Topological and Epitope Mapping of the Cellular Retinaldehyde-binding Protein from Retina*

(Received for publication, February 22, 1991)

John W. Crabb‡§, Vinod P. Gaur¶‖, Gregory G. Garwin‡, Steven V. Marx‡, Christine Chapline‡, Charles M. Johnson§, and John C. Saaril¶‖

From the ‡Protein Chemistry Facility, W. Alton Jones Cell Science Center, Lake Placid, New York, 12946 and the Departments of ¶Ophthalmology and ‖Biochemistry, University of Washington School of Medicine, Seattle, Washington 98195

Cellular retinaldehyde-binding protein (CRALBP) carries 11-cis-retinol or 11-cis-retinaldehyde as endogenous ligands and may function as a substrate carrier protein that modulates interaction of these retinoids with visual cycle enzymes. As a first approach to identifying functional domains and protein recognition sites in CRALBP, a low resolution topological and epitope map has been developed using monoclonal and polyclonal antibodies and limited proteolysis.

Fifteen peptides of 8–31 residues spanning 99% of the 316-residue bovine CRALBP were synthesized and used to prepare 13 anti-peptide polyclonal antibodies. Using a competitive ELISA procedure, peptide epitopes were classified as either accessible or inaccessible in the native protein based on the extent of their recognition by these site-specific antibodies. Use of the synthetic peptides to map the epitopes of a polyclonal antibody to intact CRALBP confirmed that the amino terminus and carboxyl terminus are immunodominant regions and hence likely to be exposed, at least in part.

Limited tryptic proteolysis of native CRALBP produced three major fragments which were shown by microsequence and Western analysis to be derived from sequential loss of short peptides from the amino terminus. None of these major fragments reacted with four monoclonal antibodies (mAbs) to intact CRALBP although each mAb immunoprecipitated native CRALBP. These results and the lack of mAb recognition of any of the synthetic peptides indicates that the amino terminus of the protein is exposed and contains part of an assembly epitope recognized by the mAbs.

Overall this study indicates that residues 1–30, 100–124, and 257–285 contain highly exposed segments in the native protein and therefore constitute potential interaction domains for CRALBP and visual cycle enzymes. Residues 30–99 and 176–229 are inaccessible in the native structure and may be involved with retinoid binding. These results provide a basis for a systematic higher resolution mutagenesis study directed toward correlating CRALBP structural domains with function.

* This work was supported in part by National Institutes of Health Grants EY02317, EY03523, EY01730, EYO6903, CA37589 and DK38639, an award from Research to Prevent Blindness, Inc., and National Science Foundation Grant DMB-8516111. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† Present address: Dept. of Neurobiology and Anatomy, Bowman Gray School of Medicine, Wake Forest University, Winston-Salem, NC 27103.

‡ To whom correspondence should be addressed.

Retinoids undergo intercellular transport and several enzymatic transformations during the course of the visual cycle (reviewed in Saari, 1980). All-trans-retinaldehyde, the product of visual pigment bleaching, is reduced to all-trans-retinol within photoreceptor cells and transported through an extracellular compartment to the retinal pigment epithelium (RPE) (Dowling, 1960) where it is esterified (Saari and Bredberg, 1989) and isomerized (Deigner et al., 1989) to 11-cis-retinol. A dehydrogenase-mediated oxidation in RPE in mammals (Lion et al., 1965) produces 11-cis-retinaldehyde, which must then traverse the extracellular compartment to gain access to the photoreceptors where regeneration of visual pigment occurs. Since retinoids are relatively water-insoluble, it is not surprising that several proteins have been discovered that specifically bind the ligands and appear to be involved in their solubilization (reviewed in Blomhoff et al., 1990; Saari, 1990). Cellular retinaldehyde-binding protein (CRALBP) is a water-soluble protein found only in retina and pineal that carries 11-cis-retinaldehyde or 11-cis-retinol as physiological ligands (Saari et al., 1982). Bound 11-cis-retinaldehyde is not affected by incubation with water-soluble reducing agents or hydroxylamine although it is readily reduced by NADH and 11-cis-retinol dehydrogenase of RPE (Saari and Bredberg, 1982), providing evidence for a specific interaction of the enzyme and binding protein. Recent evidence suggests that CRALBP also affects the partition of 11-cis-retinol between enzymatic oxidation or esterification (Saari and Bredberg, 1990), suggesting that it modulates retinoid metabolic flux. As an initial step toward identifying functional domains in CRALBP such as the retinoid-binding pocket and surface regions of the protein involved in interaction with enzymes of the visual cycle, we have pursued topological and epitope mapping of bovine CRALBP. In the present study the preparation of four anti-CRALBP mAbs and 13 CRALBP site-specific anti-peptide pAbs are described. In addition, regions of large-probe accessibility are identified in the tertiary structure of CRALBP using antibodies, proteases, protein sequencing, and peptide synthesis.

EXPERIMENTAL PROCEDURES

RESULTS

Solid-phase Synthesis and Characterization of Peptides Derived from CRALBP—Fifteen peptides of 8–31 residues each

1 The abbreviations used are: RPE, retinal pigment epithelium; CRALBP, cellular retinaldehyde-binding protein; EDAC, 1-ethyl-3(3-dimethylaminopropyl)carbodiimide HCl; ELISA, enzyme-linked immunosorbent assay; KLH, keyhole limpet hemocyanin; mAb, monoclonal antibody; pAb, polyclonal antibody; PBS, phosphate-buffered saline; PVDF, polyvinylidene difluoride; RP-HPLC, reverse-phase high-performance liquid chromatography; RT, room temperature; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

Portions of this paper (including "Experimental Procedures," Tables I and II) are presented in miniprint at the end of this paper.
and together covering 99% of the 316-amino acid bovine CRALBP were synthesized by automatic solid-phase peptide synthesis, deprotected, and removed from the solid support by HF cleavage. The peptides synthesized are identified in Fig. 1 within the primary structure of bovine CRALBP, and a summary of the synthetic yields is presented in Table I. The quality of the peptides was evaluated by RP-HPLC, amino acid analysis, and by automatic Edman degradation following RP-HPLC. Although several of the crude synthetic preparations exhibited some heterogeneity by RP-HPLC, all contained the desired peptide as a significant component and generated highly specific anti-peptide pAbs as described below.

Characterization of Anti-peptide Antibodies—A panel of 13 CRALBP anti-peptide pAbs (Table II) was prepared in rabbits as described under "Experimental Procedures." All anti-peptide pAbs recognized both the peptide antigen and intact denatured CRALBP; a summary of the antisera dilutions capable of detecting these antigens with ELISA is presented in Table II. The specificity of the anti-peptide pAbs for CRALBP was demonstrated by Western analysis of crude retinal supernatant proteins as shown in Fig. 2.

Limited Proteolysis of CRALBP—Previous purification and characterization of CRALBP indicated that the protein was extremely sensitive to proteases. The gel pattern produced by limited tryptic digestion depicted in Fig. 3A provides an example of this sensitivity and illustrates the characteristic degradation pattern observed with several proteases. The pattern of fragments observed suggests that relatively small peptides are removed sequentially from the amino and/or carboxyl terminus. A similar fragmentation pattern has also been observed with subtilisin, papain, and thermolysin (results not shown).

To determine the sites of tryptic cleavage, the proteolysis fragments were separated by SDS-PAGE, electroblotted to PVDF membrane, and the amino-terminal sequences of the fragments determined by gas-phase Edman degradation (Matsudaïra, 1987). The amino acid sequences, shown in Fig. 3B, indicate that three major fragments result from cleavages at Arg8, Lys23, and Lys46 (Crabb et al., 1988a). These results demonstrate that the amino terminus of the native protein is accessible to a macromolecular probe such as trypsin but do not rule out additional proteolysis near the carboxyl terminus. Western analysis with an anti-peptide pAb directed against carboxyl-terminal residues 286–316 (Fig. 3C) shows that the three largest fragments retain reactivity against this antibody and demonstrates that the fragments result solely from cleavages in the amino-terminal domain. Minor components of lower M, (Fig. 3B) suggest that proteolysis near the carboxyl terminus occurs more slowly and to a lesser extent.

mAb Isotyping—The initial screening for hybridomas producing anti-CRALBP mAbs was performed as described under "Experimental Procedures." Of the positive clones, four were chosen for further study based on their strong ELISA response. Isotyping by immunodiffusion revealed that these mAbs (mAb 1, 2, 10, and 11) are IgGm immunoglobulins. Qualitatively identical results were obtained with all four mAbs (as described below), and it is likely these antibodies recognize the same or a similar epitope.

mAb Immunoprecipitation—The ability of anti-CRALBP mAbs to recognize native CRALBP was assessed by immunoprecipitation. Monoclonal antibodies were added to a bovine retinal supernatant and immune complexes isolated by protein A- Sepharose chromatography and analyzed by SDS-PAGE as shown in Fig. 4. Two lanes are shown for each mAb, one with the mAb incubated with buffer alone (−) and the other with the retinal supernatant (+). mAbs 1, 10, and 11 recognize uniquely CRALBP, as evidenced by the strongly staining component present in each of the (+) lanes, corresponding in migration to purified CRALBP. mAb 2 apparently recognizes the same component although the amount of material present in the gel is considerably less. Thus, these four mAbs recognize epitopes accessible in the native protein. Light chain heterogeneity, observed in this study, has been noted by others (Stanker et al., 1985).

mAb Epitope Mapping—Initial indication that mAb 10 recognizes an amino-terminal region of CRALBP came from cDNA cloning studies. Screening of a λgt11 retinal expression cDNA library with a rabbit anti-CRALBP pAb resulted in

Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are included in the microfilm edition of the Journal that is available from Waverly Press.
purification of three clones carrying inserts coding for CRALBP residues 1-316, 221-316, and 285-316, respectively (Crabb et al., 1988b). The two clones lacking the aminoterminal coding region were not recognized by mAb 10. However, the clone containing the entire coding region of CRALBP (residues 1-316) was recognized (Fig. 5).

Limited proteolysis, Western analysis, and synthetic peptides were used to map more precisely the amino-terminal sequences associated with mAb recognition. CRALBP was treated with trypsin for 30 min at 0 °C to generate limited proteolysis fragments. Following SDS-PAGE of the fragments and intact CRALBP, the polypeptides were either stained with Coomassie Blue (Fig. 3D, two lanes, far right) or transferred to nitrocellulose and reacted with the mAbs indicated on the figure. The results demonstrate that mAbs 1, 2, 10, and 11 react with intact CRALBP but not with any of the proteolysis fragments. Since the largest tryptic fragment lacks only the aminoterminal octapeptide (Fig. 3B), the results further demonstrate that the epitope (or a component of the epitope) recognized by these mAbs is located in an aminoterminal domain, perhaps within the first eight amino acids of the protein.

The ability of trypsin-nicked CRALBP to compete for mAb binding with intact CRALBP was examined by competitive ELISA. Before ELISA, mAb 10 was incubated with varying concentrations of either native CRALBP or CRALBP that had been subjected to limited tryptic digestion. The ELISA results, shown in Fig. 6, indicate that the proteolyzed protein competes effectively with CRALBP for mAb 10 binding. In fact, the concentration of nicked CRALBP giving 50% inhibition (~10^{-8} M) is not significantly different than that found with native CRALBP (results not shown). Since a released amino-terminal peptide might be competing with native CRALBP, synthetic peptides corresponding to amino-terminal residues 1-8 and 1-17, both with and without N^\text{\textsuperscript{\neg}}-acetylation, were also examined in competitive ELISA. The results, shown in Fig. 6 for the acetylated peptides, demonstrate that these peptides do not compete with CRALBP for mAb 10 binding.

To probe further for the epitopes recognized by the mAbs, a panel of 14 synthetic peptides together covering all but 3 residues of the CRALBP polypeptide were screened by ELISA for binding by mAbs 1, 2, 10, and 11. The antigenic profile for mAb 10 shown in Fig. 7A is representative of the results obtained with all four mAbs. All of the mAbs failed to recognize any of the synthetic peptides. Overall, these results indicate that the mAbs recognize a non-linear assembly epitope associated with the amino terminus of CRALBP.

Topological Mapping with Site-specific Anti-peptide Antibodies—The ability of anti-peptide pAbs to recognize their epitopes in the native protein was determined with an accessibility ELISA. A test was designed that determined the ability of native or heat-denatured CRALBP to compete for antibody with CRALBP adsorbed to the ELISA plate. The assumption made here is that CRALBP has a more open structure as a result of binding to the polystyrene surface (see "Discussion"). The results of the test procedure are presented (Fig. 8) as the percent inhibition of the ELISA reaction resulting from preincubation of the antibodies with native or heat-denatured (10 min, 60 °C) CRALBP. Three classes of site-specific antibodies are immediately apparent. Anti-peptide antibodies to CRALBP residues 1-17, 10-30, 100-124, and 257-285 are neutralized effectively (>67% inhibition) by both native and heated CRALBP. Thus, the residues recognized within these linear peptides are accessible to their antibodies in the native protein. In contrast, antibodies to
Topological and Epitope Mapping of CRALBP

A

B

C

D

Fig. 3. Limited proteolysis of CRALBP. Panel A, native CRALBP was digested at 0 or 20 °C with trypsin (trypsin to CRALBP, 1:1000, w/w) as detailed below, analyzed by SDS-PAGE, and stained with Coomassie Blue. The high molecular weight bands are contaminants in the preparation. Lane 1, unmodified CRALBP; lane 2, 30 min, 0 °C; lane 3, 90 min, 0 °C; lane 4, 120 min, 0 °C; lane 5, 3 h, 20 °C. Panel B, sequence analysis of tryptic fragments. Native CRALBP was treated with trypsin (1:400, w/w, trypsin/CRALBP) for 1 h at 0 °C, fractionated by SDS-PAGE, electrophobted to PVDF membrane, and stained with Coomassie Blue. Analysis by Edman degradation revealed the sequences indicated. No amino acids are released from intact CRALBP because the protein is N'-acyetylated. Panel C, immunostaining with antibodies. Intact CRALBP (lanes 2 and 4) and tryptic fragments (lanes 1 and 3), generated as in panel B above, were resolved by SDS-PAGE and transferred to nitrocellulose. Immunostaining with anti-peptide antibody specific for the COOH-terminal peptide (residues 286–316) (lanes 1 and 2) indicates that the three largest proteolysis fragments retain the COOH terminus. Immunostaining with mAb 10 indicates that none of the proteolysis fragments are recognized by the mAb (lanes 3 and 4). The stained component in lane 3 is residual undigested CRALBP. Panel D, Western blotting. CRALBP was treated with trypsin (trypsin/CRALBP ratio, 1:400, w/w, 0 °C, 1 h), and following SDS-PAGE of these fragments and undegraded CRALBP, the polypeptides were either stained with Coomassie Blue (two lanes, far right) or transferred to nitrocellulose and reacted with the indicated mAbs using the peroxidase-anti-peroxidase technique. mAbs 1, 2, 10, and 11 react with undegraded CRALBP but not with any of the proteolysis fragments.

CRALBP residues 30–45, 46–70, 71–99, 176–201, and 202–229 show little competition with native CRALBP (<18% inhibition) and a dramatic increase in competition following heating of the protein. Presumably, the epitopes in these peptides are not accessible to the antibodies in the native protein structure. Finally, several anti-peptide antibodies (125–145, 149–175, 286–316) showed intermediate responses (25–43% inhibition) in that some competition was seen with...

Fig. 4. Immunoprecipitation of CRALBP with mAbs. mAbs to CRALBP were mixed with protein A-Sepharose beads following a 3-h incubation at 4 °C with supernatant of bovine retina (+) or with buffer (–). Proteins adsorbed to the beads were removed by boiling in SDS buffer and analyzed by SDS-PAGE and silver staining. The numbers are mAb designations. The results demonstrate that all four mAbs recognize native CRALBP. The upper and lower arrows indicate the migration positions of heavy and light IgG chains, respectively. The middle arrow indicates the migration position of CRALBP.

Fig. 5. Screening of CRALBP cDNA expression clones with polyclonal and monoclonal antibodies. Three CRALBP cDNA clones containing 1174-, 448-, and 265-bp inserts encoding residues 1–316, 221–316, and 285–316, respectively, were obtained from a lgt11 bovine retinal cDNA library (Crabb et al., 1988b). Duplicate lifts of the purified clones are shown following detection with either a pAb raised against intact CRALBP or mAb 10. These results show that mAb 10 only recognizes clones encoding the complete protein with the amino-terminal domain.
The pAb strongly reacts with peptides covering amino-terminal residues 1-17 and 10-30 and COOH-terminal residues 257-285 and 286-316. Moderate yet significant reaction was also observed with a peptide corresponding to residues 202-229. All the other synthetic peptides were recognized only weakly by the pAb. Since the peptide corresponding to residues 20-30 was not recognized, the major amino-terminal antigenic determinants for this pAb appear to lie within residues 1-19.

**DISCUSSION**

Several characteristics of CRALBP suggest that the protein is involved with the visual process. First, CRALBP has only been detected in tissues that respond to light, namely retina and pineal. Second, the protein carries 11-cis-retinol or 11-cis-retinaldehyde as endogenous ligands (Saari et al., 1982), retinoids whose known occurrence in mammals is confined to the neural retina and RPE. Third, CRALBP is remarkably stereoselective in recognizing the isomer of retinaldehyde involved in vision. Incubation of CRALBP-11-cis-retinol with a mixture of 9-, 11-, 13-cis- and all-trans-retinaldehydes results in binding of only 11-cis-retinaldehyde (Saari and Bredberg, 1987). Fourth, 11-cis-retinaldehyde bound to CRALBP is readily reduced by NADH and 11-cis-retinol dehydrogenase of RPE even though chemical reducing agents and purified yeast alcohol dehydrogenase are not effective (Saari and Bredberg, 1982). Finally, partition of 11-cis-retinol between enzymatic oxidation or esterification in RPE is modulated by CRALBP, suggesting that it may play a role in reaction control as well (Saari and Bredberg, 1990). A functional role for CRALBP most consistent with these properties is that of a substrate carrier protein that solubilizes the retinoid and modulates its interaction with visual cycle enzymes of RPE. Interaction of CRALBP with visual cycle enzymes is ex-
pected to involve a relatively large area of surface contact as described for other protein-protein interactions (Amit et al., 1985). Since a significant portion of the molecular surface of proteins can be inaccessible to other proteins (Novotny et al., 1986), as a first approximation, protein recognition regions in CRALBP were assumed to be associated with highly exposed surface areas. Accordingly, antibodies and proteases were employed to gain an appreciation of regions of CRALBP that could be surface oriented and involved in protein-enzyme interactions or buried and perhaps involved with retinoid binding. Our topological and epitope mapping strategies incorporated (i) identification of limited proteolysis fragments; (ii) production of four CRALBP mAbs; (iii) solid-phase synthesis of 15 CRALBP peptides covering 313 of the 316 residues in the protein; (iv) epitope mapping with the mAbs and a pAb to intact CRALBP; (v) production of 13 site-specific CRALBP anti-peptide pAbs; and (vi) determination of the accessibility of the peptide regions in the native CRALBP to the anti-peptide pAbs.

Antibody Probes of Accessible Surface—Two methods were used to determine whether an epitope was accessible to antibody in the native protein. First, a traditional approach employed immunoprecipitation of complexes formed when antibody was added to native protein. Using this approach each of the four mAbs was found to recognize the native protein (Fig. 4).

Second, an assay was devised to determine if a linear peptide epitope is accessible in the native protein. Synthetic peptides essentially covering the CRALBP sequence were used to prepare a battery of anti-peptide pAbs (Table II). An accessibility ELISA was then used to score for the effectiveness of either native or heat-denatured CRALBP in competing for antibody with unfolded protein on the surface of the ELISA plate (Fig. 8). The technique resolved the anti-peptide antibodies into three classes: those that recognized native protein, those that reacted only with denatured protein, and those with an intermediate reactivity. Those antibodies in the first class clearly recognize epitopes exposed on the surface while those antibodies that only reacted with the heated protein recognize epitopes that are either inaccessible or in an unreactive conformation in the native CRALBP structure. An intermediate response could result from several conditions such as a partially accessible epitope, a heterogenous population of native and partially denatured protein, the existence of more than one epitope on a peptide antigen, or from a kinetic phenomenon. As an initial hypothesis, we interpret the lack of anti-peptide pAb recognition of the native structure to suggest inaccessible epitopes, and intermediate reactivity to suggest a partially accessible epitope because of the size of the peptide antigens in these regions (21–31 residues).

Two features that potentially limit the amount of information that can be gained from the accessibility ELISA technique deserve note. First, the effect of heating CRALBP at 60 °C for 10 min has not been established. Second, although there is considerable evidence that a perturbation of protein structure occurs on adsorption of proteins to plastic surfaces (Soria et al., 1985; Hollander and Katchalski-Katzir, 1986; Anderson et al., 1987), the effect is protein-specific and remains uncharacterized for CRALBP. Thus, although we are unable to describe structurally these two states of denaturation, the method has proven useful as a first approximation of the accessibility of peptide regions in CRALBP and may have general applicability. Notably, the epitope of mAb 10, known to be exposed from limited proteolys and immunoprecipitation studies, was found to be accessible in the native protein using this ELISA technique, providing a verification of the method. A variant of this technique has also proven useful with a different antibody (Gaur et al., 1990), and others have studied the general phenomenon of epitope accessibility of adsorbed proteins (Soria et al., 1985; Hollander and Katchalski-Katzir, 1986).

The Exposed Amino Terminus—Limited tryptic proteolysis of CRALBP produces a graded decrease in the molecular weight of the protein, consistent with cleavage at one end of the protein (Fig. 3B). Initial tryptic cleavage is limited to the amino terminus as the three major proteolysis fragments were found to originate from cleavages at Arg⁶, Arg¹⁷, and Lys⁴⁶ and retain reactivity to a site-specific COOH-terminal anti-peptide antibody (Fig. 3C). Accordingly, the amino terminus of CRALBP is readily accessible and exposed in the native structure by this criterion. Limited proteolysis has long been used to obtain information about accessible regions of protein surface. While trypsin fragmentation patterns are presented in Fig. 3, other proteases gave similar patterns. Thus, it is unlikely that regions of exposed or disorganized structure were missed due to the specificity of trypsin for lysyl and arginyl residues.

Further evidence of the accessibility of the amino terminus was obtained with antibodies. First, anti-peptide pAbs to amino-terminal residues 1–17 and 10–30 were clearly able to recognize native CRALBP (Fig. 8). Second, the mAb epitope was accessible in the native protein as evidenced by immunoprecipitation (Fig. 4) and ELISA (Fig. 8). Screening of CRALBP cDNA clones demonstrated that mAb 10 requires a component in the amino-terminal portion of the protein for recognition (Fig. 5). Since the largest limited tryptic fragment of CRALBP is not recognized by mAb 10 (Fig. 3D), and this fragment results uniquely from proteolytic cleavage at Arg⁶ (Fig. 3B), the epitope (or a portion of the epitope) appears to be associated with the first eight amino acids of the protein.

A mAb Assembly Epitope—Most characterized protein epitopes are composed of discontinuous regions of polypeptide (Geysen et al., 1986; Barlow et al., 1986; Davies et al., 1988), and the CRALBP epitope recognized by mAb 10 fits into this category. Synthetic peptides representing amino-terminal residues 1–8 and 1–17 failed to block mAb 19 binding to the intact protein (Fig. 6) even though the mAb strongly recognized CRALBP modified by limited tryptic cleavage (Fig. 6). This implies that the CRALBP fragments generated during limited proteolysis remain associated until more severe denaturing conditions are established, a condition also reported in other limited proteolysis studies (Richards and Vithayathil, 1959). Epitope mapping with the synthetic peptides further demonstrated that the mAbs did not recognize any of the 14 CRALBP synthetic peptides that together covered 99% of the 316 residue protein (Fig. 7A). Although mAb 10 recognizes CRALBP on a Western blot after SDS-PAGE, this does not rule out an assembly epitope because the protein structure may have been fully denatured by SDS or may have partially reassembled following transfer to nitrocellulose.

Others have reported renaturation of protein tertiary structure following SDS-PAGE and Western blotting (Laurent et al., 1987) and, with another protein, we have observed the recovery of enzyme activity following SDS-PAGE and electrophotoblotting to PVDF membrane (Chapline et al., 1989).

We conclude that CRALBP residues 1–8 likely include a component of an assembly epitope although it is possible that the amino-terminal domain promotes assembly of the mAb epitope from nearby and distal regions of the protein sequence.

The COOH Terminus—This portion of CRALBP is clearly less accessible to macromolecular probes than the amino
terminals. Trypsin cleavage at the COOH terminus occurred minimally and only after modification at the amino terminus despite trypsin sensitive residues at Lys₁₅⁴, Lys₁₇⁸, and Arg₁₇⁸. A pAb generated against intact CRALBP recognized peptides corresponding to residues 257–285 and 286–316 (Fig. 7B). However, antibodies to these peptides showed relatively stronger reactivity with residues 257–285 than residues 286–316 (Fig. 8) in the native protein. We conclude that the COOH terminus is immunogenic yet less susceptible to cleavage with trypsin because the carboxyl-terminal 31 residues are only partially exposed.

*Other Exposed Regions*—The only evidence for accessibility of other portions of CRALBP was obtained with the antibody to peptide 110–124 (Fig. 8), which was nearly as reactive with the native protein as the heat-denatured protein.

*Inaccessible Regions*—Several anti-peptide antibodies were unable to bind to native CRALBP but reacted completely with the heated protein (Fig. 8). Anti-peptide antibodies to residues 30–45, 46–70, and 46–99 near the amino terminus and to residues 176–201 and 202–229 near the COOH terminus were particularly unreactive in the native structure. Also, the antibody to residues 202–229 only partially recognized the heated protein. More drastic denaturation than heating at 60 °C may be required to fully expose this region of CRALBP.

The reactivity of the pAb to intact CRALBP toward the set of synthetic peptides also indicates that large blocks of sequence from residues 20–201 and 229–256 were not recognized (Fig. 7B). Since the emulsification employed as part of the immunization procedure (Bunt-Milam and Saari, 1983) would be expected to partially denature CRALBP, the regions of sequence not recognized by this pAb are either not immunogenic or are inaccessible even after emulsification. Lack of immunogenicity seems unlikely since these residues generated anti-peptide antibodies (Fig. 2 and Table II). More likely, the segments not recognized by the pAb were inaccessible even in the partially denatured protein. The cores of most proteins are substantid, hydrophobic, and important for folding and stability (Bowis et al., 1990). Notably, the core of CRALBP likely serves to insulate the hydrophobic, light-sensitive retinoid ligand from photoisomerization and from the aqueous environment (Saari and Bredberg, 1987).

How do the present results correlate with current structure predictions for CRALBP? The most useful approach for obtaining a rough picture of the three-dimensional structure of CRALBP would be to compare it to a homologous protein for which the crystal structure is known. Unfortunately, this is presently impossible since CRALBP is not structurally related to any known protein sequence or tertiary structure. As an alternative approach, Fig. 9 presents a composite profile of the potential for helix and β-sheet secondary structure (Chou and Fasman, 1978) aligned with a hydropathy prediction (Kyte and Doolittle, 1982) and the location of our anti-peptide antibody binding regions. The hydropathy profile predicts two major hydrophilic clusters near the amino terminus of CRALBP and the present report confirms that this region is immunogenic and exposed to water-soluble high molecular weight probes. The COOH terminus is also predicted to be hydrophilic, and our results demonstrate its antigenicity. Other exons have been studied by analyzing the surface composition of 12 proteins that most continuous epitopes lie in loops that protrude from the protein structure, with a few also in helices but none in β-sheets (Barlow et al., 1986). Our results are consistent with this in that secondary structure predictions for CRALBP (Fig. 9 and Crabb et al., 1988b) appear to favor helices and β-turns for the amino- and carboxyl-terminal domains accessible to large probes in the native structure. β-

Sheet predictions appear to predominate for the central regions of CRALBP which also are much less accessible to antibodies and proteases. Interestingly, the synthetic peptide corresponding to CRALBP residues 202–229 was recognized by the pAb to intact CRALBP but in the native structure this region is inaccessible to the site-specific antibody. Apparently this in case significant unfolding of CRALBP occurred during immunization.

Additional clues to the domain structure of CRALBP may be provided by the exon/intron structure of the CRALBP gene (Intres et al., 1990; Crabb et al., 1991). The complete sequence of the 13-kilobase human CRALBP gene has recently been determined, and several of its eight exons can be correlated reasonably well with potential protein domains. Amino-terminal CRALBP residues 4–46 and carboxyl-terminal residues 265–316 are encoded by separate exons and may
The present results indicate that the amino terminus of CRALBP provides useful tools for probing functional domains and efforts are underway to build upon these results. The anti-CRALBP antibodies outlined here provide low resolution topographic map of CRALBP. The present results provide a low resolution topographic map of CRALBP. The anti-CRALBP antibodies employed both site-specific antibodies and site-directed mutagenesis. Such studies should enhance understanding of the molecular basis for the interactions of CRALBP with 11-cis-retinaldehyde and with other proteins in the visual cycle.

Acknowledgments—We thank Vijay Sarthy for assistance in the preparation of the monoclonal antibodies. The expert technical assistance of S. Goldflam in preparing CRALBP cDNA clones is gratefully acknowledged. We are also grateful to M. LaDuke for art work and photography and to V. Oliver, J. Lamb, and J. Seng for secretarial assistance.

REFERENCES


Richards, F. M., and Vithayathil, P. J. (1959) J. Biol. Chem. 234, 1459-1465

Continued on next page.
Preparation of Monoclonal Antibodies - Three-week-old female BALB/c mice received an initial immunization (IP) injection of 50 μg of purified CRALBP subcutaneously in an equal volume of complete Freund's adjuvant. Two boosters of 10 μg of CRALBP were given at 3-week intervals. The last booster was followed by a 1-week rest period. Spleen cells of the mice were then fused with the fusion agent. Hybridomas were screened for antibody activity in the immune response to the antigen. The hybridomas were cloned by limiting dilution on a microtiter plate and a total of 15 clones was obtained.

SDS-PAGE and Western Analyses - The gel system employed for the evaluation of the limited proteolysis fragments from CRALBP was that of Laemmli (1970) using 10% polyacrylamide slabs. Gels were stained with either Coomassie blue or silver according to the methods of Blum and Dainiak (1977) or the method of Patrick et al. (1982). Immuno blotting of PVDF membranes for the detection of CRALBP fusion fragments was performed as described by Fazekas de St Groth et al. (1988).

Protein Purification - Thezyme (1992) provided a useful method for making a good quality peptide. It was demonstrated that in presence of the fluorophores, the fluorescence was enhanced, and that the fluorophores were not removed by the procedure. The concentration of the peptide was determined using the molar absorptivity at 280 nm.

Immunoprecipitation - The procedure was adapted from that described by Horvath (1984). Briefly, polypeptide antigens were coated onto the inner wall of 15 cm × 5 cm Nunc culture bottles and incubated for 1 hour. The reaction was then stopped by the addition of 50 μl of 20% trichloroacetic acid and the precipitate was removed by centrifugation. The supernatant was then mixed with 50 μl of 20% trichloroacetic acid to remove any unbound protein. The precipitate was then dissolved in 0.1 N NaOH and the absorbance at 280 nm was determined.

Limited Proteolysis - The time course of proteolysis of CRALBP with trypsin was determined at pH 7.5 by use of a CHALP trap ratio of 1:10,000, 1:300, and 1:100 for CRALBP and the same for the peptide, respectively. The reaction mixture was incubated at 37°C for 24 hours in the presence of 5 μg of trypsin. The reaction was stopped by the addition of 20% trichloroacetic acid and the protein concentration was measured by UV absorbance at 280 nm. The absorbance at 280 nm was determined.

Immunoprecipitation - The procedure was adapted from that described by Horvath (1984). Polypeptide antigens were coated onto the inner wall of 15 cm × 5 cm Nunc culture bottles and incubated for 1 hour. The reaction was then stopped by the addition of 50 μl of 20% trichloroacetic acid and the precipitate was removed by centrifugation. The supernatant was then mixed with 50 μl of 20% trichloroacetic acid to remove any unbound protein. The precipitate was then dissolved in 0.1 N NaOH and the absorbance at 280 nm was determined.
### Table 1. Summary of Peptide Synthesis Yields

<table>
<thead>
<tr>
<th>Synthetic Peptide</th>
<th>CRALBP Residues</th>
<th>Average Coupling Yield (%)</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>S-8-R</td>
<td>1-8</td>
<td>99.7</td>
<td>47</td>
</tr>
<tr>
<td>S-17-L</td>
<td>1-17</td>
<td>99.1</td>
<td>43</td>
</tr>
<tr>
<td>V-31-G</td>
<td>10-39</td>
<td>99.3</td>
<td>35</td>
</tr>
<tr>
<td>G-31-G</td>
<td>30-38</td>
<td>99.4</td>
<td>36</td>
</tr>
<tr>
<td>C-35-G</td>
<td>35-42</td>
<td>99.4</td>
<td>37</td>
</tr>
<tr>
<td>K-25-Q</td>
<td>46-73</td>
<td>99.3</td>
<td>59</td>
</tr>
<tr>
<td>A-29-F</td>
<td>71-96</td>
<td>99.3</td>
<td>25</td>
</tr>
<tr>
<td>P-23-P</td>
<td>120-145</td>
<td>99.7</td>
<td>35</td>
</tr>
<tr>
<td>L-21-T</td>
<td>149-175</td>
<td>99.8</td>
<td>25</td>
</tr>
<tr>
<td>M-36-E</td>
<td>175-201</td>
<td>97.9</td>
<td>79</td>
</tr>
<tr>
<td>N-38-S</td>
<td>205-229</td>
<td>98.5</td>
<td>36</td>
</tr>
<tr>
<td>H-27-M</td>
<td>225-256</td>
<td>98.6</td>
<td>49</td>
</tr>
<tr>
<td>L-29-P</td>
<td>257-285</td>
<td>98.6</td>
<td>51</td>
</tr>
<tr>
<td>S-31-F</td>
<td>285-316</td>
<td>98.7</td>
<td></td>
</tr>
</tbody>
</table>

- **a.** Average coupling yields based on ninhydrin analysis of peptide-resin aliquots taken from every cycle of synthesis.
- **b.** Quantified by FPLC amino acid analysis of the crude peptide except where indicated.
- **c.** Quantified by dry weight of the crude peptide.

### Table 2. Summary of Anti-Peptide Antibodies

<table>
<thead>
<tr>
<th>Peptide</th>
<th>CRALBP Residues</th>
<th>Peptide</th>
<th>Antibody Titers</th>
</tr>
</thead>
<tbody>
<tr>
<td>S17L</td>
<td>1-17</td>
<td>S17L</td>
<td>+++</td>
</tr>
<tr>
<td>V21G</td>
<td>10-39</td>
<td>V21G</td>
<td>+++</td>
</tr>
<tr>
<td>D19Q</td>
<td>30-45</td>
<td>D19Q</td>
<td>+++</td>
</tr>
<tr>
<td>K25Q</td>
<td>46-70</td>
<td>K25Q</td>
<td>++</td>
</tr>
<tr>
<td>A25I</td>
<td>71-96</td>
<td>A25I</td>
<td>+++</td>
</tr>
<tr>
<td>E21G</td>
<td>120-145</td>
<td>E21G</td>
<td>+++</td>
</tr>
<tr>
<td>T27V</td>
<td>149-175</td>
<td>T27V</td>
<td>+++</td>
</tr>
<tr>
<td>L23E</td>
<td>175-201</td>
<td>L23E</td>
<td>++</td>
</tr>
<tr>
<td>N25S</td>
<td>253-279</td>
<td>N25S</td>
<td>+++</td>
</tr>
<tr>
<td>E27F</td>
<td>271-295</td>
<td>E27F</td>
<td>++</td>
</tr>
<tr>
<td>L25P</td>
<td>297-321</td>
<td>L25P</td>
<td>+++</td>
</tr>
<tr>
<td>S21F</td>
<td>295-316</td>
<td>S21F</td>
<td>+++</td>
</tr>
</tbody>
</table>

*Antibody dilutions yielding significant ELISA reactivity with the peptide antigen or intact CRALBP: 1:10,000 → 1:50,000 dilution; 2: 1:5,000 → 1:10,000 dilution; 3: 1:10,000 → 1:50,000 dilution; 4: 1:50,000 → 1:100,000 dilution; 5: 1:100,000 → 1:500,000 dilution; 6: 1:500,000 → 1:1,000,000 dilution.*