N-TERMINAL SEQUENCE HOMOLOGY AMONG RETINOID-BINDING PROTEINS FROM BOVINE RETINA

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Received 1 May 1981

1. Introduction

Bovine retina contains at least 4 proteins which form relatively stable, one-to-one complexes with retinoids including rhodopsin, the membrane bound visual pigment, and 3 water soluble proteins [1–3]. The characteristics of the latter 3 proteins are quite similar in that each is acidic, 2 are of equal molecular mass and the third has a molecular mass twice the value of the others. These proteins, distinct from the serum retinol-binding protein (SRBP, M_r 21 000) [4], have been termed cellular retinol-binding protein (CRBP, M_r 16 500) [5], cellular retinoic acid-binding protein (CRABP, M_r 16 500) [5] and cellular retinal-binding protein (CRALBP, M_r 33 000) [3]. CRABP and CRBP have been purified to apparent homogeneity from several tissues in addition to retina [6–9] whereas CRALBP appears to be a retina-specific protein [10]. Although serum and cellular retinoid-binding proteins would seem to form a family of structurally related proteins, no evidence for this has appeared. Immunological studies have revealed that antibodies directed toward bovine retinal CRALBP fail to cross-react with bovine retinal CRBP, CRABP, bovine SRBP or bovine opsin [11]. In addition antibodies against rat SRBP have been shown not to react with rat liver CRBP by two laboratories [8,12] and antibodies directed against rat liver CRBP have been shown not to react with either rat SRBP or rat liver CRABP [13]. The amino acid sequence of rat liver CRBP was reported not to display any homology with SRBP although, surprisingly, significant homology was evident with myelin protein P_2 [14]. This communication again addresses the question of whether the retinoid-binding proteins are structurally related. Amino-terminal amino acid sequence data is presented which demonstrates for the first time that homology does exist between CRBP and CRABP from bovine retina.

Abbreviations: SDS, sodium dodecyl sulfate; PTH, phenylthiohydantoin; PTC, phenylthiocarbamyl; HPLC, high performance liquid chromatography; PEC, pyridylethylcysteine; CRBP, cellular retinol-binding protein; CRABP, cellular retinoic acid-binding protein; CRALBP, cellular retinal-binding protein; SRBP, serum retinol-binding protein

2. Materials and methods

2.1. *Preparation of binding proteins*

CRBP, CRABP and CRALBP were purified to electrophoretic homogeneity from frozen cattle retinas (Hormel) as in [15]. The initial steps used in the purification of bovine SRBP have been described [16]. An additional purification procedure was required to bring this protein to a state of homogeneity suitable for primary structural studies. Fractions containing SRBP obtained from chromatography on DEAE-cellulose columns [16] were pooled and pumped onto a column (0.9 × 60 cm) of hydroxylapatite (BioRad HTP) pre-equilibrated in 20 mM Tris–HCl (pH 7.5), 80 mM NaCl, at a flow rate of 30 ml/h. After washing the column with 2 vol. of this buffer, SRBP was eluted with a linear gradient of sodium phosphate (0–100 mM) in the same buffer (total volume of gradient, 500 ml). Each of the binding proteins exhibited a single band in SDS–polyacrylamide slab gel electrophoresis [17] and, except for CRALBP, a single amino terminal amino acid in Edman degradation.

SRBP was pyridylethylated by a modification of the procedure in [18]. About 1 mg salt-free protein was reduced in 0.3 M *N*-ethylmorpholinium formate (pH 8.3), 2% β-mercaptoethanol under a N_2 barrier

for 2 h at 45°C. 4-Vinyl pyridine was then added to 6% (v/v) and the alkylation allowed to proceed with mixing at 45°C under N_2. After 2 h the 4-vinyl pyridine concentration was raised to 12% (v/v) and reaction conditions maintained for 1 h. After addition of β-mercaptoethanol to 12% (v/v) followed by 2 vol. of acetone, pyridylethylated SRBP was pelleted by centrifugation, washed 3 times with acetone and dried in vacuo.

2.2. *Protein sequence determination*

Manual Edman degradation was done as in [19]. At prolyl and glycyl residues cleavage times were extended 6 and 4 min, respectively, to compensate for the relatively slower rates of cleavage [19] and thus minimize out-of-step degradation. Conversion of thiazolinone amino acids to phenylthiohydantoin (PTH) amino acids was carried out in 1 N methanolic–HCl at 50°C for 20 min. PTH-amino acids were separated on a Beckman Ultrasphere ODS column using a Beckman Model 332 high-performance liquid chromatography system and identified and quantified spectrophotometrically at 269 nm as in [20]. PTH-dehydrothreonine was also detected at 320 nm. Thin-layer chromatography was employed as a second method of PTH-amino acid identification [19]. Sequence analysis of each of the binding proteins was performed at least twice on quantities ranging from 6–30 nmol. Amino acid analyses [21] were performed on a Beckman 120B amino acid analyzer modified to give increased sensitivity [22]. All chemicals were obtained commercially in the highest purity available.

3. Results and discussion

The amino-terminal sequences of the first 29–30 amino acids from CRBP and CRABP from bovine retina and bovine SRBP from bovine serum were determined by manual Edman degradation of the unfragmented proteins. No PTH-amino acids were generated above background during sequence analyses of CRALBP. Neither 5 cycles of Edman degradation on 30 nmol CRALBP nor 3 cycles on 12 nmol was productive, indicating that this retina-specific protein contains a blocked amino-terminus. Yields of PTH-amino acids obtained from sequence analyses of CRBP, CRABP and SRBP were quantified by HPLC and are presented in table 1. Repetitive yields averaged ~90% for analyses of CRBP and CRABP and ~91% for analysis of SRBP. Overlap or lag averaged ~1.1%/cycle for all 3 analyses.

Extensive sequence homology exists among the SRBPs from bovine, human and rabbit serum [22,23]. The amino terminal sequence of 29 residues of bovine SRBP is identical to that reported for rabbit SRBP [14] and, except for an Ala/Ser interchange at position 21, is also identical to that reported for human SRBP [23,24]. The extent of sequence homology observed in the amino terminal sequences of bovine rabbit and human SRBP is somewhat surprising in light of the observation that antibodies to the human protein do not cross-react with the SRBP from rat serum [25].

Significant sequence homology also exists between CRBP and CRABP from bovine retina (fig.1); the two

Table 1
Yields of PTH-amino acids[a]

Cycle	CRABP		CRBP		SRBP	
0	30 nmol		10 nmol		20 nmol	
1	Pro[b]	10.1	Pro[b]	1.0	Glu	10.6
2	Asn	17.1	Val	3.0	Arg	5.7
3	Phe	14.6	Asp	5.5	Asp	8.1
4	Ala	8.8	Phe	5.0	Pec[e]	2.1
5	Gly[c]	9.2	Thr[d]		Arg	4.4
6	Thr[d]		Gly[c]	2.7	Val	2.7
7	Trp	4.6	Tyr	2.2	Ser[d]	
8	Lys	4.0	Trp	1.4	Ser[d]	
9	Met	8.0	Lys	1.0	Phe	4.2
10	Arg	2.3	Met	1.5	Arg	1.3
11	Ser[d]		Leu	0.8	Val	1.7
12	Ser[d]		Ala	0.6	Lys	1.6
13	Glu	4.7	Asn	1.0	Glu	2.1
14	Asn	4.1	Glu	1.0	Asn	1.9
15	Phe	3.9	Asn	0.8	Phe	1.7
16	Asp	3.7	Phe	0.8	Asp	2.2
17	Glu	2.8	Glu	0.6	Lys	1.3
18	Leu	2.1	Glu	0.7	Ala	1.0
19	Leu	2.6	Tyr	0.6	Arg	0.7
20	Lys	1.1	Leu	0.3	Phe	1.0
21	Ala	1.2	Arg	0.2	Ala	0.8
22	Leu	1.1	Gly[c]	0.3	Gly[c]	0.8
23	Gly[c]	1.3	Leu	0.2	Thr[d]	
24	Val	1.2	Asp	0.3	Trp	0.3
25	Asn	1.0	Val	0.2	Tyr	0.4
26	Ala	1.0	Asn	0.2	(Ala)[f]	
27	Met	0.7	Val	0.4	Met	0.5
28	Leu	0.8	Ala	0.3	Ala	0.7
29	Arg	0.4	Leu	0.2	Lys	0.3
30			Arg	0.2		
Repetitive yield[g]	90%		90%		91%	

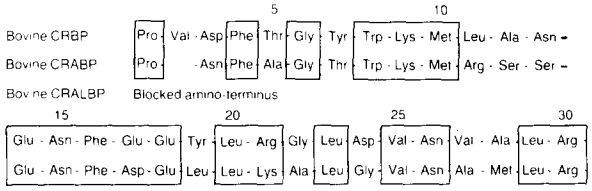

Fig.1. Comparison of the amino-terminal sequences of cellular retinol-binding protein (CRBP) and cellular retinoic acid-binding protein (CRABP) and cellular retinal-binding protein (CRALBP) from bovine retina. Numbering is based on the sequence of CRBP. Sequence identities and Glu/Asp and Arg/Lys interchanges are enclosed in boxes. A blank space indicates the introduction of a gap in the sequence.

proteins contain identical amino acid residues in sixteen of the first 29–30 positions (after insertion of a single gap in the CRABP sequence). Of the remaining 13 positions, ten sequence differences may be accounted for by a single base change in the nucleotide sequence and often reflect conservative interchanges such as Glu/Asp at position 17 and Arg/Lys at position 21. (Numbering is based on the CRBP sequence as shown in fig.1.)

Comparison of the partial amino acid sequences of the cellular retinoid-binding proteins with the serum retinol-binding proteins reveals some sequence similarity. The pentapeptide, Phe–Ala–Gly–Thr–Trp, is common to rabbit [14] and bovine SRBP (residues 20–24) and CRABP (residues 3–7) and yields the best alignment of the available sequence data based on the statistical methods in [26]. These and other similarities in the gapless alignment of residues 4–25 of CRBP and 3–24 of CRABP with residues 20–41 of rabbit [14] and human SRBP [23,24] are statistically significant*. Analysis of additional sequence data will be required in order to evaluate this observation.

Comparison of the partial sequences of rat liver CRBP [14] and bovine retinal CRBP reveals a striking amount of homology; identical residues are found in 27 of the first 30 positions (90%). Rask et al. [14] first reported that rat liver CRBP and myelin P_2 protein appear to be homologous. Our study of the amino-terminal sequence of bovine retinal CRABP shows that this protein also exhibits significant homology with myelin protein P_2 [27]. Sequence identities exist in 18 of the first 30 residues of bovine retinal CRABP and myelin P_2 (60%). The significance of this observation is obscure at present. Although the cellular retinoid-binding proteins exhibit entirely different binding specificities and appear to differ in function, these partial sequence data provide the first evidence that CRBP and CRABP belong to a family of structurally related proteins which probably evolved from a common ancestral gene.

[a] Quantitative results of the amino terminal sequence analyses of CRABP (cellular retinoic acid-binding protein) and CRBP (cellular retinol-binding protein) from bovine retina and bovine SRBP (serum retinol-binding protein). PTH-amino acids and PTC-amino acids were quantified by HPLC [19]. Yields were normalized to 100 percent injection and corrected for overlap and background as follows:

corrected yield = (yield at n) + (yield at $n+1$) − (yield at $n-1$)

where, n=cycle number. In correcting the yields of adjacent identical residues, the same amount of background was subtracted from each residue, namely that amount existing at the cycle immediately preceding the pair. The same amount of overlap was also added to each residue, namely 1/2 the amount existing at the cycle immediately following the pair. TLC was used as a second method of identification [18]

[b] Sum of PTH-Pro and PTC-Pro-methyl ester; [c] Sum of PTH-Gly and PTC-Gly-methyl ester

[d] Not quantified; PTH-dehydrothreonine was detected at 320 nm

[e] PTH-pyridylethylcysteine; [f] Tentative assignment

[g] The repetitive yields were calculated as a linear regression/least squares fit of all data except that for PTH-Pro, -Ser and -Thr. Overlap or lag as defined in [28], averaged 1.1%/cycle for all 3 analyses and was calculated as a linear regression/least squares fit of all data except that for PTH-Pro, -Ser, -Thr and that of adjacent identical residues

Acknowledgements

Mrs Lucille Bredberg and Mr Gregory Garwin provided expert assistance in the preparation of the retinoid-binding proteins. Conversations with Dr K. A. Walsh and Mr L. H. Ericsson were helpful during the course of these studies. We owe special thanks to Dr David C. Teller for providing statistical analyses of the amino acid sequence similarities and to Dr George E. Tarr for providing a preprint of his HPLC method of identifying PTH amino acids and for valuable discussions. This study was supported in part by USPHS

* Statistical analyses of the amino acid sequence similarities according to the methods described by DeHaen et al. [26] were performed by Dr David Teller, Department of Biochemistry, University of Washington School of Medicine, Seattle, Washington

grants EY 02317, EY 00343 and EY 01730. J. W. C. was supported by Institutional National Research Service Award EY 07013 from the National Eye Institute.

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