^{-1}$ at 383 nm). The photosen-

sivities of 11-*cis*-retinal in presence of ethanol, CRALBP, and R234W were determined with the photosensitivity being defined as the product of the quantum yield and the molar extinction coefficient of the substrate.

Dynamic Light Scattering. DLS analysis has been carried out on a DynaPro Plate reader (Wyatt Technology). The protein concentration was set at 1 mg/mL, and analyses were performed using Dynamics 5.25 software provided by the manufacturer.

Crystallization. Hexagonal crystals of wild-type CRALBP in complex with 11-*cis*-retinal were obtained at 20 °C by the sitting-drop, vapor-diffusion method, in 1.0 M K/Na tartrate, 0.1 M Tris-HCl (pH 7.0), 0.2 M Li₂SO₄. The crystals belong to space group P6₃22 with *a* = 71.92 Å, *b* = 71.92 Å, *c* = 303.20 Å, α = 90°, β = 90°, γ = 120°. Monoclinic crystals of R234W in complex with 11-*cis*-retinal were obtained using the same method in a condition of 20% (wt/vol) PEG 3000, 0.1 M Hepes (pH 7.5), 0.2 M NaCl. The crystals belong to the space group C2 with *a* = 87.93 Å, *b* = 57.88 Å, *c* = 75.15 Å, α = 90°, β = 122.846°, γ = 90°.

Data Collection and Structure Solution. Crystals were flash-cooled in a nitrogen stream at 110 K after 15% glycerol was added. Data for wild-type CRALBP were collected at 100 K at the beamline ID29 (European Synchrotron Radiation Facility, France). Data for the R234W mutant were collected at beamline X06SA (Swiss Light Source, Paul Scherrer Institute, Switzerland) at 100 K. The

structure was solved by SAD using a single crystal of selenomethionine-labeled R234W diffracting to 1.7 Å (32). Data were integrated and scaled with the x-ray detector software for processing single-crystal monochromatic diffraction data (XDS) (50). Selenium positions were determined by heavy-atom substructure search (32, 51). The SAD data set was submitted to a software suite for improvement and objective interpretation of crystallographic electron density maps and automatic construction and refinement of macromolecular models (ARP/wARP) (52), and the primary model was refined using the phenix.refine program from the PHENIX program package (51). The final R234W model contained residues 57–306, 11-*cis*-retinal, and 374 water molecules. The structure of wild-type CRALBP subsequently was solved by molecular replacement using the atomic model of R234W and the program Phaser (53) and was refined to 3.0-Å resolution using the program phenix.refine. Coordinates and structure factors of both structures have been deposited in the RCSB Protein Data Bank with ID codes 3HX3 and 3HY5.

ACKNOWLEDGMENTS. We thank Dr. Rosalie K. Crouch, Storm Eye Institute, Medical University of South Carolina, for the generous gift of 11-*cis*-retinal. We thank Prof. Ulrich Baumann and Prof. Bernhard Erni for comments on the manuscript. Data collection was performed at the Swiss Light Source, Beamline X06DA (PXIII), Villigen, Switzerland, and at the European Synchrotron Radiation Facility, Beamline ID29, Grenoble, France. Financial support was supplied by the Berne University Research Foundation.

- Burstedt MS, Sandgren O, Holmgren G, Forsman-Semb K (1999) Bothnia dystrophy caused by mutations in the cellular retinaldehyde-binding protein gene (RLBP1) on chromosome 15q26. *Invest Ophthalmol Visual Sci* 40:995–1000.
- Burstedt MS, et al. (2001) Ocular phenotype of Bothnia dystrophy, an autosomal recessive retinitis pigmentosa associated with an R234W mutation in the RLBP1 gene. *Archives of Ophthalmology* 119:260–267.
- Maw MA, et al. (1997) Mutation of the gene encoding cellular retinaldehyde-binding protein in autosomal recessive retinitis pigmentosa. *Nat Genet* 17:198–200.
- Morimura H, Berson EL, Dryja TP (1999) Recessive mutations in the RLBP1 gene encoding cellular retinaldehyde-binding protein in a form of retinitis punctata albescens. *Invest Ophthalmol Visual Sci* 40:1000–1004.
- Katsanis N, et al. (2001) Fundus albipunctatus and retinitis punctata albescens in a pedigree with an R150Q mutation in RLBP1. *Clin Genet* 59:424–429.
- Eichers ER, et al. (2002) Newfoundland rod-cone dystrophy, an early-onset retinal dystrophy, is caused by splice-junction mutations in RLBP1. *Am J Hum Genet* 70:955–964.
- Futterman S, Saari JC, Blair S (1977) Occurrence of a binding protein for 11-*cis*-retinal in retina. *J Biol Chem* 252:3267–3271.
- Saari JC, Bredberg DL (1987) Photochemistry and stereoselectivity of cellular retinaldehyde-binding protein from bovine retina. *J Biol Chem* 262:7618–7622.
- McBee JK, Van Hooser JP, Jang GF, Palczewski K (2001) Isomerization of 11-*cis*-retinoids to all-*trans*-retinoids in vitro and in vivo. *J Biol Chem* 276:48483–48493.
- Saari JC, et al. (2001) Visual cycle impairment in cellular retinaldehyde binding protein (CRALBP) knockout mice results in delayed dark adaptation. *Neuron* 29:739–748.
- Trevino SG, Villazana-Espinoza ET, Muniz A, Tsin AT (2005) Retinoid cycles in the cone-dominated chicken retina. *J Exp Biol* 208:4151–4157.
- Futterman S, Saari JC (1977) Occurrence of 11-*cis*-retinal-binding protein restricted to the retina. *Invest Ophthalmol Visual Sci* 16:768–771.
- Fishman GA, et al. (1999) Variation of clinical expression in patients with Stargardt dystrophy and sequence variations in the ABCR gene. *Archives of Ophthalmology* 117:504–510.
- Saari JC, Bredberg DL, Noy N (1994) Control of substrate flow at a branch in the visual cycle. *Biochemistry* 33:3106–3112.
- Travis GH, Golczak M, Moise AR, Palczewski K (2007) Diseases caused by defects in the visual cycle: Retinoids as potential therapeutic agents. *Annu Rev Pharmacol Toxicol* 47:469–512.
- Stecher H, Gelb MH, Saari JC, Palczewski K (1999) Preferential release of 11-*cis*-retinol from retinal pigment epithelial cells in the presence of cellular retinaldehyde-binding protein. *J Biol Chem* 274:8577–8585.
- Muniz A, Villazana-Espinoza ET, Thackeray B, Tsin AT (2006) 11-*cis*-Acyl-CoA:retinol O-acyltransferase activity in the primary culture of chicken Muller cells. *Biochemistry* 45:12265–12273.
- Wu Z, et al. (2006) CRALBP ligand and protein interactions. *Adv Exp Med Biol* 572:477–483.
- Rual JF, et al. (2005) Towards a proteome-scale map of the human protein-protein interaction network. *Nature* 437:1173–1178.
- Nawrot M, et al. (2004) Cellular retinaldehyde-binding protein interacts with ERM-binding phosphoprotein 50 in retinal pigment epithelium. *Invest Ophthalmol Visual Sci* 45:393–401.
- Wu Z, Hasan A, Liu T, Teller DC, Crabb JW (2004) Identification of CRALBP ligand interactions by photoaffinity labeling, hydrogen/deuterium exchange, and structural modeling. *J Biol Chem* 279:27357–27364.
- Wu Z, et al. (2003) Mapping the ligand binding pocket in the cellular retinaldehyde binding protein. *J Biol Chem* 278:12390–12396.
- Golovleva I, et al. (2003) Disease-causing mutations in the cellular retinaldehyde binding protein tighten and abolish ligand interactions. *J Biol Chem* 278:12397–12402.
- Ryan MM, Temple BR, Phillips SE, Bankaitis VA (2007) Conformational dynamics of the major yeast phosphatidylinositol transfer protein sec14p: Insight into the mechanisms of phospholipid exchange and diseases of sec14p-like protein deficiencies. *Mol Biol Cell* 18:1928–1942.
- Bateman A, et al. (2002) The Pfam protein families database. *Nucleic Acids Res* 30:276–280.
- Meier R, Tomizaki T, Schulze-Briese C, Baumann U, Stocker A (2003) The molecular basis of vitamin E retention: Structure of human alpha-tocopherol transfer protein. *J Mol Biol* 331:725–734.
- Stocker A, Tomizaki T, Schulze-Briese C, Baumann U (2002) Crystal structure of the human supernatant protein factor. *Structure (London)* 10:1533–1540.
- D'Angelo I, Welti S, Bonneau F, Scheffzek K (2006) A novel bipartite phospholipid-binding module in the neurofibromatosis type 1 protein. *EMBO Reports* 7:174–179.
- Sha B, Phillips SE, Bankaitis VA, Luo M (1998) Crystal structure of the *Saccharomyces cerevisiae* phosphatidylinositol-transfer protein. *Nature* 391:506–510.
- Schaaf G, et al. (2008) Functional anatomy of phospholipid binding and regulation of phosphoinositide homeostasis by proteins of the sec14 superfamily. *Mol Cell* 29:191–206.
- Derewenda ZS, Vekilov PG (2006) Entropy and surface engineering in protein crystallization. *Acta Crystallogr D* 62:116–124.
- Terwilliger TC (2003) SOLVE and RESOLVE: Automated structure solution and density modification. *Methods Enzymol* 374:22–37.
- Kleywegt GJ, Jones TA (1994) Detection, delineation, measurement and display of cavities in macromolecular structures. *Acta Crystallogr D* 50(Pt 2):178–185.
- Noy N (2000) Retinoid-binding proteins: Mediators of retinoid action. *Biochem J* 348:481–495.
- Palczewski K, et al. (2000) Crystal structure of rhodopsin: A G protein-coupled receptor. *Science* 289:739–745.
- Nakamichi H, Okada T (2006) Crystallographic analysis of primary visual photochemistry. *Angew Chem Int Ed* 45:4270–4273.
- Salgado GF, et al. (2004) Deuterium NMR structure of retinal in the ground state of rhodopsin. *Biochemistry* 43:12819–12828.
- Sugihara M, Buss V, Entel P, Elstner M, Frauenheim T (2002) 11-*cis*-retinal protonated Schiff base: Influence of the protein environment on the geometry of the rhodopsin chromophore. *Biochemistry* 41:15259–15266.
- Saari JC, Bunt-Milam AH, Bredberg DL, Garvin GG (1984) Properties and immunocytochemical localization of three retinoid-binding proteins from bovine retina. *Vision Research* 24:1595–1603.
- Eisenberg D, McLachlan AD (1986) Solvation energy in protein folding and binding. *Nature* 319:199–203.
- Matsumura M, Becktel WJ, Matthews BW (1988) Hydrophobic stabilization in T4 lysozyme determined directly by multiple substitutions of Ile 3. *Nature* 334:406–410.
- Fishman GA, et al. (2004) Novel mutations in the cellular retinaldehyde-binding protein gene (RLBP1) associated with retinitis punctata albescens: Evidence of inter-familial genetic heterogeneity and fundus changes in heterozygotes. *Archives of Ophthalmology* 122:70–75.
- Nakamura M, Lin J, Ito Y, Miyake Y (2005) Novel mutation in RLBP1 gene in a Japanese patient with retinitis punctata albescens. *American Journal of Ophthalmology* 139:1133–1135.
- Saari JC, Nawrot M, Stenkamp RE, Teller DC, Garvin GG (2009) Release of 11-*cis*-retinal from cellular retinaldehyde-binding protein by acidic lipids. *Molecular Vision* 15:844–854.
- Min KC, Kovall RA, Hendrickson WA (2003) Crystal structure of human alpha-tocopherol transfer protein bound to its ligand: Implications for ataxia with vitamin E deficiency. *Proc Natl Acad Sci USA* 100:14713–14718.
- Cavaller L, et al. (1998) Ataxia with isolated vitamin E deficiency: Heterogeneity of mutations and phenotypic variability in a large number of families. *Am J Hum Genet* 62:301–310.
- Phillips SE, et al. (1999) Yeast Sec14p deficient in phosphatidylinositol transfer activity is functional in vivo. *Mol Cell* 4:187–197.
- Lemmon MA (2008) Membrane recognition by phospholipid-binding domains. *Nat Rev Mol Cell Biol* 9:99–111.
- Van Duyn GD, Standaert RF, Karplus PA, Schreiber SL, Clardy J (1993) Atomic structures of the human immunophilin FKBP-12 complexes with FK506 and rapamycin. *J Mol Biol* 229:105–124.
- Kabsch W (1988) Evaluation of single crystal X-ray diffraction data from a position sensitive detector. *J Appl Crystallogr* 21:916–924.
- Adams PD, et al. (2004) Recent developments in the PHENIX software for automated crystallographic structure determination. *Journal of Synchrotron Radiation* 11:53–55.
- Perrakis A, Morris R, Lamzin VS (1999) Automated protein model building combined with iterative structure refinement. *Nature Structural Biology* 6:458–463.
- Qian B, et al. (2007) High-resolution structure prediction and the crystallographic phase problem. *Nature* 450:259–264.
- Delano ML (2002) *The PyMOL Molecular Graphics System* (Delano ML, Palo Alto, CA).