Disease-causing Mutations in the Cellular Retinaldehyde Binding Protein Tighten and Abolish Ligand Interactions*[S]

Received for publication, July 19, 2002, and in revised form, January 16, 2003
Published, JBC Papers in Press, January 20, 2003, DOI 10.1074/jbc.M207300200

Irina Golovleva,a,b Sanjoy Bhattacharyya,a Zhiping Wu,c,d Natacha Shaw,e Yanwu Yang,f Khurshid Andrabhi, Karen A. West,a Marie S. I. Burstedt,f Kristina Forsman,a,h Gösta Holmgren,a Ola Sandgren,a Noa Noy,g Jun Qin, and John W. Crabb,c,d,f

From the Departments of aMedical Biosciences and Ophthalmology, Umeå University, S-901 85 Umeå, Sweden, the bCole Eye Institute, and cLerner Research Institute, Cleveland Clinic Foundation, Cleveland, Ohio 44195, the dDivision of Nutritional Sciences, Cornell University, Ithaca, New York 14853, and the eDivision of Chemical Biology, Cleveland State University, Cleveland, Ohio 44115

Mutations in the human cellular retinaldehyde binding protein (CRALBP) gene cause retinal pathology. To understand the molecular basis of impaired CRALBP function, we have characterized human recombinant CRALBP containing the disease causing mutations R233W or M225K. Protein structures were verified by amino acid analysis and mass spectrometry, retinoid binding properties were evaluated by UV-visible and fluorescence spectroscopy and substrate carrier functions were assayed for recombinant 11-cis-retinol dehydrogenase (rRDH5). The M225K mutant was less soluble than the R233W mutant and lacked retinoid binding capability and substrate carrier function. In contrast, the R233W mutant exhibited solubility comparable to wild type rCRALBP and bound stoichiometric amounts of 11-cis- and 9-cis-retinol with at least 2-fold higher affinity than wild type rCRALBP. Holo-R233W significantly decreased the apparent affinity of rRDH5 for 11-cis-retinol relative to wild type rCRALBP. Analyses by heteronuclear single quantum correlation NMR demonstrated that the R233W protein exhibits a different conformation than wild type rCRALBP, including a different retinoid-binding pocket conformation. The R233W mutant also undergoes less extensive structural changes upon photoisomerization of bound ligand, suggesting a more constrained structure than that of the wild type protein. Overall, the results show that the M225K mutation abolishes and the R233W mutation tightens retinoid binding and both impair CRALBP function in the visual cycle as an 11-cis-retinol acceptor and as a substrate carrier.

Mutations in the human gene RLBP1 encoding the cellular retinaldehyde-binding protein (CRALBP) cause retinal pathology and have been associated with autosomal recessive retinitis pigmentosa (1), Bothnia dystrophy (2, 3), retinitis punctata albescens (4), fundus albipunctatus (5), and Newfoundland rod-cone dystrophy (6). These diseased phenotypes are all characterized by photoreceptor degeneration and night blindness (delayed dark adaptation) but differ in age of onset, rate of progression, and severity. The molecular basis for the clinical differences in these related retinal dystrophies is not well understood and no effective therapies exist for the pathology resulting from impaired CRALBP function.

CRALBP is an abundant, 36-kDa protein in the cytosol of the retinal pigment epithelium (RPE) and Muller cells of the retina where it carries endogenous 11-cis-retinol and 11-cis-retinal (7). The CRALBP ligand binding cavity is mapped in the accompanying report (8). In vivo studies (9) show that CRALBP serves as a major 11-cis-retinol acceptor in the isomerization step of the visual cycle (7, 10, 11), stimulating the enzymatic isomerization of all-trans- to 11-cis-retinol in the rod visual cycle. However, CRALBP appears to function within an RPE protein complex (12) and to serve multiple functions. In vitro, CRALBP facilitates the oxidation of 11-cis-retinol to 11-cis-retinal by 11-cis-retinol dehydrogenase (12, 13), retards 11-cis-retinol esterification in the RPE by lecithin:retinol acyltransferase (13), and is required for hydrolysis of endogenous RPE 11-cis-retinyl ester (14).

Six CRALBP mutations have been linked with retinal pathology, including three missense mutations (R150Q, M225K, and R233W), a frameshift mutation and two predicted splice junction alterations (1, 2, 4, 6). Recombinant CRALBP (rCRALBP) containing the R150Q mutation lacks the ability to bind 11-cis-retinal and exhibits low solubility (1). Toward a better understanding of the molecular basis of retinal pathology associated with RLBP1 gene defects, we report here characterization of rCRALBP containing the M225K or R233W disease-causing mutations (Fig. 1).

EXPERIMENTAL PROCEDURES

Materials—11-cis-Retinal was obtained from the NEI, National Institutes of Health, and 9-cis-retinal was purchased from Sigma. Tritic...
ated 11-cis-retinol was produced by reduction of 11-cis-retinal with NaB[3H]4 (15).

Mutagenesis and Production of rCRALBP Mutants M225K and R233W—Mutant rCRALBP cDNA carrying either the R233W or M225K substitutions were created using The QuikChange site-directed mutagenesis method (Stratagene). Briefly, WT human CRALBP cDNA in the pET19b vector (16) was cleaved with XbaI and HindIII and the coding region subcloned into pBlueSK. The following complimentary oligonucleotides were used to substitute a Lys for a Met at residue 225 (underlined) in mutant M225K: sense, 5'-GAAGATGGTGGACAAGCT-CCAGGATTCCTT-3'; antisense, 5'-AAGGAATCCTGGAGCTTGTCCA-CCATCTTC-3'. To substitute a Trp for an Arg at residue 233 (underlined) in the R233W mutant, complimentary oligonucleotides were also used: sense, 5'-ATTCCTTCCCAGCCTGGTTCAAAGCCATCC-3'; antisense, 5'-GGATGGCTTTGAACCAGGCTGGGAAGGAAT-3'. Each mutagenesis mix was transformed into Escherichia coli strain XL1-Blue (Stratagene), mutant clones identified by restriction mapping with NspI for M225K and MspI for R233W, were amplified, cleaved with XbaI and HindIII, and ligated back into expression vector pET19b (Novagen). Each insert was sequenced in both directions using the ABI PRISM Dye Terminator Cycle Sequencing kit and the model 377 DNA sequencer (PerkinElmer Life Sciences, Applied Biosystems). WT and rCRALBP mutants M225K and R233W were expressed in E. coli strain BL21(DE3)LysS with a N-terminal His tag and purified using nickel-nitrilotriacetic acid-agarose columns (Qiagen) (16). Recombinant protein was quantified according to Bradford (17) using WT rCRALBP previously quantified by amino acid analysis for the standard reference protein.

Mass Spectrometry, Amino Acid Analysis, and Electrophoresis—The masses of the intact mutant proteins were determined by LC-ESMS using a PerkinElmer Life Sciences Sciex API 3000 triple quadrupole electrospray mass spectrometer, a Vydac C4 column (1.0 × 150 mm), an Applied Biosystems model 140D high-performance liquid chromatography system and aqueous acetonitrile/trifluoroacetic acid solvents at a flow rate of 50 µl/min (18, 19). Phenylthiocarbamyl amino acid analysis was performed with an Applied Biosystems model 420H/130/920 automated system and vapor phase HCl hydrolysis (20). Purified rCRALBP mutants M225K and R233W (~100 pmol each) were digested overnight with trypsin, and the peptide digests were analyzed with a PE Biosystems Voyager DE Pro MALDI-TOF mass spectrometer using α-cyano-4-hydroxycinnamic acid as matrix (21, 22). SDS-PAGE was performed.
on 10% or 12% acrylamide gels using the Bio-Rad Mini-Protein II system (8).

Analysis of Retinoid Binding Function—Retinoid labeling of purified apo-rCRALBP with 11-cis-retinal or 9-cis-retinal, removal of excess retinoid, bleaching, and analysis by UV visible spectroscopy and fluorescence spectroscopy were performed in the dark, under dim red illumination as previously described (8, 19).

Analysis of 11-cis-Retinol Dehydrogenase Activity—Human recombinant 11-cis-retinol dehydrogenase (rRDH5) was expressed in Hi-5 insect cells using a baculovirus vector kindly provided by Dr. K. Palczewski (12, 23) and purified to apparent homogeneity by nickel affinity chromatography. rRDH5 oxidation activity was measured at pH 7.5 (8, 24) and reduction activity was measured at pH 5.5 (25) using purified mutant or WT rCRALBP or equimolar amounts of free 11-cis-retinol or 11-cis-retinal as substrate (8). Control assays with free retinoid as substrate were done in the absence of any carrier protein.

Solution State Heteronuclear Single Quantum Correlation NMR—\(^{15}\)N uniformly labeled WT and mutant R233W rCRALBP were prepared by biosynthetic incorporation in E. coli strain BL21(DE3)LyS3 grown in defined minimal media (8, 26). Purified mutant and WT rCRALBP with bound 11-cis-retinal (~0.3 mM) were adjusted to 8% D\(_2\)O (v/v) and transferred to 250 \(\mu\)l of microcell NMR tubes (Shigemi Inc., Allison Park, PA) (8). All NMR experiments were performed at 25 °C with a Varian INOVA 500-MHz spectrometer equipped with a triple resonance probe. Sensitivity enhanced two-dimensional \(^1\)H-\(^{15}\)N heteronuclear single quantum correlation experiments were recorded using water-flipback for water suppression. Data was processed on a Sun UltraSPARC workstation using NMRPipe and Pipp software (8, 27, 28). Holo-protein preparations were maintained in the dark or under dim red illumination to prevent retinoid isomerization.

**RESULTS**

**Expression and Structural Integrity of rCRALBP Mutants M225K and R233W**—WT and mutant rCRALBPs were produced in bacteria and SDS-PAGE of the crude soluble bacterial lysates and re-suspended pellet fractions showed that the M225K mutant was less soluble than the R233W rCRALBP mutant (Fig. 2). The R233W mutant was present in the soluble lysate fraction in amounts comparable to that of the WT protein (Fig. 2). The purified mutant proteins were characterized...
by amino acid analysis (Table I) and by LC ESMS and the determined compositions and intact masses found to be in excellent agreement with the sequence calculated values (M225K, M\text{obs} = 39,110 \pm 3, M\text{calc} = 39,107; R233W, M\text{obs} = 39,145 \pm 4, M\text{calc} = 39,140). About 73% of each mutant protein sequence was confirmed by MALDI-TOF MS peptide mass mapping, including the peptides containing the M225K and R233W substitutions (Supplemental Fig. S1).

**Table II**

| rCRALBP Preparation | Mean Apparent $K_d$ (nM) |
|----------------------|--------------------------|
|                      | 11-cis-Retinal | 9-cis-Retinal |
| Wild type            | 21.0 ± 3.2 (n = 5) | 53.3 ± 9.6 (n = 7) |
| R233W                | 10.3 ± 2.0 (n = 3) | 24.3 ± 5.7 (n = 3) |

**Table III**

| Reduction of 11-cis-retinal to 11-cis-retinol | $K_a$ (μM) | $V_{max}$ (nmol/min/mg) |
|-----------------------------------------------|------------|------------------------|
| Mutant R233W (n = 6)                          | 12.5 ± 0.5 | 475 ± 28               |
| Mutant M225K (n = 6)                          | 5.8 ± 0.6  | 367 ± 61               |
| WT CRALBP (n = 6)                             | 1.7 ± 0.3  | 411 ± 44               |
| Free 11-cis-retinal (n = 4)                   | 6.0 ± 0.2  | 440 ± 40               |

**Oxidation of 11-cis-retinol to 11-cis-retinal**

| Mutant R233W (n = 4) | 10.8 ± 0.5 | 129 ± 09 |
| Mutant M225K (n = 4) | 6.8 ± 1    | 123 ± 15 |
| WT CRALBP (n = 4)    | 2.5 ± 0.3  | 143 ± 10 |
| Free 11-cis-retinol (n = 4) | 7.5 ± 0.3 | 117 ± 3 |

**Discussion**

Functionally impaired CRALBP was first associated with autosomal recessive retinitis pigmentosa (arRP) in 1997 when the missense mutation R150Q was found in the RLBP1 gene from a family in India (1). Retinitis pigmentosa is a family of inherited diseases with many forms and causative genes and classification of the disease types continue to evolve (29). Since 1997, five other recessive defects in the RLBP1 gene have been found to cause retinal dystrophies, including the two missense mutations M225K and R233W associated with retinitis punctata albescens and Bothnia dystrophy (2, 5). Pathological mutations in the CRALBP gene have now been associated with other phenotypes and detected in pedigrees from Europe, the Middle East, Newfoundland, and India (1–6). RLBP1 gene defects are thought to be a rare cause of retinal disease (4); however, in northern Sweden the high prevalence of Bothnia dystrophy caused by the R233W mutation constitutes a significant medical problem for which therapies are sought (2, 3). To better understand the molecular basis of retinal pathology associated with impaired CRALBP and possibilities for therapeutic intervention, we have pursued structure-function studies of the mutant rCRALBP containing the disease causing substitutions M225K and R233W.

The primary structural integrity of the purified M225K and R233W mutant recombinant proteins was confirmed by amino acid analysis and mass spectrometry. In contrast to the largely insoluble R150Q rCRALBP associated with arRP (1), the
R233W mutant exhibits solubility comparable to that of WT rCRALBP, whereas the M225K mutant is less soluble than WT rCRALBP but significantly more soluble than the R150Q mutant. With regard to retinoid binding properties, UV-visible spectral analysis revealed that the M225K mutant resembled the R150Q mutant and completely lacked the ability to bind cis-retinoids (1). In contrast, mutant R233W bound stoichiometric amounts of 11-cis- or 9-cis-retinal based on absorbance spectral ratios (19).

Fluorescence titrations yielded apparent equilibrium dissociation constants for the R233W mutant that demonstrated nanomolar affinities for 9-cis- and 11-cis-retinal that were about 2-fold tighter than determined for WT rCRALBP. The variability of the retinoid affinity data (relative standard error of the mean was ~15–23%) was within the limits of experimental error of the titration methodology and due, in part, to the low aqueous solubility of retinoids and variable apo-protein stability. Furthermore, the protein concentration (0.5 μM) used in the fluorescence titrations was significantly higher than the apparent $K_d$ values, and, under these conditions, $K_d$ values

---

**Fig. 4.** Heteronuclear single quantum correlation NMR spectra of uniform $^{15}$N-labeled WT and mutant R233W rCRALBP. HSQC NMR spectra for WT holo-rCRALBP (blue) and holo-R233W rCRALBP (red) were recorded separately in the dark then overlaid. These experiments correlate directly bonded $^1$H-$^{15}$N pairs within the protein structures and show that substantial conformational differences exist between the WT and mutant R233W holo-proteins. Met-222 in the WT spectra does not overlay with any resonance in the R233W spectra, demonstrating that the proteins differ in retinoid binding pocket conformation. Trp-233 in the R233W spectra was assigned by an additional characteristic Trp resonance in the downfield chemical shift region relative to WT rCRALBP. Other residue assignments were determined elsewhere (8).

**Fig. 5.** Heteronuclear single quantum correlation NMR spectra of uniform $^{15}$N-labeled mutant R233W and WT rCRALBP before and after bleaching. The $^1$H-$^{15}$N correlation spectrum for the proteins with bound 11-cis-retinal was recorded in the dark (red), and the sample was then exposed to bleaching illumination and re-analyzed (blue) by the same $^1$H-$^{15}$N correlation experiment. A, mutant R233W HSQC spectra. The vast majority of the resonances overlay in both experiments, indicating very little conformational change occurs upon ligand isomerization in the R233W ligand binding pocket. B, WT rCRALBP HSQC spectra. Although most residues remain unchanged upon bleaching (8), more chemical shift changes are apparent than in the R233W spectra.
These data are consistent with the notion that CRALBP affects the holo-R233W mutant as substrate, reflecting lower affinity retinoid (12). An even higher mutant approximated that for the enzyme reaction with free 11-cis-R233W mutations decrease the apparent affinity of rRDH5 for demonstrating that rCRALBP harboring either the M225K or the analyses performed here with purified recombinant proteins rCRALBP and its R233W mutant may therefore be larger than 12402 including different retinoid-binding cavity conformations. Furthermore, structural NMR. HSQC NMR spectra showed that the three-dimensional structure of the R233W mutant with bound ligand properties of the R233W mutant were obtained by two-dimensional with CRALBP mutations. CRALBP serves as the major gand binding pocket. The results of this study implicate im-

pears to disrupt critical interactions within the retinoid bind-

ing cavity, perhaps by opening the hydrophobic region to greater solvent accessibility, which in turn precludes specific ligand interaction and lowers M225K protein solubility. The function of CRALBP in the visual cycle depends upon the rapid association and dissociation of retinoid from the ligand binding pocket. The results of this study implicate impairment of both retinoid binding and release as causes of the night blindness and retinal pathology reported for human patients with CRALBP mutations. CRALBP serves as the major 11-cis-retinol acceptor in the isomerization reaction of the rod visual cycle, therefore the lack of retinoid binding by M225K rCRALBP significantly slows the enzymatic conversion of all-trans to 11-cis-retinol, as observed for the CRALBP knockout mouse (9). Binding of 11-cis-retinol by apoCRALBP, coupled with its oxidation to 11-cis-retinal by RDH5, provides a strong driving force for isomerization (13, 33). Interactions with other proteins likely facilitate the release of ligand from the CRALBP binding pocket, promoting catalytic rather than stoichiometric retinoid binding (9, 12). The R233W mutation results in tighter rCRALBP retinoid binding and lower RDH5 affinity for rCRALBP bound retinoid. The overly tight retinoid binding caused by the R233W mutation appears to hinder the rapid release of ligand, resulting in a "full house" effect that impairs 11-cis-retinol acceptor function and slows the isomerization of all-trans to 11-cis-retinol.

Acknowledgment—We thank Dr. John C. Saari for useful discussions and for reviewing the manuscript prior to publication.

REFERENCES

1. Maw, M. A., Kennedy, B., Knight, A., Bridges, R., Roth, K. E., Mani, E. J., Mukkadan, J. K., Nancarrow, D., Crabb, J. W., and Denton, M. J. (1997) Nat. Genet. 17, 198–200.
2. Burstedt, M. S., I, Sandgren, O., Holmgren, G., and Forsman-Semb, K. (1999) Invest. Ophthalmol. Vis. Sci. 40, 995–1000.
3. Burstedt, M. S., Sandgren, O., Forsman-Semb, K., Golovleva, I., Janunger, T., Wachtmeister, L., and Sandgren, O. (2001) Arch. Ophthalmol. 119, 260–267.
4. Marmorra, H., Benson, E. L., and Dryja, T. P. (1999) Invest. Ophthalmol. Vis. Sci. 40, 1000–1004.
5. Katsanis, N., Shroyer, N. F., Lewis, R. A., Cavender, J. C., Al-Rajhi, A. A., Jabluk, M., and Lupski, J. R. (2003) Clin. Genet. 60, 424–429.
6. Eichers, E. R., Green, J. S., Stockton, D. W., Jackman, C., Whelan, J., McNamara, J. A., Johnson, G. J., Lupski, J. R., and Katsanis, N. (2002) J. Am. Human Genet. 70, 955–964.
7. Saari, J. C. (1994) in The Retinoids (Sporn, M. A., Roberts, A. B., and Goodman, D. S., eds) pp. 351–385, Raven Press, Ltd., New York.
8. Wu, Z., Yang, Y., Shaw, N., Bhattacharya, S., Yan, L., West, K., Roth, K., Noy, N., Qin, J., and Crabb, J. W. (2000) J. Biol. Chem. 275, 12980–12986.
9. Saari, J. C., Navrot, M., Kennedy, B. N., Hurley, J. B., Gwinn, G. H., Huang, J., and Crabb, J. W. (2001) Neuron 29, 739–748.
10. Crosthwaite, S. R., Chader, G. J., Wigger, B., and Pepperberg, D. R. (1996) Photobiology 64, 613–621.
11. Rando, R. R. (2001) Chem. Rev. 101, 1881–1896.
12. Bhattacharya, S. K., Wu, Z., Jin, Z., Yan, L., Miyagi, M., West, K., Navrot, M., Saari, J. C., and Crabb, J. W. (2000) FEBS J. 16, A14.
13. Saari, J. C., Bredberg, D. L., and Noy, N. (1994) Biochemistry 33, 3106–3112.
14. Stecher, H., Gelb, M. H., Saari, J. C., and Palczewski, K. (1999) J. Biol. Chem. 274, 8577–8585.
15. Garwin, G. G., and Saari, J. C. (2000) Methods Enzymol. 316, 313–324.
16. Crabb, J. W., Carlson, A., Chen, Y., Goldflam, S., Intres, R., West, K. A., Hulmes, J. D., Kapron, J. T., Luck, L. A., Horwitz, J., and Bok, D. (1998) Protein Sci. 7, 746–757.
17. Bradford, M. M. (1976) Anal. Biochem. 72, 248–254.
18. Crabb, J. W., Chen, Y., Goldflam, S., West, K., and Kapron, J. (1998) Methods Mol. Biol. 89, 91–104.
19. Crabb, J. W., Nie, Z., Chen, Y., Hulmes, J. D., West, K. A., Kapron, J. T., Ruuska, S. E., Noy, N., and Saari, J. C. (1998) J. Biol. Chem. 273, 12712–20729.
20. Crabb, J. W., West, K. A., Dodson, W. S., and Hulmes, J. D. (1997) in Current Protocols in Protein Science, Unit 11.9, Supplement 7 (Coligan, J. E., Floegel, H. L., Smith, J. A., and Speicher, D. W., eds) pp. 11.9.1–11.9.42, John Wiley & Sons, Inc., New York.
21. West, K. A., Yan, L., Miyagi, M., Crabb, J. S., Marmorstein, A. D., Marmorstein, L., and Crabb, J. W. (2001) Exp. Eye Res. 73, 479–491.
22. Miyagi, M., Sakaguchi, H., Darrow, R. M., Yan, L., West, K. A., Aukar, K. S., Stuehr, D. J., Hollyfield, J. G., Organisciak, D. T., and Crabb, J. W. (2002) Mol. Cell. Proteomics 1, 293–303.
23. Jang, G. F., McBee, J. K., Alekseev, A. M., Haeseleer, F., and Palczewski, K. (2000) J. Biol. Chem. 275, 28128–28138.
24. Futterman, S., and Saal, L. D. (1961) J. Biol. Chem. 236, 1652–1657.
25. Luck, L. A., Barrows, S. A., Venters, R. A., Kapron, J., Roth, K. A., Paradis, S. A., and Crabb, J. W. (1997) in Techniques in Protein Chemistry VIII (Mashak, D. ed) pp. 439–448, Academic Press, San Diego, CA.
26. Delaglio, F., Grzesiek, S., Vuister, G. W., Zhu, G., Pfeifer, J., and Bax, A. (1995) J. Biomol. NMR 6, 277–295.
27. Garrett, D. S., Powers, R., Renhennoh, A. M., and Clore, G. M. (1991) J. Magn. Reson. 95, 214–220.
28. Phelan, J. K., and Bok, D. (2000) Mol. Vis. 6, 116–124, http://www.molvis.org/molvis/v6/v6a16.
29. Noy, N. (1999) in Handbook of Experimental Pharmacology (Nau, H., and Blaner, W. S., eds) Vol. 139, pp. 39–113, Springer-Verlag, Heidelberg.
30. Dong, D., Ruuska, S., Levinthal, D. J., and Noy, N. (1999) J. Biol. Chem. 274, 25689–25698.
31. Budhu, A. S., Gillilan, R., and Noy, N. (2001) J. Mol. Biol. 305, 939–949.
32. McLee, J. K., Kuksa, V., Alvarez, R., de Lera, A. B., Prezado, O., Haeseleer, F., Sokal, I., and Palczewski, K. (2000) Biochemistry 39, 11370–11380.
33. Kennedy, B. N., Huang, J., Saari, J. C., and Crabb, J. W. (1998) Mol. Vis. 4, 14, http://www.molvis.org/molvis/v4/v4p14.
34. Crabb, J. W., Andrahi, K., Shroyer, N., Wu, Z., Bhattacharya, S., West, K., Burstedt, M., Sandgren, O., Noy, N., and Golovleva, I. (2001) Invest. Ophthalmol. Vis. Sci. 42, 3524 (Abstr. 8655).