The Complete Primary Structure of the Cellular Retinaldehyde-binding Protein from Bovine Retina*

(Received for publication, June 17, 1988)

John W. Crabb§, Charles M. Johnson‡, Steven A. Carr¶, Lyman G. Armes¶, and John C. Saari¶

From the §W. Alton Jones Cell Science Center, Lake Placid, New York 12946, the ‡Department of Physical and Structural Chemistry, Smith Kline and French Laboratories, King of Prussia, Pennsylvania 19406, 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 and 11-cis-retinaldehyde as endogenous ligands and may be a functional component of the visual cycle. The complete amino acid sequence of CRALBP from bovine retina has been determined by direct microanalysis of the protein. Bovine CRALBP contains 316 residues in a single amino-terminal-blocked chain corresponding to a molecular weight of 36,421, inclusive of the blocking group. Overlapping peptides were generated by cleavage of lysyl, arginyl, methionyl, glutamyl, and one tryptophanyl bond and sequenced by gas-phase Edman degradation. Analysis of amino-terminal arginyl and methionyl peptides by fast atom bombardment mass spectrometry identified the N*-blocking group as an acetyl moiety, and tandem mass spectrometry provided the sequence of the first 9 residues. Comparison of CRALBP with other known protein sequences reveals no significant structural relatedness. The present results provide a basis for relating CRALBP domains with physiological function and for the future development of a more detailed three-dimensional model of the interaction of 11-cis-retinaldehyde with protein.

Four water-soluble retinoid-binding proteins have been purified from bovine retina. These proteins form noncovalent complexes with specific endogenous retinoids and are thought to play a role in the transport, metabolism, and function of vitamin A (1–3). Previous structural studies from our laboratories have described the NH₂-terminal sequence similarity of cellular retinol-binding protein and cellular retinoic acid-binding protein (4), partial characterization of interphotoreceptor retinoid-binding protein (5), and the complete amino acid sequence of cellular retinoic acid-binding protein (6). Cellular retinaldehyde-binding protein (CRALBP)§ carries 11-cis-retinaldehyde and/or 11-cis-retinol as endogenous ligands, has only been found in visual tissue, and is likely to be a functional component of the visual cycle, perhaps serving as a stereoselective agent and/or substrate carrier protein (7, 8). As part of our continuing study of the role of retinoid-binding proteins in the physiology of the retina, we have determined the primary structure of bovine retinal CRALBP. NH₂-terminal analysis of CRALBP is hindered by the presence of a blocking group (4). A combination of fast atom bombardment (FAB-MS) and tandem mass spectrometry (MS/MS) coupled with amino acid analysis has provided the identity of the N*-blocking group and NH₂-terminal sequence. The strategy used in the direct microanalysis of the protein and evidence in support of the complete amino acid sequence of bovine CRALBP are presented here.

EXPERIMENTAL PROCEDURES

RESULTS

The strategy used to determine the complete amino acid sequence of CRALBP from bovine retina is shown in Fig. 1. The single amino-terminally acetylated polypeptide chain contains 316 residues, corresponding to a molecular weight of 36,421 (including the N*-acyetyl group), about 10% larger than the 33,000 determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (19). The CRALBP sequence is in agreement with the amino acid composition determined experimentally (Table I). The majority of the sequence of bovine CRALBP was determined by Edman degradation of peptides generated by cleavage at lysyl residues. The alignment of the lysyl peptides was obtained by cleavage of CRALBP at methionyl, arginyl, and glutamyl bonds and by one tryptophanyl peptide generated during cyanogen bromide cleavage. The N*-acyetyl blocking group and the sequence of the first 9 residues were determined by mass spectral analysis of the NH₂-terminal arginyl and methionyl peptides. The COOH terminus was identified both by carboxypeptidase Y digestion and by Edman degradation of lysyl and arginyl peptides lacking a COOH-terminal lysine or arginine, respectively.

Lysyl Peptides—The sequence of 281 residues was determined by Edman degradation of 13 peptides that were generated by treatment of pyridylethyl CRALBP with endoprotease Lys-C and purified by RP-HPLC (Fig. 2). Lysyl peptides are numbered K1, K2, etc. from the amino terminus except for peptides containing uncleaved lysyl peptide bonds (e.g. K3/5 and K12/13). Purification yields and amino acid compositions are shown in Table II. Sequence analyses of peptides from another endoprotease Lys-C digest (Fig. 3) of

*This work was supported in part by United States Public Health Service Grants EY-06603, EY-02317, and CA-37589 and by RJR Nabisco. 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.

§To whom correspondence should be addressed.

¶The abbreviations used are: CRALBP, cellular retinaldehyde-binding protein; CPr, carboxypeptidase Y; FAB-MS, fast atom bombardment-mass spectrometry; Gdn-HCl, guanidine hydrochloride; MS/MS, tandem mass spectrometry; PEC, (pyridylethyl)lysine; PTC, phenylthiocarbamyl; PTH, phenylthiohydantoin; RP-HPLC, reverse phase-high performance liquid chromatography; SDS, sodium dodecyl sulfate; TPCK, N-tosyl-L-phenylalanine chloromethyl ketone.

¶ Portions of this paper (including "Experimental Procedures," Figs. 2-11, and Tables I–IV) are presented in miniprint at the end of this paper. 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.

18678
Protein Sequence Analysis of CRALBP from Bovine Retina

Ac-SEGACTFMVVEEQELARQLERLTTKDHGVPFGPCSLRHTLQKADKELNEKCTREETAEKVRLEQLQELVARAAASQELAVAERVQXKDSAFLRF1RARKFHVGRAY

FIG. 1. Summary of the proof of the sequence of bovine CRALBP. The determined sequences of specific peptides are indicated by a solid line. Prefixes K, R, E, M, and CBW denote peptides generated by cleavage at lysyl, arginyl, glutamyl, methionyl, and tryptophanyl residues, respectively. Peptides are numbered sequentially from the amino terminus except where an uncleaved residue gives an overlap (e.g. K3/5). All peptide sequences were proven by Edman degradation except R1 and M1 where tandem mass spectrometry was used. Ac denotes an acetyl group that was identified by FAB-MS.

$[^{14}C]$carboxymethyl CRALBP corroborated the identity of cysteine at residues 36, 137, 180, and 198. Unambiguous sequences were determined as shown in Fig. 1 for each lysyl peptide except blocked peptide K1 and dipeptide K14.

Arginyl Peptides—Succinylated and pyridylethylated CRALBP was digested with trypsin and peptides purified by RP-HPLC (Fig. 4). Several of the arginyl peptides used in the proof of the CRALBP structure required rechromatography before unambiguous sequences could be determined (Fig. 5). Purification yields and amino acid compositions are shown in Table III. The sequence of 188 residues was determined by analysis of 15 arginyl peptides (Fig. 1), and overlaps were obtained between K2 through K6 and between K15 through K17.

Cyanogen Bromide Peptides—Succinylated and pyridylethylated CRALBP was cleaved at methionyl residues with cyanogen bromide, and peptides were purified by RP-HPLC at neutral pH (Fig. 6). Except for the tripeptide M5, the other five cyanogen bromide peptides were recovered, in addition to a tryptophanyl peptide (labeled CBW) generated during the cleavage. Peptides M2 and M3 required rechromatography prior to sequence analysis (Fig. 6, inset). Purification yields and amino acid compositions of selected methionyl peptides are shown in Table IV. The sequence of 109 residues was determined from analyses of the cyanogen bromide peptides, yielding strong overlaps between R2 through R4 and K2, between K8 and K9, and between K11 and K13. In addition, CBW linked K12/13 to K15 by a 2-residue overlap.

Glutamyl Peptides—To obtain the final overlaps, succinylated and carboxymethylated CRALBP was digested with Staphylococcus aureus V8 protease and the peptides purified by RP-HPLC (Fig. 7). Glutamyl peptides E18 and E31 required rechromatography (Fig. 8, top and middle) in order to obtain unambiguous sequence results. Amino acid compositions and yields of selected glutamyl peptides are shown in Table IV. The sequence of 85 residues was determined from...
four glutamyl peptides (Fig. 1), yielding strong overlaps between M1 and M2 and R1 and R2/3, between K5 and K7, between K7 and K8 and between K9 and K11. Glutamyl peptide E2/5 was also isolated (Fig. 8, bottom) and sequenced from a V8 protease subfragment of lysyl peptide K1.

Identification of the NH2-terminal Structure—FAB-MS revealed the chemical nature of the N*-blocking group and corroborated the composition of arginyl peptide R1. The FAB mass spectrum of 400 pmol of the blocked peptide R1 exhibited two sets of [(M+H)*/(M+Na)°] peaks at m/z 743/765 and 866/888, respectively (Fig. 9A). No sequence-informative fragments were observed. The arithmetical difference between the intense signal at m/z 866 and the molecular weight value 824 calculated for a peptide with the amino acid composition of R1 (Table I) strongly suggested R1 to be an acetyl-blocked octapeptide with a composition of Glu,Ser,Gly,Arg, Thr,Ala,Phe. The signal at m/z 743 is apparently unrelated to the blocked peptide and of unknown origin.

The sequence of the 8-residue blocked R2 peptide was determined by MS/MS using a VG ZAB SE-4F four-sector magnetic deflection mass spectrometer (10, 15). The first mass analyzer selected the [(M+H)°] ion produced by FAB ionization of 500 pmol of arginyl peptide R1. The daughter ion spectrum generated in the second mass analyzer (Fig. 10) exhibited a complete series of sequence fragments originating from the carboxyl terminal (W, X, Y, Z) and extending to the acetylated amino terminal. In addition, a complete series of acylum fragments (Bn) from the amino terminal is also present. These fragments establish the sequence of the first 8 amino acids of R1 to be acetyl-Ser-Glu-Gly-Ala-Gly-Thr,Ala,Phe. The signal at m/z 743 is apparently unrelated to the blocked peptide and of unknown origin.

Mass analysis of blocked methionyl peptide M1 (500 pmol) corroborated the results obtained with the blocked arginyl peptide. The FAB mass spectrum of M1 (Fig. 9B) exhibits two molecular weight-related clusters at m/z 1010 and 1038. These correspond respectively to the [(M+H)°] ion for a peptide molecular weight of 1009 and the [(M+H)°] ion for a formylated peptide of the same composition. The latter are common in the spectra of peptides obtained from CNBr digests employing formic acid as the solvent. The observed molecular mass of 1009 was 61 daltons more than the 948 expected for the 9-residue NH2-terminal acetyl, COOH-terminal homoserine peptide. Apparently ethanolamine (molecular weight 61) added to the dry CNBr peptides, improving peptide solubility and RP-HPLC separation (11), and reacted with the COOH-terminal homoserine actone to yield homoserine ethanalamide and higher molecular weight compounds. The sequence of peptide M1 was determined by MS/MS. Again, a complete NH2-terminal B series was obtained with masses identical to those observed for peptide R1 with the addition of an intense fragment corresponding to Bn (spectrum not shown). Similarly, the series of COOH-terminal fragments, W, X, Y, and Z were all observed offset to higher mass by 144 daltons, the incremental mass of the homoserine ethanolamide residue. These data establish the sequence of the NH2-terminal CNBr fragment of CRALBP as acetyl-Ser-Glu-Gly-Ala-Gly-Thr,Ala,Phe,Arg-Met.

Identification of the COOH Terminus—Carboxypeptidase Y digestion of intact pyridylethylated CRALBP suggested the COOH-terminal sequence to be ...Thr-Ala-Phe-COOH (Fig. 11). Only one lysyl peptide lacking lysine (K17) was isolated, and this peptide was recovered in high yield (about 62%); its COOH-terminal sequence, as determined by Edman degradation, agrees with the carboxypeptidase Y-deduced sequence. Edman degradation of arginyl peptide R22 also supports the COOH-terminal sequence of CRALBP to be ...Thr-Ala-Phe-COOH. Peptide R22 was isolated as K17/R22 apparently due to both incomplete succinylation and incomplete trypic digestion.

**DISCUSSION**

Direct proof of the complete structure of CRALBP from bovine retina was derived from overlapping peptides generated by cleavage at lysyl, arginyl, methionyl, and glutamyl residues. One tryptophanyl peptide produced during cyanogen bromide cleavage was also utilized in establishing an overlap. Fast atom bombardment and tandem mass spectrometry provided the data necessary to assign the first 9 residues and to determine that the protein is N*-acyethyl blocked. These results highlight the sensitivity (pico- to nanomole level) and efficacy of mass spectrometry in the microcharacterization of post-translationally modified proteins (15). Eighty-nine percent of the 316 amino acid residues in the protein were identified in more than one peptide. The weakest parts of the direct structural determination were the variable phenylthiocarbamyl amino acid analyses and the 2-residue overlap between peptides CBW and K15. The complete sequence was independently confirmed, however, by analysis of the cDNA encoding bovine CRALBP (21).

Compared with other protein sequences, CRALBP belongs neither to the superfamilies of lipophilic-binding proteins that includes cellular retinol-binding protein, cellular retinoid acid-binding protein, peripheral nerve myelin P2 protein, and several fatty acid-binding proteins (22), nor to the superfamily that includes serum retinol-binding protein (23). Furthermore, CRALBP is not related to other retinoid-binding proteins such as interphotoreceptor retinoid-binding protein (24), rhodopsin, and the cone visual pigments (25). In fact, CRALBP exhibits no structural relatedness with any other presently known protein sequence in the Protein Identification Resource database. Given the finite number of proteins and the ever accelerating rate at which new protein sequences are determined/deduced, it is likely that a protein superfamily including CRALBP will become evident in the not distant future (26).

No information is yet available concerning the retinoid-binding site in CRALBP, though most likely the water-insoluble ligand is sequestered within a hydrophobic domain. Hydropathy in CRALBP estimated according to Hopp and Woods (17) agrees in general with that predicted by Kyte and Doolittle (16); however, the strongest uninterrupted hydrophobic region is predicted by Hopp and Woods and falls between residues 238 and 250 (21). Secondary structure predictions suggest that this hydrophobic domain contains more β-sheet than α-helix (21). Crystallization of CRALBP with bound 11-cis-retinaldehyde and analysis by x-ray diffraction could lead to the identification of the retinoid-binding site. Recent work suggests a common structural motif in three proteins of the retinol-binding protein superfamily (27). Compared with rhodopsin and other membrane-bound visual pigments, the water solubility of the CRALBP-retinoid complex indeed renders it a reasonable candidate for crystallization. In this regard, CRALBP may provide a useful three-dimensional structural model for the interaction of 11-cis-retinaldehyde with protein.

The physiological role of CRALBP is not yet fully understood. However, several lines of evidence suggest that the protein is a functional component of the visual cycle. First,
CRALBP has only been found in retina and pineal, both light-sensitive tissues (1). Second, the endogenous ligands bound by CRALBP are 11-cis-retinol and 11-cis-retinaldehyde, retinoids that are only known to function in the visual process. Third, CRALBP interacts specifically with the visual cycle enzymes 11-cis-retinol dehydrogenase (7) of retinal pigment epithelium, catalyzing the reduction of 11-cis-retinaldehyde to CRALBP, and retinyl ester synthase, yielding retinyl esters (28). Finally, CRALBP is able to select 11-cis-retinaldehyde from a mixture of vitamin A stereoisomers and, relative to rhodopsin, protect it from photoisomerization, suggesting a role for the protein in the generation of 11-cis-retinoids (8).

These results establish the complete primary structure of bovine CRALBP. Determination of the three-dimensional structure of CRALBP and structure-function correlations facilitated by the present results should provide more definitive clues to the cellular role of this protein.

Acknowledgments—The expert technical assistance of G. Garwin and D. L. Bredberg in the purification of CRALBP is gratefully acknowledged. We are also grateful to M. LaDuke for art work and photography and to J. Lamb for secretarial assistance.

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**Protein Sequence Analysis of CRALBP from Bovine Retina**

Fig. 2 RP-HPLC of lysyl peptides. Approximately 250 μg (4.4 nmol) of porcine retinal CRALBP was digested with endoproteinase-Lys-C (98 μg) in 0.2 M tris (pH 7.5) 0.1% TFA, pH 8.0, and fractionated on a 9p Vydac C18 column (1.6 x 250 mm) at 460 nm. Fractions were collected at intervals indicated and peak fractions collected by hand. Absorbance (214 nm and 270 nm) and tryptophan fluorescence (350 nm excitation, 380 nm emission) were monitored simultaneously. Solvent A was 0.1% trifluoroacetic acid in H2O and solvent B was H2O protonative containing about 0.01% trifluoroacetic acid. Rapid identification of cysteine containing peptides by absorbence at 270 nm and fluorescence was sought for the design of appropriate aliphatic aminopeptidase DNA screening probes. Peptides are numbered as in Figure 1.

Fig. 3 Identification of cysteine-containing lysyl peptides. About 220 μg (5.5 nmol) of 14C-labeled methyl-CRALBP was digested with endoproteinase-Lys-C and fractionated on a 9p Vydac C18 column as described in Figure 2 and measured for 14C-radioactivity (shaded bars). Cysteine-containing peptides were sequenced and the PTH-amino acids monitored for radioactivity. Peptide numbers are numbered as in Figure 1, cysteines are numbered according to residue position in the complete polypeptide (i.e., C36, C137, C180 and C198).
Protein Sequence Analysis of CRALBP from Bovine Retina

Fig. 4 RP-HPLC of arginyl peptides. Approximately 148 pg of carboxymethylated CRALBP was digested with TPCK trypsin (4% w/v) and injected onto a 5 μm Vydac C18 column (4.6 x 250 mm) at 1 ml/min. Arginyl peptides were eluted using the gradient indicated and collected by hand: Solvent A, 0.1% trifluoroacetic acid in H2O; solvent B, 84% acetonitrile containing about 0.1% trifluoroacetic acid. Flow rate, 1 ml/min. Peptides are numbered as in Figure 1.

Fig. 5 Rechromatography of selected arginyl peptides. Several arginyl peptides from Figure 4 were rechromatographed by RP-HPLC for sequence analysis. Rechromatography conditions are as described in Figure 4. (A) R8, (B) R13, (C) R11, (D) R12 and R15, (E) R19, and (F) R21.
Protein Sequence Analysis of CRALBP from Bovine Retina

Fig. 6 HP-HPLC of tryptic bromide peptides. About 150 µg (1.5 nmol) of native bromide CRALBP was digested with tryptic bromide and dried as described in Experimental Procedures. The peptide mixture was dissolved in solvent B and injected onto a 5% Waters gel-filtration 25 cm column (0.8 x 20 cm) at 60°C. Peptides were eluted at a flow rate of 1 ml/min. The peptide fraction M2 + M3 was rechromatographed using the same column and solvent system (inset). Methionyl peptides are numbered as in Figure 1. CBM denotes a tryptic peptide generated by CBM.

Fig. 7 RP-HPLC of glutamic peptides. Approximately 170 µg (4.7 nmol) of native CRALBP was digested with trypsin, desalted, and analyzed by RP-HPLC using a 4.6 x 250 mm, 5 µm, C18 reversedphase column at a flow rate of 1 ml/min. The peptide fraction M2 + M3 was rechromatographed using the same column and solvent system as in Figure 6. Glutamic peptides were eluted at 50 min with an Applied Biosystems Model 165microbore HPLC system and collected at 20 minute intervals. Peptide numbering and solvent are as in Figures 1 and 7, respectively.
Protein Sequence Analysis of CRALBP from Bovine Retina

Fig. 6: Final purification of selected trypsin peptides. Chromatography of peptides 1B (4A) and (3) B) were rechromatographed on silica gel for sequence analysis. (2) Purification using a three-step procedure (see text). The three-step procedure involves elution of the peptide with 6% acetic acid, 6% acetic acid, and 6% acetic acid, respectively, with charge retention and resolution of the COOH-terminal ends.

Fig. 9: Identification of the COOH-terminal group. Positive ion FAB mass spectra of the COOH-terminal blocked (A) arginine peptide 61 (100 pmol) and (3) methionine peptide 61 (100 pmol).

Ac-Ser-Gly-Ala-Gly-Thr-Phe-Arg

Fig. 10: Tandem mass spectrum analysis of the COOH-terminal group of the blocked arginine peptide 81 (50 pmol) with (M+H) = 861.4. Fragment ion mass spectrometry in agreement with Wolfert and Schuster (28) for trypsin digestion. The most abundant ions are marked and correspond to COOH-terminal fragments. Similarly, X1, Y1, and M1 correspond to fragments arising by cleavage of the COOH-terminal arginine residue and removal of COOH-terminal groups, respectively, with charge retention.

m/z
Protein Sequence Analysis of CRALBP from Bovine Retina

Table 1

| Peptide | Residues | Peptide Destuction, Incomplete Hydrolysis, Not Determined. | wcovemd per 1000 HPLC (1000) | PEC |
|---------|----------|----------------------------------------------------------|-------------------------------|-----|---|
|         |          |                                                          |                               |     |   |

Fig. 1

Moles Amino Acid Released per Mole Protein

Molar Batches Released from Bovine Retina

Table 2

| Peptide | Molar Batches Released per Mole Protein | Molar Batch Released per Mole Protein | Molar Batch Released per Mole Protein | Molar Batch Released per Mole Protein |
|---------|----------------------------------------|--------------------------------------|--------------------------------------|--------------------------------------|
|         |                                        |                                      |                                      |                                      |

Note on Table 2:
1. Values for amino acids released at different pH levels are given in Table 2.
2. Values with an asterisk (*) indicate the presence of interfering compounds.
3. Values in parentheses ( ) represent the percentage of amino acids released.
4. Values without an asterisk indicate the absence of interfering compounds.
5. Values with a question mark (?) indicate further analysis is required.
6. Values with a plus sign (+) indicate partial hydrolysis.

Graphical Representation of Molar Batches Released per Mole Protein

- Moles Amino Acid Released per Mole Protein
- Molar Batches Released from Bovine Retina
- Values for amino acids released at different pH levels
- Values with an asterisk (*) indicate the presence of interfering compounds.
- Values in parentheses ( ) represent the percentage of amino acids released.
- Values without an asterisk indicate the absence of interfering compounds.
- Values with a question mark (?) indicate further analysis is required.
- Values with a plus sign (+) indicate partial hydrolysis.

Graphical Representation of Molar Batches Released from Bovine Retina

- Moles Amino Acid Released per Mole Protein
- Molar Batches Released from Bovine Retina
- Values for amino acids released at different pH levels
- Values with an asterisk (*) indicate the presence of interfering compounds.
- Values in parentheses ( ) represent the percentage of amino acids released.
- Values without an asterisk indicate the absence of interfering compounds.
- Values with a question mark (?) indicate further analysis is required.
- Values with a plus sign (+) indicate partial hydrolysis.
### Table III

| Peptide   | R1 | R2/3 |
|-----------|----|------|
|           | 1-8| 9-23 |

### Table IV

| Peptide   | R1 | R2/3 |
|-----------|----|------|
|           | 1-9| 10-15 |

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### Notes:

- a. Residues per peptide by phenylalanine amino acid analysis (5 N HCl, 150°C, 1 h). Values are from a single analysis and are reported for partial destruction, incomplete hydrolysis, or contamination from solvents (e.g., neat and hist). Values less than 0.1 are not reported. Numbers in parentheses are from the sequence shown in Figure 1.
- b. Not quantified due to interfering compounds.
- c. Amino acid residues per residue by phenylalanine amino acid analysis (5 N HCl, 150°C, 1 h) are reported for partial destruction, incomplete hydrolysis, or contamination from solvents (e.g., neat and hist). Values less than 0.1 are not reported. Numbers in parentheses are from the sequence shown in Figure 1.
- d. Not quantified due to interfering compounds.
- e. Amino acid residues per residue by phenylalanine amino acid analysis (5 N HCl, 150°C, 1 h) are reported for partial destruction, incomplete hydrolysis, or contamination from solvents (e.g., neat and hist). Values less than 0.1 are not reported. Numbers in parentheses are from the sequence shown in Figure 1.