The Primary Structure of Rat Liver Cellular Retinol-binding Protein*

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The complete amino acid sequence of a cellular retinol-binding protein (CRBP) has been determined for the first time. The primary structure of rat liver CRBP was elucidated by analyses of cyanogen bromide fragments and peptides obtained by tryptic and thermolytic digestions. The single polypeptide chain of rat CRBP consists of 134 amino acid residues. Under reducing conditions, CRBP exists as a monomer, but, in the absence of reducing agents, dimers and multimers of the protein emerge. This is explained by the observation that CRBP contains 3 cysteines, one of which seems to be highly reactive. Whether CRBP contains a disulfide bond is not yet established.

The present data extend the previously described homology between CRBP and a family of low molecular weight proteins, all members of which may bind hydrophobic ligands. Since some of these proteins apparently display intracellular transport functions, a similar role for CRBP is envisaged.

Vitamin A is transported in plasma from its storage sites in the liver to various epithelial tissues by retinol-binding protein (RBP)1. The transport of vitamin A in plasma has been extensively studied from both biochemical and physiological aspects (for reviews, see Refs. 1 and 2).

In contrast, only limited information is available about the storage and metabolism of vitamin A in the liver and in epithelial cells. The presence of two vitamin A-binding proteins with ligand specificities for retinol (cellular retinol-binding protein (CRBP)) and for retinoic acid (cellular retinoic acid-binding protein (CRABP)) has been demonstrated in the cytosol of several types of cells (3). Both proteins have been isolated and found to have molecular weights of approximately 15,000 (4–8). The NH2-terminal sequences of CRBP and CRABP were found to be highly homologous to each other (9, 10). Unexpectedly, a similar degree of homology was also found between these two proteins and the myelin protein P2 (11), a protein without any known connection to vitamin A. More recently, the fatty acid-binding Z-protein was shown to be a member of this protein family (12).

The intracellular vitamin A-binding proteins have been suggested to mediate, directly or indirectly, the effects of vitamin (3). Further studies of the physiological role of these proteins would be facilitated if more were known about their molecular characteristics. We are therefore trying to elucidate the primary and tertiary structures of the intracellular binding proteins. We report here the primary structure of rat CRBP.

EXPERIMENTAL PROCEDURES*

RESULTS

NH2-terminal Sequence Analyses of CRBP and CNBr Fragments Thereof—Automated NH2-terminal sequence analysis of reduced and S-carboxymethylated CRBP allowed the identification of the first 45 amino acid residues (Figs. 1 and 9). One of the 5 methionines present in CRBP (cf. Table I) was located at position 10.

Cleavage with CNBr resulted in two large and three small fragments (CB1–CB5) which were isolated by gel chromatography (Fig. 2). The amino acid compositions of the fragments are given in Table I together with that of the intact CRBP chain. A comparison of the amino acid compositions of the CNBr fragments with the NH2-terminal sequence of CRBP showed that CB1 constitutes the NH2-terminal 10 residues of CRBP.

One CNBr fragment, CB3, lacked homoserine. Carboxypeptidase digestion of this fragment and of intact CRBP (Fig. 3), established CB5 as the COOH-terminal fragment. CB2–CB5 were subjected to NH2-terminal sequence analyses. Amino acid residues were identified in a total of 91 positions (Fig. 1, Table II, and Fig. 9). The sequence determinations demonstrated that CB1 is followed by the long fragment CB2 in the sequence.

Isolation and Analyses of Tryptic and Thermolytic Peptides of CRBP. Deduction of the Primary Structure of CRBP—Five peptides, T1–T5, were isolated from a tryptic digest of reduced and S-carboxymethylated CRBP by gel and ion-exchange chromatography as well as by HPLC (Figs. 4 and 5, and Table III). The NH2-terminal part of T2 and virtually the complete sequences of T1 and T3–T5 were determined by automated Edman degradation (Table IV). The sequence information on T1 and T2 enabled the alignment of CNBr fragments CB3 and CB4, and confirmed the order of all CNBr fragments, as outlined in Fig. 9. The sequence determination of T3 revealed the presence of a Met-Thr bond (residues 83–84). The difficulty to cleave such a bond with CNBr (15) explains why five

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The abbreviations used are: RBP, retinol-binding protein; CRBP, cellular retinol-binding protein; CRABP, cellular retinoic acid-binding protein; HPLC, high pressure liquid chromatography; PTH, phenylthiohydantoin; TFA, trifluoroacetic acid.

1 Portions of this paper (including all of "Experimental Procedures," part of "Results," Figs. 1–8, and Tables I–VI) 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 available from the Journal of Biological Chemistry, 9650 Rockville Pike, Bethesda, MD 20814. Request Document No. 84M-2997, cite the authors, and include a check or money order for $7.60 per set of photocopies. Full size photocopies are also included in the microfilm edition of the Journal that is available from Waverly Press.
CNBr fragments were obtained and not six, as expected from the number of methionines present in CRBP (cf. Table I).

The amino acid sequence of the COOH-terminal part of CB4 was obtained by analyses of the tryptic peptides T3 and T4. Sequence determination of peptide T5 confirmed the sequence of CB5 and extended it up to the COOH-terminal residue of CRBP.

A number of thermolytic peptides of CRBP were isolated by gel chromatography followed by HPLC (Figs. 6-8) and subjected to amino acid analyses. Two peptides, TL1 and TL2, were chosen for amino acid sequence determinations (Tables V and VI). The sequences of TL1 and TL2 overlapped those of the tryptic peptides T3-T5, and thus provided the information needed to establish the primary structure of CRBP (Fig. 9).

**DISCUSSION**

CRBP is composed of 134 amino acid residues. The calculated molecular weight, 15,700, agrees with that determined by physical methods. Thus, Ong and Chytil (4) estimated the molecular weight of CRBP to be 14,600 by averaging the results from three different methods, whereas Liou et al. (8) measured the apparent molecular weight of CRBP from a sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis at 15,900.

CRBP contains 3 cysteine residues, but it is not known if a disulfide bond exists in the native molecule. CNBr fragmentation of unreduced CRBP shows that the most COOH-terminal cysteine residue can form a disulfide bond (Fig. 2B). This bond could, however, have been formed during preparation of the protein.

Under nonreducing conditions, newly isolated CRBP migrates as a monomer on sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Upon prolonged storage or repeated thawing and freezing, dimers and multimers of the protein are formed. An interchain disulfide is likely to be involved in this process, since the dimer disappears upon reduction. This indicates that at least 1 of the cysteine residues is exposed. Additionally, one indication that 1 cysteine residue is located at the surface of the molecule is the observation that the organomercurial compound p-chloromercuriphenylsulfonic acid reacts with native CRBP (16).

P-Chloromercuriphenylsulfonic acid treatment abolishes the binding of retinol to CRBP (16). The abolished interaction is reversible, as a reducing agent restores the binding capacity. Although it is possible that 1, or more, of the cysteine residues is located at the retinol-binding site, it is also possible that the inhibition of retinol-binding by p-chloromercuriphenylsulfonic acid is due to steric hindrance or to an induced conformational change in the CRBP molecule. Further work is needed to clarify the status of the cysteine residues and their possible involvement in the binding of retinol to CRBP.

Partial sequence data showed that CRBP is homologous to CRABP and protein P2 (9-11). Later, Takahashi et al. (12) noted that the Z-protein belongs to the same protein family. The data now available allow a more comprehensive analysis of the relatedness of these proteins. In the following paper (17), describing the complete amino acid sequence of bovine CRABP, such an analysis is presented. One should point out, however, that no significant homology was found between CRBP and plasma RBP.

The molecular functions of the four proteins, CRBP, CRABP, protein P2, and the Z-protein, are not clearly defined. However, it is well established that the Z-protein binds fatty acids (18) and it has been suggested that it interacts with several microsomal enzymes, thereby influencing the biosynthesis of cholesterol (19). Protein P2 is a peripheral nerve membrane protein, which most probably interacts with lipid components in the myelin layer (20). Accordingly, all hitherto identified members of this protein family bind hydrophobic low molecular weight substances and it seems rea-
reasonable to suggest that CRBP and CRABP might also be able to interact with membranes as do protein P2 and possibly the Z-protein. No continuous stretch of hydrophobic amino acid residues longer than a few residues is present in either the CRBP sequence or in that of protein P2 (Fig. 9). However, a hydrophobic surface might very well be created by the three-dimensional folding of the peptide chain. This will be further clarified by the determination of the three-dimensional structure of CRBP, well under way (21).

It might be inferred from the sequence comparisons with protein P2 and the Z-protein that CRBP has evolved from an ancestor cytosol lipid-binding protein with the ability to interact with membrane structures. One biological role of CRBP might accordingly be to store vitamin A in the cytoplasm and to transport the vitamin between cellular organelles. A role of CRBP as a cellular storage and transport protein for vitamin A implies the ligand saturation of CRBP to be high under normal nutritional conditions. This seems to be the case. CRBP isolated from bovine pigment epithelial cells is completely saturated with retinol (22).

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Primary Structure of Rat CRBP

SUPPLEMENTARY INFORMATION TO
THE PRIMARY STRUCTURE OF RAT LIVER CELLULAR RETINOL-BINDING PROTEIN

By
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EXPERIMENTAL PROCEDURES

Materials

CRBP was purified from rat liver, essentially as described (4).

Chemical Modifications

Reduction and 5-carboxymethylation was carried out as in (3). Maleylation was performed by dissolving the protein to a concentration of 5 mg/ml in 0.2 M sodium bicarbonate, 5% guanidine-HCl, pH 8, and 1% iodoacetamide. After 15 min at room temperature, 50 µl of 100 mM iodoacetamide was added and the mixture was incubated for 15 min. Column chromatography and gel electrophoresis were performed on a Bio-Rad apparatus using 12% gels and 5% stacking gels. The gels were stained with Coomassie Brilliant Blue R-250. The amino acid analysis was performed using an Applied Biosystems 400A. The results were calculated as moles of amino acid per mole of protein.

RESULTS

The protein was purified by ion-exchange chromatography on CM-Sephadex C-50. The purified protein was eluted with a linear gradient of NaCl from 0.05 M to 0.5 M. The fractions containing the pure protein were pooled and dialyzed against 0.01 M sodium phosphate, pH 7.5. The protein was then concentrated by lyophilization.

The amino acid composition of the CRBP is given in Table 1. The values are the average of at least three determinations.

| Amino Acid | CRBP1 | CRBP2 | CRBP3 |
|------------|-------|-------|-------|
| Asp        | 1.0   | 1.2   | 0.9   |
| Glu        | 2.5   | 3.0   | 2.8   |
| Arg        | 1.2   | 1.2   | 1.2   |
| Lys        | 1.8   | 2.0   | 2.1   |
| His        | 0.8   | 0.8   | 0.8   |
| Ser        | 5.5   | 6.0   | 6.2   |
| Thr        | 3.5   | 3.6   | 3.7   |
| Ile        | 2.5   | 3.2   | 3.2   |
| Val        | 1.5   | 1.6   | 1.6   |
| Thr        | 0.6   | 0.6   | 0.6   |
| Asp        | 0.8   | 0.8   | 0.8   |
| Glu        | 2.5   | 2.5   | 2.5   |
| Arg        | 1.2   | 1.2   | 1.2   |
| Lys        | 3.0   | 3.0   | 3.0   |
| Ser        | 3.5   | 3.5   | 3.5   |
| Thr        | 2.5   | 2.5   | 2.5   |
| Ile        | 2.5   | 2.5   | 2.5   |
| Val        | 2.0   | 2.0   | 2.0   |

The values are the average of at least three determinations.
Primary Structure of Rat CRBP

The COOH-terminal sequences of CRBP-fragments CB5 and of intact, reduced and alkylated CRBP were analyzed by digestions with carboxypeptidase A and by gas chromatography of amino acids released by acid hydrolysis. The COOH-terminal sequences of CB5 were determined from the primary structure of rat CRBP (Tables I and II) and showed an identity of 70% with the intact protein. The sequence of CB5 was identical to the sequence of CB5 described by others. The COOH-terminal sequence of CB5 was identical to the sequence of CB5 described by others. The sequence of CB5 was identical to the sequence of CB5 described by others.

Isolation and characterization of tryptic peptides of intact and reduced CRBP

Reduced, [14C]labeled and alkylated CRBP (300 pmol) were digested with trypsin and separated on a column of Sephadex G-75 (Fig. 4). Fractions were combined as indicated and lyophilized. Pool A and B were further purified by ion-exchange chromatography on Bio-Gel-porase (Fig. 5 A-C) and pool C by reversion chromatography on a Ultrogel AcA-54 column (Fig. 6).

Five tryptic peptides (T1-T5) were isolated. Peptides T1 and T2 were obtained in pure form, as judged from the amino acid composition data and the results from other experiments. Chromatography of pool C on a C8 column resulted in two major, closely migrating, peaks (Fig. 6, B). Both peaks contained material with identical amino acid composition (peptide T4). Edman degradation of the first peak gave a single amino acid sequence, indicating a homogeneity of at least 94% (peptide T1).

Peptides T2 and T3, isolated by ion-exchange chromatography, were not obtained in completely pure form. Sequence analysis of these two peptides revealed a cross-contamination of approximately 10%. This contamination did not, however, result in any ambiguity in the sequence assignments for peptides T2 and T3.

The amino acid compositions of all isolated tryptic peptides are shown in Table III and the results from the sequence determinations in Table IV.

![Figure 4](image-url)  
**Figure 4**: Chromatography of tryptic peptides of [14C]labeled and alkylated CRBP on a column of Sephadex G-75 (10 x 1.7 cm) equilibrated in 37% propionic acid. Fractions of 1 ml were collected every 1 min.

![Figure 5](image-url)  
**Figure 5**: Chromatography of pool B from Fig. 4 on a column of Bio-Gel-porase (10 x 1 cm) equilibrated with 0.02 M Tris-HCl, pH 7.4, and eluted with a linear gradient of 0.05 to 0.2 M NaCl. Fractions of 1 ml were collected every 4 min. --- absorption at 200 nm; -- radioactivity.

![Figure 6](image-url)  
**Figure 6**: Chromatography of pool C from Fig. 4 on a column of Ultrogel AcA-54. The conditions were the same as in Fig. 5.

Table III: Amino acid compositions of tryptic peptides of alkylated CRBP. The integral values in parentheses are based on the sequence.

| Peptide | T1 | T2 | T3 | T4 | T5 |
|---------|----|----|----|----|----|
| Isoleucine | 2.9 (3) | 1.8 (1) | 2.7 (4) | 2.9 (3) |
| Histidine | 0.8 (1) | 1.5 (2) | 0.8 (1) | 0.7 (1) |
| Arginine | 1.0 (1) | 1.6 (1) | 1.0 (1) | 1.0 (1) |
| Cys-Derivative | 0.4 (4) | 4.9 (4) | 0.5 (1) | 1.4 (1) |
| Glutamic Acid | 0.5 (4) | 4.9 (4) | 0.5 (1) | 1.4 (1) |
| Threonine | 1.5 (1) | 1.9 (2) | 1.2 (1) | 0.9 (1) |
| Serine | 1.1 (3) | 1.3 (1) | 1.1 (3) | 1.1 (3) |
| Glutamic Acid | 2.4 (2) | 4.4 (4) | 4.0 (4) | 4.1 (4) |
| Tyrosine | 2.9 (1) | 2.4 (2) | 2.5 (3) | 2.1 (2) |
| Alanine | 0.9 (1) | 1.5 (1) | 0.6 (1) | 1.0 (1) |
| Methionine | 0.7 (1) | 1.6 (1) | 0.8 (1) | 0.9 (1) |
| Lysine | 5.0 (4) | 1.4 (1) | 0.9 (1) | 1.0 (1) |
| Leucine | 0.4 (1) | 1.4 (1) | 1.6 (1) | 2.3 (2) |
| Tyrosine | 0.6 (1) | 1.8 (2) | 0.6 (1) | 0.6 (1) |
| Tryptophan | (1) | (1) | (1) | (1) |

*Peptides were hydrolyzed for 24 h. The threonine and serine values are corrected for decomposition by using the correction factors 0.36 and 0.02 respectively.*

*Not determined.*

The integral values in parentheses are based on the sequence.
Primary Structure of Rat CRBP

Table IV. Automated Edman degradation of tryptic peptides TL-75

| Amount T1 | T2 | T3 | T5 | T6 | T7 | T8 |
|-----------|----|----|----|----|----|----|
| Applied 16 pmol | 25 pmol | 10 pmol | 20 pmol | 25 pmol | 25 pmol | 25 pmol |
| Degree | Alanine | Yield | Alanine | Yield | Alanine | Yield | Alanine | Yield | Alanine | Yield |
| cycle | (nmol) | (nmol) | (nmol) | (nmol) | (nmol) | (nmol) | (nmol) | (nmol) | (nmol) | (nmol) |
| 1 | Lys 4.6 | 36 | Tyr 3.0 | 28 | Val 2.6 | 24 | Glu 1.7 | 20 | Val 1.9 | 16 |
| 2 | Arg 3.3 | 22 | Gly 2.9 | 21 | Ser 2.3 | 17 | Asp 1.7 | 16 | Ser 1.9 | 15 |
| 3 | Lys 3.3 | 20 | Pro 2.4 | 20 | Gln 2.1 | 18 | Arg 1.8 | 16 | Gln 1.9 | 15 |
| 4 | Lys 3.3 | 20 | Lys 2.4 | 20 | Lys 2.2 | 18 | Lys 1.8 | 16 | Lys 1.8 | 15 |
| 5 | Lys 3.3 | 20 | Leu 2.4 | 20 | Leu 2.2 | 18 | Leu 1.8 | 16 | Leu 1.8 | 15 |
| 6 | Lys 3.3 | 20 | Lys 2.4 | 20 | Lys 2.2 | 18 | Lys 1.8 | 16 | Lys 1.8 | 15 |
| 7 | Lys 3.3 | 20 | Lys 2.4 | 20 | Lys 2.2 | 18 | Lys 1.8 | 16 | Lys 1.8 | 15 |
| 8 | Lys 3.3 | 20 | Lys 2.4 | 20 | Lys 2.2 | 18 | Lys 1.8 | 16 | Lys 1.8 | 15 |
| 9 | Lys 3.3 | 20 | Lys 2.4 | 20 | Lys 2.2 | 18 | Lys 1.8 | 16 | Lys 1.8 | 15 |
| 10 | Lys 3.3 | 20 | Lys 2.4 | 20 | Lys 2.2 | 18 | Lys 1.8 | 16 | Lys 1.8 | 15 |

Isolation and characterization of thermolytic peptides.

Reduction and alkylation CRBP (500 pmol) was digested with thermolysin and subsequently size-fractionated on a Sephadex G-75 column (Fig. 6). In order to obtain peptides covering the tryptic peptides T1, T4 and T5, peptides containing the sequence Arg-Tyr were therefore combined as indicated in Table V. Automated Edman degradation of peptides T1 and T5, followed by HPLC (Fig. 8A) or directly by HPLC (Fig. 8A), the amino acid compositions of two of the isolated peptides, T1 and T5, are shown in Table V, and the results from the Edman degradation in Table VI.

Table V. Amino acid composition of the thermolytic peptides T1 and T2. The integral values in parentheses are based on the sequence.

| Amino Acid | T1 (mol%) | T2 (mol%) |
|------------|-----------|-----------|
| Lysine     | 3.0 (2)   | 1.0 (1)   |
| Arginine   | 1.1 (1)   | 1.0 (1)   |
| Threonine  | 1.1 (1)   | 2.0 (1)   |
| Glutamic acid | 3.0 (4) | 2.0 (2)   |
| Alanine    | 1.0 (1)   | 1.0 (1)   |
| Valine     | 1.0 (1)   | 0.8 (1)   |
| Methionine | 1.0 (1)   | 1.0 (1)   |
| Tryptophan | (1)       | (1)       |

% Edym determined for 24 h. The threonine and serine values are corrected for decomposition by using the correction factors 0.86 and 0.93 respectively.

Yields are calculated as mol peptides isolated/total protein subjected to cleavage.

Table VI. Automated Edman degradation of the thermolytic peptides T1 and T2

| Amount applied | T1 15 pmol | T2 44 pmol |
|----------------|------------|------------|
| Degradation cycle | Alanine | Yield (nmol) | Alanine | Yield (nmol) |
| 1 | Val 5.3 | Leu 11 | 2 | Glu 3.4 | Gln 12 |
| 2 | Lys 3.3 | Met 12 | 3 | Gly 3.7 | Arg 11 |
| 4 | Gly 3.7 | Arg 11 | 5 | Gln 2.4 | Ala 0.3 |
| 6 | Lys 1.6 | Gln 12 | 7 | Gln 2.5 | Glt 12 |
| 8 | Arg 1.1 | Glu 12 | 9 | Lys 1.6 | Gln 12 |
| 10 | Gly 2.4 | Arg 11 | 11 | Lys 1.6 | Gln 12 |
| 12 | Thr 0.6 | Lys 11 |

% Edym determined.