Cellular retinaldehyde-binding protein (CRALBP) carries 11-cis-retinal and/or 11-cis-retinol as endogenous ligands in the retinal pigment epithelium (RPE) and Müller cells of the retina and has been linked with autosomal recessive retinitis pigmentosa. Ligand interactions determine the physiological role of CRALBP in the RPE where the protein is thought to function as a substrate carrier for 11-cis-retinol dehydrogenase in the synthesis of 11-cis-retinal for visual pigment regeneration. However, CRALBP is also present in optic nerve and brain where its natural ligand and function are not yet known. We have characterized the interactions of retinoids with native bovine CRALBP, human recombinant CRALBP (rCRALBP) and five mutant rCRALBPs. Efforts to trap and/or identify a Schiff base in the dark, under a variety of reducing, denaturing, and pH conditions were unsuccessful, suggesting the lack of covalent interactions between CRALBP and retinoid. Buried and solvent-exposed lysine residues were identified in bovine CRALBP by reductive methylation of the holoprotein followed by denaturation and reaction with [3H]acetic anhydride. Radioactive lysine residues were identified by Edman degradation and electrospray mass spectrometry following proteolysis and purification of modified peptides. Human rCRALBP mutants K152A, K221A, and K294A were prepared to investigate possible retinoid interactions with buried or partially buried lysines. Two other rCRALBP mutants, I162V and Q210R, were also prepared to identify substitutions altering the retinoid binding properties of a random mutant. The structures of all the mutants were verified by amino acids and mass spectral analyses and retinoid binding properties of all the mutants were verified by amino acids and mass spectral analyses and retinoid binding properties of a random mutant. The data implicate Gln-210 and Lys-221 as components of the CRALBP retinoid binding cavity and are discussed in the context of ligand interactions in structurally or functionally related proteins with known crystallographic structures.

The cellular retinaldehyde-binding protein (CRALBP) is thought to play a fundamental role in vitamin A metabolism in the retina and retinal pigment epithelium (RPE). Notably, mutations in the human CRALBP gene can result in autosomal recessive retinitis pigmentosa (1). In vitro CRALBP serves as a substrate carrier protein for enzymes of the mammalian visual cycle, modulating whether 11-cis-retinol (11-cis-Rol) is stored as an ester in the RPE or oxidized by 11-cis-Rol dehydrogenase to 11-cis-retinal (11-cis-Ral) for visual pigment regeneration (2). In the RPE and Müller cells of the retina, CRALBP carries endogenous 11-cis-retinoids, the isomers of vitamin A utilized for phototransduction. However, CRALBP is not always associated with a retinoid ligand and more than one physiological role for the protein appears likely (3). The protein is also present in ciliary body, cornea, pineal gland, optic nerve, brain, transiently in iris, but not in the rod and cone photoreceptors. CRALBP is expressed in developing retina and RPE before the tissues contain 11-cis-retinoids or the enzyme responsible for generating 11-cis-retinoids (3). Apparently the protein serves functions unrelated to visual pigment regeneration in brain and tissues not involved in the visual cycle and may bind ligands other than retinoids.

CRALBP was first detected in retina about 20 years ago and shown to carry 11-cis-Rol and 11-cis-Ral as endogenous ligands (4, 5). Structure function studies have defined ligand stereoselectivity and photosensitivity (6), developed a topological and epitope map (7), established in vitro evidence for a substrate carrier function in RPE (8, 9) and produced human recombini-
nant CRALBP (10, 11). The primary structures of bovine (12), human (13, 14), and mouse CRALBP (15) have been determined and are ~87% identical in protein sequence. Investigation of cis-elements and transcription factors required for tissue specific expression of CRALBP are in progress (16).

As part of ongoing efforts to better understand the normal functions of CRALBP, we are characterizing CRALBP-ligand interactions and the retinoid binding pocket. Recent studies have localized the retinoid-binding domain to the C-terminal region (17) and characterized ligand interactions by NMR (18) and circular dichroism (11). The report linking mutations with autosomal recessive retinitis pigmentosa also demonstrated that rCRALBP mutant R150Q lacks retinoid binding function (1). Here we demonstrate that 11-cis-Ral does not appear to be covalently attached to CRALBP. We further show by chemical modification, site-directed mutagenesis, and retinoid binding analyses that Gln-210 and Lys-221 are likely components of the CRALBP retinoid binding pocket.

EXPERIMENTAL PROCEDURES

Materials—Native bovine CRALBP was purified from frozen bovine dark retina according to Lawson, Lawrence, and Bredberg (19). Human recombinant CRALBP (rCRALBP) was produced in Escherichia coli as described elsewhere (10, 11). 11-cis-Ral was obtained from the National Eye Institute, NIH through R. Crouch and 9-cis-Ral was purchased from Sigma.

Analysis of Covalent versus Noncovalent CRALBP Ligand Interactions—To explore the possibility that 11-cis-Ral was covalently attached to CRALBP by a Schiff base linkage, CRALBP was labeled with [3H]11-cis-Ral as described previously (9) and mixed in the dark (7.5 μM final concentration of CRALBP) at room temperature with either 50 mM NaBH₄ in 50 mM Tris, pH 7.4, or 300 mM borane dimethylamine in 5 mM MOPS, 30 mM sodium acetate, 4 mM sodium phosphate, pH 4.0. Various denaturants were then added in the dark to give the following final conditions: pH 4, pH 10, 1% SDS, 1% Triton X-100, 8 μM urea, and 50% ethan. After 20 min of further incubation at room temperature, an equal volume of ice-cold ethanol was added followed by 3 volumes of hexane. The upper phase was removed, and the extraction with hexane repeated. Radioactivity was determined in the combined upper phases by liquid scintillation counting. Extraction of the retinoid from the protein in the absence of reducing and denaturing agents served as the control. For some experiments, the hexane-urea was dried in a stream of flowing argon, dissolved in 68% aqueous acetonitrile, and analyzed by reverse phase HPLC (5). In a second approach exploring the possibility of a Schiff base linkage, a solution of CRALBP complexed with 11-cis-Ral was prepared in 10 mM MOPS buffer at pH 7 and a UV-visible absorption spectrum obtained. SDS and HCl were added to the solution under red illumination to give final concentrations of 1% SDS and 0.5% HCl. A second spectrum was then obtained and a difference spectrum generated.

Identification of Solvent-accessible and -inaccessible Lysine Residues by Chemical Modification—Reductive methylation of native bovine CRALBP with bound 11-cis-Ral was performed essentially according to Longstaff and Rando (20). The CRALBP retinoid complex was incubated overnight in the dark with 2 mM formaldehyde and 20 mM pyridine/borane, 10 mM PIPES at pH 6.5. After 24 h, fresh formaldehyde and pyridine/borane were added. Spectral analysis after 48 h of methylation demonstrated no change in the 425- and 280-nm absorbance, indicating the protein-retinoid complex was still intact. The protein was then denatured in the light by dialysis against 6 M guanidine HCl, 0.1 M borate, pH 9, and acetylated with 4 mM [3H]acetic anhydride for 30 min at room temperature followed by exhaustive dialysis to remove background [3H] (21). To identify acetylation sites, the modified apoprotein was then denatured in the light by dialysis against 6 M guanidine HCl and 1% SDS and pH 4, respectively. A second spectrum was then obtained and a difference spectrum generated. UV-visible absorption spectrum obtained. SDS and HCl were added to the protein in the Ni-NTA spin column buffer containing 250 mM imidazole. Excess retinoid was removed from apoprotein preparations by liquid scintillation counting. Total acetylation was found to be about 1.2-fold molar excess over CRALBP) was incubated with the clarified bacterial lysate for 15 min at 4 °C in the dark prior to spin column purification. For retinoid labeling of purified apo-rCRALBP, either 11-cis-Ral or 9-cis-Ral (1.2-fold molar excess over CRALBP) was incubated for 15 min at 4 °C with the protein in the Ni-NTA spin column buffer containing 250 mM imidazole. Excess retinoid was removed from apo-protein preparations prior to UV-visible absorbance measurements by molecular sieve chromatography using Sephadex G-15. Retinoids were added in a concentrated solution (1–2 mg/ml) in ethanol (11). Bleaching of rCRALBP samples and retinoid binding measurements by scanning UV-visible spectrophotometry (with either a Hitachi U-2000 spectrophotometer or a Hewlett Packard 8452A diode array spectrophotometer) were as described previously (11). The operation of the Cary 11-cis-Ral-labeled rCRALBP (εmax/ε280 = 3.2) and for 9-cis-Ral-labeled rCRALBP (εmax/ε280 = 2.2) approximate the spectral ratio values for rCRALBP fully saturated with these retinoids (11). Observed absorbance spectral ratios (A280/A260 and A280/A240) were used to estimate rCRALBP retinoid binding stoichiometries.

Fluorescence spectroscopy was also used to evaluate apo wild type...
and apo mutant rCRALBs interactions with retinoid by monitoring the quenching of tryptophan fluorescence upon ligand binding. The amount of quenching is a function of the location and orientation of the ligand relative to the tryptophan residues. The protein was excited at 280 nm, and tryptophan fluorescence emission monitored at 340 nm (using a SPEX models fluorolog 2 spectrofluorometer). Titrations with 11-cis- and 9-cis-Ral were carried out by monitoring the decrease in the intrinsic fluorescence of the aequorin (0.5 μM rCRALP) and analyzed by fitting the data to an equation derived from simple binding theory as described elsewhere (24).

**Amino Acid Analysis, Edman Degradation, Electrophoresis, and Protein Quantification—Phenylthiocarbamyl amino acid analysis was performed using an Applied Biosystems model 420H/130/920 automated analysis system (25), and Edman degradation was performed using an Applied Biosystems gas phase protein sequencer model 470/120/900 (12). To identify [3H]acetyl-lysine, sequencer fractions from each Edman cycle were analyzed for tritium by liquid scintillation counting. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed on 12% acrylamide gels according to Laemmli using a Mini-Protein II slab gel system (Bio-Rad). Apo-rCRALBP was quantified using the Bio-Rad protein assay (26) calibrated with rCRALBP previously quantified by amino acid analysis.

**Mass Spectrometry—ESMS and liquid chromatography ESMS (LC-ESMS) were performed with a Perkin-Elmer Sciex API 300 (Concord, Ontario, Canada) fitted with an articulated ion spray plenum and an atmospheric pressure ionization source (27). Initial tuning and calibration was with a standard mixture of polypropylene glycol from Perkin-Elmer Sciex. Resolution was adjusted to about 50 valley between adjacent isotope peaks in a singly charged cluster, allowing singly charged ions to be identified by apparent spacing between peaks and doubly charged ions to be distinguished from those with higher charge states. Nitrogen was used as the nebulization gas (at 40 p.s.i.) and curtain gas and was supplied from a Dewar (XL-45, Taylor Wharton) of liquid nitrogen (Merriam-Graves, Claremont, NH). LC-ESMS were acquired in positive ion mode at an orifice potential of 50 V. For LC-ESMS of intact proteins a scan range of m/z 800–1750 was used with 0.1 atomic mass unit steps and a scan time of 7.5 s. Analysis of peptides a scan range of m/z 250–2200 was used with 0.25 atomic mass unit steps and a scan time of 6 s. Synthetic peptides of known mass were used to verify correct calibration. RP-HPLC for LC-ESMS was performed at a flow rate of 5 μl/min on a Perkin-Elmer C18 capillary column (0.5 × 150 mm) using an Applied Biosystems model 140 HPLC system and aqueous acetonitrile/trifluoroacetic acid solvents. To verify the structures of the mutant rCRALBP (40 μg) were reduced with dithiothreitol and alkylated with iodoacetamide in the presence of 8 M urea, 400 mM ammonium bicarbonate, pH 8. The carboxymethylated proteins were digested with trypsin (Promega) in 1 M urea, 50 mM ammonium bicarbonate (2.5% trypsin by weight, overnight at 37 °C) and the tryptic digest (4 μg) analyzed by LC-ESMS. A portion (50%) of the mutant I162V tryptic digest was further fragmented with endoproteinase Asp-N (Boehringer Mannheim) (2% protease by weight, 6 h at 37 °C) under the same conditions and analyzed by LC-ESMS. Following subtilisin digestion of modified bovine CRALBP, portions of select chromatography fractions containing [3H]acetyl-lysine were dried, resuspended in 0.2–5% formic acid, 50% methanol and analyzed by infusion at 5 μl/min to confirm the presence of modified amino acids.

**RESULTS

**CRALBP Ligand Interactions Are Noncovalent—Attempts to trap a putative Schiff base were based on previous studies (8, 28) demonstrating that the aldehyde of 11-cis-Ral was not accessible to water soluble reagents when bound to CRALBP. Reducing agents (NaBH₄, borane dimethylamine) were added in the dark to a solution of [3H]11-cis-Ral bound to CRALBP and then protein denaturants were added in the dark. Following a 20-min incubation period, tryptophan was extracted into hexane and radioactivity determined. The results of these experiments are shown in Fig. 1. Nearly quantitative amounts of retinoid were extracted from CRALBP regardless of the denaturant or reducing agent (Fig. 1). HPLC analysis of the extracted retinoid from several of the treatments at neutral pH showed that [3H]11-cis-Ral was the only retinoid present (not shown). Treatments at low pH resulted in considerable destruction of the retinoid, but again 11-cis-Ral was the major product detected. If the CRALBP was bleached with white light in the presence of the reducing agents, the product of the reaction was all-trans-Ral, as reported previously (5). In another approach to identify a Schiff base, the absorption spectrum of CRALBP complexed with [3H]11-cis-Ral was incubated with 50 mM NaBH₄, (panel A) or 300 mM borane dimethylamine (panel B) under red illumination. The indicated denaturants or additives were then added, and the incubation continued for 20 min at room temperature. Retinoids were then extracted into hexane and radioactivity determined. Samples marked CRALBP contain the binding protein without denaturant or reducing agent.

![Fig. 1. Extraction of retinoids following treatment of CRALBP with reducing agents and denaturants. CRALBP complexed with [3H]11-cis-Ral was incubated with reducing agents at pH 7. The products were analyzed by RP-HPLC (panel A) or SDS-PAGE (panel B).](image)

**Identification of Buried and Solvent-exposed Lysine Residues in CRALBP—Native bovine CRALBP was reductively methylated then denatured in 6 M guanidine HCl, radioactively acetylated, fragmented with subtilisin, and the peptides fractionated by RP-HPLC (Fig. 3A). Approximately 87% of the radioactivity applied to the HPLC was accounted for in the chromatography fractions (Fig. 3B). Acetylated residues were identified by sequence and mass spectrometric analysis of the radioactive chromatography fractions (Fig. 3B). Radioactive lysine residue 221 was identified in chromatography fractions 10 and 22 within peptide DLRK (residues 215–222); it was acetylated but not methylated based on ESMS analysis and therefore was completely accessible to solvent (observed mass = 703.5, calculated mass = 703.3). Radioactive lysine residue 152 was identified in chromatography fractions 10, 27, 28, and 30 within peptide residues 152–154, 148–152, 148–155, and 148–156. Radioactive lysine residue 294 was identified in chromatography fractions 35, 42, and 46 within peptide residues 287–294, 287–296, and 287–298. Peptides containing lysine
residues 152 and 294 were found by ESMS to exhibit masses either about 42 or 56 Da greater than the calculated masses for the unmodified peptides, consistent with the presence of either N-acetyl-lysine or N-acetyl-N-methyl-lysine (not shown). In addition, during Edman analysis, the phenylthiohydantoin amino acid encountered at the lysine positions with the larger mass modification eluted later than phenylthiohydantoin-acetyl-lysine (not shown). These results suggest that residues 152 and 294 may be partially inaccessible to solvent. Thirteen of the 16 lysine residues were not recovered in peptides with substantial radioactivity and are considered to be methylated and fully exposed to solvent (Fig. 4). Lysine residues fully accessible to solvent appear to exist in bovine CRALBP at positions 27, 46, 48, 54, 90, 103, 185, 204, 235, 254, 258, 260, and 298.

Mutagenesis Strategy and Structural Verification of rCRALBP Site-directed Mutants—A previous report (17) described a three point rCRALBP mutant with reduced retinoid binding capability that resulted from random PCR misincorporation and contained mutations at residues 9 (Met to Thr), 162 (Ile to Val) and 210 (Gln to Arg). Positions 162 and 210 are within the retinoid-binding domain (17), and therefore rCRALBP mutants I162V and Q210R were prepared to determine which of these substitutions influenced retinoid binding. To probe the possibility that lysine may reside within the retinoid binding pocket and influence ligand interactions (as in visual pigments and CRBP), buried or partially buried lysine residues (Fig. 4) were substituted with alanine and mutant rCRALBPs K152A, K221A, and K94A produced for retinoid binding studies. Typical SDS-PAGE profiles of purified rCRALBP mutants are shown in Fig. 5. All intact mutants were structurally characterized by amino acid analysis (Table I) and electrospray mass spectrometry (Table II) and found to exhibit compositions and molecular weights in excellent agreement with the known values. Each mutation was confirmed by identification of peptides containing the relevant substitutions (Table II).

Retinoid Binding Analyses by UV-visible Spectroscopy—Retinoid binding analyses performed by labeling rCRALBP with either 11-cis-Ral or 9-cis-Ral in bacterial lysates followed by protein purification are shown in Figs. 6-8. Wild type rCRALBP absorption spectra (Fig. 6) exhibit the characteristic chro-
morphic maxima at 425 nm for bound 11-cis-Ral and at 400 nm for bound 9-cis-Ral. Upon exposure to light, the chromophore absorbance maxima shift to ~380 nm because of the production of unbound all-trans-Ral. rCRALBP mutant I162V was found to bind both 11-cis-Ral and 9-cis-Ral in a manner similar to wild type rCRALBP; mutant Q210R exhibits wild type-like binding with 11-cis-Ral but reduced binding with 9-cis-Ral (Fig. 7). rCRALBP mutants K152A, K221A, and K294A also exhibit wild type-like binding with 11-cis-Ral, and mutants K152A and K294A exhibit wild type binding with 9-cis-Ral (Fig. 8). Of the three lysine mutants, only K221A exhibits reduced binding with 9-cis-Ral (Fig. 8). Retinoid binding analyses with 11-cis-Ral and 9-cis-Ral were also performed with apo-rCRALBP by labeling the protein with retinoid after purification. Results of UV-visible spectral analyses of the apoprotein preparations agree overall with the results in Figs. 6–8. Approximate binding stoichiometries obtained with the apo-rCRALBP preparations for n = 2 measurements were: wild type, 1.0 mol 11-cis, 0.6 mol 9-cis; mutant I162V, 1.1 mol 11-cis, 1.0 mol 9-cis; mutant Q210R, 0.5 mol 11-cis, 0.3 mol 9-cis; mutant K152A, 0.8 mol 11-cis, 0.7 mol 9-cis; mutant K221A, 0.8 mol 11-cis, 0.3 mol 9-cis; and mutant K294A, 1.0 mol 11-cis, 0.8 mol 9-cis.

**Retinoid Binding Analyses by Fluorescence Spectroscopy**—Equilibrium dissociation constants ($K_d$) of complexes of wild type and mutant rCRALBP with 11-cis-Ral and 9-cis-Ral were measured with multiple apoprotein preparations by fluorescence titrations monitored by following the decrease in the intrinsic fluorescence of the proteins upon ligand binding. Apparent $K_d$ values extracted from the titration data were in the nanomolar range for both retinoids (Table III). Consistent with an earlier report (6), the fluorescence titrations (Table III) corroborate a lower affinity of wild type rCRALBP for 9-cis-Ral ($K_d$ ~53 nM) than for 11-cis-Ral ($K_d$ ~21 nM). The titrations also show that the affinity of rCRALBP mutants Q210R and K221A for 9-cis-Ral ($K_d$ ~70 nM) is reduced relative to the wild type protein. Extraction of binding sites from the titration data yielded average number of binding sites = 0.4 ± 0.05 for both 11-cis-Ral (n = 38) and 9-cis-Ral (n = 32).
The CRALBP Retinoid Binding Pocket

TABLE I
Amino acid compositions of rCRALBP mutants

Compositions were determined by phenylthiocarbamyl amino acid analysis of the purified mutant proteins and average error determined as described elsewhere (25). Cys and Trp were not determined. WT represents the wild type fusion rCRALBP (10, 11).

| Amino acid | WT    | I162V | Q210R | K152A | K221A | K294A |
|------------|-------|-------|-------|-------|-------|-------|
| Asx (D + N)| 29    | 27.6  | 29.5  | 26.3  | 25.9  | 28.2  |
| Glx (E + Q)| 57    | 58.4  | 56.4  | 54.1  | 56.6  | 53.3  |
| Ser (S)    | 17    | 17.4  | 17.4  | 17.0  | 17.8  | 16.8  |
| Gly (G)    | 21    | 25.0  | 21.2  | 25.1  | 24.8  | 24.6  |
| His (H)    | 17    | 14.1  | 17.0  | 12.6  | 14.5  | 16.2  |
| Arg (R)    | 20    | 18.4  | 21.0  | 20.2  | 18.2  | 20.4  |
| Thr (T)    | 15    | 15.0  | 15.2  | 15.0  | 15.2  | 14.7  |
| Ala (A)    | 23    | 32.8  | 23.0  | 33.0  | 32.2  | 31.6  |
| Pro (P)    | 15    | 17.9  | 13.3  | 16.5  | 19.3  | 16.0  |
| Tyr (Y)    | 10    | 7.6   | 10.1  | 7.4   | 8.3   | 8.4   |
| Val (V)    | 20    | 22.2  | 20.3  | 23.0  | 24.6  | 21.8  |
| Met (M)    | 7     | 6.4   | 6.8   | 6.6   | 7.0   | 7.4   |
| Ile (I)    | 14    | 12.9  | 12.9  | 14.8  | 13.4  | 14.1  |
| Leu (L)    | 30    | 37.6  | 29.9  | 35.1  | 39.3  | 32.3  |
| Phe (F)    | 24    | 25.8  | 24.0  | 23.5  | 25.7  | 20.2  |
| Lys (K)    | 16    | 14.7  | 16.1  | 15.3  | 14.8  | 15.0  |
| Average error | 13%  | 1%    | 12%   | 14%   | 9%   |

TABLE II
Masses of human rCRALBP mutants and mutant peptides

Masses were determined by LC-ESMS as described under "Experimental Procedures." Calculated masses are chemical average masses except for those less than 2000, which are monoisotopic. Error refers to the difference between the observed and calculated masses. Residues refer to the fusion human rCRALBP structure (which contains an N-terminal His-tag extension). Mutant designations reflect substitution positions in the native protein. Mutant peptides are from tryptic digestion unless marked with an asterisk (*). Peptide I162V* was from digestion with trypsin and endoprotease Asp-N. Substituted amino acid residues are shown in bold italics within peptide sequences.

| Residues | Calculated mass | Observed mass | Error | Sequence |
|----------|----------------|---------------|-------|----------|
| Intact mutant |               |               |       |          |
| I162V    | 1–339          | 39096         | 39100 | 4        |
| Q210R    | 1–339          | 39138         | 39142 | 4        |
| K152A    | 1–339          | 39053         | 39055 | 2        |
| K221A    | 1–339          | 39053         | 39057 | 4        |
| K294A    | 1–339          | 39053         | 39056 | 3        |
| Mutant peptide |             |               |       |          |
| I162V    | 179–208        | 3708.3        | 3707.6| 0.7      |
|          |                | VVMLFNWENWQSQEITFDEILQAYCFILEK |
| I162V*   | 179–195        | 2084.4        | 2084.5| 0.1      |
|          |                | VVMLFNWENWQSQEITF |
| Q210R    | 228–233        | 738.5         | 738.5 | 0.2      |
|          |                | GFTMQGR |
| K152A    | 174–175        | 580.3         | 580.5 | 0.2      |
|          |                | DAYGR |
| K221A    | 244–256        | 1479.7        | 1480.0| 0.3      |
|          |                | AVMVDNLQDSFPAR |
| K294A    | 288–321        | 3717.0        | 3717.3| 0.3      |
|          |                | VFVHGDDLGSFYQEIDENILPSDFGGTLFPAYDGK |

Fig. 6. Wild type rCRALBP absorption spectra. UV-visible absorption spectra are shown for purified wild type fusion rCRALBP before and after exposure to bleaching illumination. Approximate binding stoichiometries are ~1.0 mol 11-cis (maximum 425 nm) and ~1.0 mol 9-cis (maximum 400 nm). Upon bleaching, the chromophore absorbance maxima shift to ~380 nm because of photoisomerization of the retinoid and production of unbound all-trans-Ral.

chemical modification (Fig. 3), all but 1 (i.e. Lys-221) are solvent accessible. The lysines at positions 152 and 294 may be located in flexible regions of the molecule as peptides containing these residues were recovered with double modifications (Fig. 4), suggesting they may be partially exposed. An earlier topological study demonstrated that residues 30–99 and 176–229 in native bovine CRALBP were not accessible to several specific antipeptide antibodies (7). Current observations are of higher resolution and supplement the previous study.

Retinoid Binding Properties of rCRALBP Mutants—Two rCRALBP mutants (I162V and Q210R) were prepared to identify substitutions altering the retinoid binding properties of a PCR misincorporation mutant (17) and three others (K152A, K221A, and K294A) to investigate possible retinoid interactions with buried or partially buried lysine residues (Figs. 3 and 4). The structural integrity of all the mutant proteins was confirmed (Tables 1 and 2; Fig. 5) and their retinoid binding properties evaluated by UV-visible and fluorescence spectrom-
etly. When labeled with retinoid before purification, all of the rCRALBP mutants bound stoichiometric amounts of 11-cis-Ral like the wild type protein, indicating that the proteins were not grossly misfolded (Figs. 6–8). However, of the 5 mutants tested, Q210R and K221A exhibited altered UV-visible absorption spectra when presented with 9-cis-Ral and bound markedly less than stoichiometric amounts of 9-cis-Ral (~0.3 mol of retinoid/mol of protein). Essentially the same UV-visible spectral results were obtained whether the retinoid labeling was performed in bacterial lysates or with the apoproteins. The physiological significance of CRALBP’s ability to bind 9-cis-Ral is not yet understood; however, it was shown previously that bovine CRALBP exhibits greater relative affinity for 11-cis-than 9-cis-Ral (6). The lower binding stoichiometries observed with Q210R and K221A and 9-cis-Ral are likely because of losses of retinoid during protein chromatography and reflect reduced affinity for the 9-cis-isomer (Table III). These results implicate Gln-210 and Lys-221 as potential components of the CRALBP retinoid binding pocket. In other recent studies, ligand-dependent rCRALBP conformational changes have been demonstrated by NMR and shown to be associated in part with Met residues by 13C NMR (11, 18). The close proximities of Gln-210 with Met-208 and Lys-221 with Met-222 further support the possible association of Gln-210 and Lys-221 with the retinoid binding cavity.

Overall, the apparent equilibrium dissociation constants determined for wild type apo-rCRALBP (Table III) reveal lower affinities for 9-cis-Ral than for 11-cis-Ral and for the Q210R and K221A mutants, even lower affinities for interactions with 9-cis-Ral. The lower affinity for the 9-cis-isomer suggests that the Q210R and K221A mutations have changed the structural integrity of the ligand binding pocket. The apparent Kd values determined for wild type rCRALBP (~21 nM for 11-cis-Ral and ~53 nM for 9-cis-Ral) are similar in magnitude to Kd values reported for other protein-retinoid interactions. For example, for binding with all-trans-Rol, Kd ~10–40 nM have been reported for CRBP (33). For delipidated interphotoreceptor RBP, Kd ~50 nM have been reported for binding with all-trans-Rol and Kd ~29–36 nM for binding with 11-cis-Ral (24). Notably, the low solubility and lability of retinoids in aqueous solution contribute to the difficulty and variability in retinoid affinity measurements (33). Variable protein stability also cannot be ruled out as a contributing factor in the present measurements as apo-CRALBP is structurally less stable than the holoprotein (9, 11). Furthermore, it is difficult to accurately assess the affinity of protein-retinoid complexes when the Kd is significantly lower than the protein concentration required for the measurement. In these cases, Kd values extracted from fluorescence titrations can reflect upper limits rather than precise estimates (34, 35).

TABLE III
Equilibrium Dissociation Constants of rCRALBP with Retinoids

| rCRALBP preparation | 11-cis-Ral | 9-cis-Ral |
|---------------------|-----------|-----------|
| Wild type           | 21.0 ± 3.2 (n = 8) | 53.3 ± 9.6 (n = 7) |
| I162V               | 18.7 ± 2.5 (n = 8) | 26.9 ± 11.1 (n = 3) |
| Q210R               | 34.2 ± 10.5 (n = 8) | 71.0 ± 19.7 (n = 7) |
| K152A               | 16.7 ± 3.7 (n = 6) | 26.0 ± 9.5 (n = 3) |
| K221A               | 36.9 ± 12.9 (n = 6) | 70.1 ± 23.0 (n = 6) |
| R294A               | 39.3 ± 5.1 (n = 5) | 58.3 ± 12.5 (n = 6) |
| Average             | 27.7 ± 3.4 (n = 38) | 56.2 ± 7.1 (n = 32) |

Mean Kd values ± S.E. are shown for n titrations. Average values are from the combined fluorescent titration data for all wild type and mutant rCRALBP preparations and reflect the overall greater affinity of rCRALBP for 11-cis-Ral than for 9-cis-Ral.
two proteins are likely to be similar. In vitro, PITP catalyzes exchange between membrane bilayers of phosphatidyl-inositol and phosphatidylcholine and in vivo is required for vesicle budding from the Golgi complex (37). The ligand binding cavity of PITP is lined with hydrophobic residues that make van der Waals contacts with the acyl chains of two molecules of the detergent n-octyl-β-D-glucopyranoside in the crystal structure. Consistent with the current results, CRALBP residues Gln-210 and Lys-221 are located within a segment of sequence that exhibits high homology to part of the ligand binding cavity of PITP (Fig. 9).

Ligand Interactions in Other Retinoid-binding Proteins—No Schiff base linkage or other covalent bonds attach retinoid to the cellular retinol-binding proteins (CRBP and CRBP II), which bind 1 mol of all-trans-Ral or all-trans-Ral per mol of protein and also exhibit red shifted chromophore absorbance. CRBP and CRBP II serve intracellular transport and substrate carrier functions and belong to a family of small intracellular lipid and retinoid-binding proteins containing about 130 amino acids (33). X-ray crystallographic structures have been determined for both CRBP and CRBP II and detailed comparisons with other known structures are available (33, 39, 40). CRBP and CRBP II exhibit the β-clam motif common to the lipid-binding protein family and are more flattened in shape than the classic β-barrel structure of serum retinol-binding protein (RBP). Although CRALBP shares no significant sequence homology with RBP or the other cellular retinoid-binding proteins, the three-dimensional motifs characteristic of RBP and CRBP tolerate significant sequence diversity, allowing in the case of CRBP and insect fatty acid-binding protein, superimposable crystal structures with only 12% sequence identity (40). Notably, 23 residues are within about 5 Å of retinoid in the ligand binding cavities of CRBP and CRBP II, including Gln and Lys residues critical for retinoid binding (33, 39, 40). The ε-amino group of Lys-40 in CRBP and CRBP II is within van der Waals distance of bound all-trans-Ral and may participate in electrostatic interactions with the ε electrons of the isoprene arm of the retinoid, which is in a solvent inaccessible environment. Amino-aromatic hydrogen bonding interactions in CRBP between Gln-108 and Phe-4 have been proposed as the basis for the protein’s higher affinity for all-trans-Ral than for all-trans-Ral. The specific roles of Lys-221 and Gln-210 in CRALBP are demonstrated to be important in CRALBP interactions with 9-cis-Ral, and likely to be components of the retinoid binding pocket.

CRALBP mutation R150Q has been associated with autosomal recessive retinitis pigmentosa and shown to abolish 11-cis-Ral binding as well as to significantly decrease rCRALBP solubility (1). Notably, a single Arg residue can modulate ligand specificity and affinity in the cellular retinoid-acid-binding protein, intestinal fatty acid-binding protein (41), and CRBP II. Mutating Arg-111 to Gln in CRBP destroys the protein’s ability to bind retinoic acid (42). In FABP, substituting corresponding Arg-106 with Gln reduces the protein’s affinity for fatty acids and converts the molecule to a retinoid-binding protein. In CRBP II, Gln-108 occupies the corresponding position, which when substituted with Arg, converts the molecule to a fatty acid-binding protein with decreased affinity for retinoids (43). Whether CRALBP Arg-150 is part of the ligand binding pocket is not clear; however, this residue appears to play an important stabilizing role in the CRALBP structure.

In summary, the lack of a covalent bond between CRALBP and retinoid is consistent with the proposed physiological function(s) of CRALBP, in which rapid association and dissociation of the ligand would appear important. The apparent substrate carrier function of CRALBP in the RPE resembles that of CRBP more than serum RBP or the visual pigments; however, a precise determination of the structure of the CRALBP ligand binding cavity awaits crystallographic analysis. In this study, we have found that Gln-210 and Lys-221 influence CRALBP retinoid interactions. These residues are likely to be components of the retinoid binding pocket and may participate in key noncovalent interactions. The function of CRALBP in tissues such as cornea, ciliary body, and oligodendrocytes of the brain and optic nerve and whether ligands other than cis-retinoids associate with the protein remain to be determined.

Acknowledgments—We gratefully acknowledge Valerie Oliver and Marina LaDuke for manuscript preparation.

REFERENCES

1. Maw, M. A., Kennedy, B., Knight, A., Bridges, R., Roth, K. E., Mani, E. J., Mukkadan, J. K., Nanovskaya, D., Crabb, J. W., and Denton, M. J. (1997) Nat. Genet. 17, 198–200
2. Saari, J. C. (1994) in The Retinoids (Sporn, M. A., Roberts, A. B., and Goodman, D. S., eds), pp. 351–383, Raven Press, Ltd., New York.
3. Saari, J. C., Huang, J., Possin, D. E., Fariss, R. N., Leonard, J., Garvin, G. G., Crabb, J. W., and Milam, H. A. (1997) Glia 21, 259–268
4. Futterman, S., Saari, J. C., and Blair, S. (1997) Endocr. J. 44, 279–291
5. Saari, J. C., Bredberg, L., and Garvin, G. W. (1982) J. Biol. Chem. 257, 13329–13333
6. Saari, J. C., and Bredberg, D. L. (1987) J. Biol. Chem. 262, 7618–7622
7. Crabb, J. W., Gaur, P. V., Garvin, G. G., Marx, S. V., Chapline, C., Johnson, C. M., and Saari, J. C. (1991) J. Biol. Chem. 266, 16674–16683
8. Saari, J. C., and Bredberg, D. L. (1982) Biochemistry 21, 266–272
9. Saari, J. C., Bredberg, D. L., and Noy, N. (1994) Biochemistry 33, 3106–3112
10. Crabb, J. W., Chen, Y., Goldflam, S., West, K., and Kapron J. (1998) in Methods in Molecular Biology, Vol 89, Retinoid Protocols (Redfern, C., ed), pp. 91–104, Humana Press, Totowa, New Jersey.
11. Crabb, J. W., Carlson, A., Chen, Y., Goldflam, S., Intres, R., West, K. A., Halun, J. D., Kapron, J. T., Luck, L. A., Horwitz, J., and Bok, D. (1998) Proc. Natl. Acad. Sci. U.S.A. 95, 1076–1080
12. Crabb, J. W., Johnson, C. M., Carr, S. A., Armes, L. G., and Saari, J. C. (1988) J. Biol. Chem. 263, 18678–18687
13. Intres, R., Goldflam, S., Cook, J. R., and Crabb, J. W. (1994) J. Biol. Chem. 269, 25411–25418
14. Kennedy, B. N., Goldflam, S., Harris, S. E., and Saari, J. C. (1988) J. Biol. Chem. 263, 18688–18692

Fig. 9. Comparison of PITP ligand-binding pocket sequence with CRALBP. Yeast PITP residues, shown on black background, line the ligand binding cavity in the crystal structure (37). Homologous sequence from human CRALBP (38) includes residues Gln-210 and Lys-221, demonstrated to be important in CRALBP interactions with 9-cis-Ral, and likely to be components of the retinoid binding pocket.
Ploegh, H. L., Smith, J. A., and Speicher, D. W., eds), pp. 11.9.1–11.9.42, John Wiley & Sons, Inc., New York
26. Bradford, M. M. (1976) Anal. Biochem. 72, 248–254
27. Kapron, J. T., Hilliard, G., Lakins, J., Tenniswood, M., West, K. A., Carr, S. A., and Crabb, J. W. (1997) Protein Sci. 6, 1–14
28. Stubbs, G. W., Saari, J. C., and Futterman, S. (1979) J. Biol. Chem. 254, 8529–8533
29. Jager, S., Paleczewski, K., and Hofmann, K. P. (1996) Biochemistry 35, 2901–2908
30. Nathans, J. (1992) Biochemistry 31, 4923–4931
31. Zhakovsky, E. A., Robinson, P. R., and Oprian, D. D. (1991) Science 251, 558–560
32. Asenjo, A. B., Rim, J., and Oprian, D. D. (1994) Neuron 12, 1131–1138
33. Ong, D. E., Newcomer, M. E., and Chytil, F. (1994) in The Retinoids (Sporn, M. A., Roberts, A. B., and Goodman, D. S., eds), pp. 283–317, Raven Press, Ltd., New York
34. Norris, A. W., and Li, E. (1998) in Methods in Molecular Biology, Vol 89, Retinoid Protocols (Redfern, C. P. F., ed), pp. 123–139, Humana Press, Totowa, New Jersey
35. Kersten, S., Dawson, M. I., Lewis, B. A., and Noy, N. (1996) Biochemistry 35, 3816–3824
36. Allenby, G., Boquel, M. T., Saunders, M., Kazmier, S., Speck, J., Rosenberger, M., Lovey, A., Kastner, P., Grippo, J. F., Chambon, P., and Levin, A. A. (1993) Proc. Natl. Acad. Sci. U.S.A. 90, 30–34
37. Sha, B., Phillips, S. E., Bankaitis, V. A., and Luo, M. (1998) Nature (Lond.) 39, 506–510
38. Salama, S. R., Cleves, A. E., Malehorn, D. E., Whitters, E. A., and Bankaitis, V. A. (1996) J. Bacteriol. 172, 4510–4521
39. Banazak, L., Winter, N., Xu, Z., Bernslez, D. A., Cowan, S., and Jones, T. A. (1994) in Advances in Protein Chemistry, Vol 45 (Schumate, V., ed), pp. 89–149, Academic Press, San Diego
40. Newcomer, M. E. (1995) FASEB J. 9, 229–239
41. Jacoby, I. V., Miller, K. R., Toner, J. J., Bauman, A., Cheng, L., Li, E., and Cistola, D. P. (1993) Biochemistry 32, 872–878
42. Zhang, J., Liu, Z.-P., Jones, T. A., Giersch, L. M., and Sambrook, J. F. (1992) Proteins Struct. Funct. Genet. 13, 87–99
43. Cheng, L., Qian, S.-J., Rotthschild, C., d’Avigou, A., Lefkowith, J. B., Gordon, J. I., and Li, E. (1991) J. Biol. Chem. 266, 24404–24412
44. Kapron, J.T., Chen, Y., West, K. A., Dodson, W.S., Bredberg, L., Saari, J.C., and Crabb, J. W. (1995) Protein Sci. 4, Suppl. 2, 147
45. Crabb, J.W., Chen, Y., Kapron, J. T., West, K. A., Bredberg, D. L., and Saari, J. C. (1996) Invest. Ophthal. Visual Sci. 37, (Suppl. 862) 3691