Partial amino acid sequence of human plasma retinol-binding protein. Isolation and alignment of the five cyanogen bromide fragments and the amino acid sequences of four of the fragments

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Abstract Studies are reported on the primary structure of human retinol-binding protein (RBP), the specific plasma transport protein for vitamin A. The protein consists of a single polypeptide chain of 186–187 amino acids. RBP was cleaved by cyanogen bromide into five fragments, CB-I (27 residues), CB-II (25 residues), CB-III (20 residues), CB-IV (15 residues), and CB-V (99–100 residues). The cyanogen bromide fragments were isolated, their compositions were determined, and they were aligned after studies that included the tryptic digestion of maleylated, reduced, and carboxymethylated RBP and subsequent enzymatic digestion of some of the resulting tryptic peptides. The amino acid sequences of four of the five cyanogen bromide fragments were determined, and the sequence of almost two-thirds of the NH_2-terminal portion of the RBP molecule was determined as: H_2N-Glu-Arg-Asp-Cys-Arg-Val-Ser-Ser-Phe-Arg-Val-Lys-Glu-Asn-Phe-Asp-Lys-Ala-Arg-Phe-Ser-Gly-Thr-Trp-Tyr-Ala-Met-Ala-Lys-Lys-Asp-Pro-Glu-Gly-Leu-Phe-Leu-Gln-Asp-Asx-Ile-Val-Ala-Glu-Phe-Ser-Val-Asx-Glx-Gly-Thr-Met-Ser-Ala-Thr-Ala-Gly-Lys-Arg-Val-Arg-Leu-Leu-Asn-Asn-Trp-Asp-Val-Cys-Ala-Asp-Met-Val-Gly-Thr-Phe-Thr-Asp-Thr-Glu-Asp-Pro-Ala-Lys-Phe-Lys-Met-Lys-Tyr-Trp-Gly-Val-Ala-Ser-Phe-Leu-Gln-Lys-Gly-Asn-Asp-Asx-His-Trp-Ile-Val-Asp-Thr-Asx-Thr-Tyr-Tyr-Ala-Val-Glu-Tyr-Cys-Ser-Arg---.—**Kanda, Y., and D. S. Goodman.** Partial amino acid sequence of human plasma retinol-binding protein. Isolation and alignment of the five cyanogen bromide fragments and the amino acid sequences of four of the fragments. *J. Lipid Res.* 1979. **20:** 865–878.

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Vitamin A is transported in plasma in the form of the lipid alcohol retinol, bound to a specific transport protein, plasma retinol-binding protein (RBP) (1, 2). Human RBP is a single polypeptide chain with a molecular weight of approximately 21,000, α_1-mobility on electrophoresis, and a single binding site for one molecule of retinol. RBP interacts strongly with plasma prealbumin and normally circulates in plasma as a 1:1 molar RBP–prealbumin complex (1–3). RBP normally circulates mainly as the holo-protein, containing a molecule of bound retinol. The usual level of RBP in plasma is about 40–50 μg/ml and that of prealbumin is about 200–300 μg/ml (4). RBP has been isolated and partly characterized from the serum of several species in addition to man (5–11).

The vitamin A transport system provides an interesting model for the study of protein–protein and protein–ligand interactions and of the characteristics of a specific lipid transport system. Considerable information is now available about the chemistry and metabolism of RBP (see (11) for review). Studies in the rat have shown that the secretion of RBP by the liver is a highly regulated process, and is regulated particularly by the vitamin A status of the animal (12–14). In addition, small soluble intracellular binding proteins with ligand specificity for retinol, but distinct from plasma RBP, have been isolated from rat liver and testis cytosol (15, 16).

Information about the partial NH_2-terminal amino acid sequence of human RBP has been reported previously (17, 18) from studies involving the direct automated sequence analysis of the intact protein. We now report the cyanogen bromide cleavage of the protein and the isolation, composition, and alignment of the five unique fragments so obtained. The amino acid sequence of almost two-thirds of the NH_2-terminal portion of the molecule is also reported.

Abbreviations: RBP, retinol-binding protein; RCM-RBP, reduced and *S*-carboxymethylated RBP; PITC, phenylisothiocyanate; PTH, 3-phenyl-2-thiohydantoin.

MATERIALS AND METHODS

Isolation of RBP

RBP was isolated from the urine of Japanese patients with chronic cadmium poisoning and tubular proteinuria; such patients excrete relatively large amounts of low molecular weight proteins, including RBP, in their urine (19, 20). The use of this source of RBP permitted us to isolate much larger amounts of RBP than would have been feasible technically if human serum had been used as the starting material. The methods employed for the isolation of RBP were similar to those used previously in our laboratory for the purification of RBP from serum (1, 3, 6).

Materials

Trypsin (L-1-tosylamido-2-phenylethyl chloromethyl ketone-treated), chymotrypsin, and carboxypeptidase A and B (diisopropyl phosphofluoridate-treated) were purchased from Worthington Biochemical Corp., Freehold, NJ. Sephadex (G-50 and G-25) and DEAE-cellulose (DE-52) were obtained from Pharmacia Fine Chemicals Inc., Piscataway, NJ, and Dowex 50 X 4 was from Bio-Rad (Richmond, CA). Cyanogen bromide and maleic anhydride were purchased from Eastman Organic Chemicals, Rochester, NY; the maleic anhydride was recrystallized from chloroform before use. Iodoacetic acid was recrystallized from petroleum ether together with iodo-[^{14}C]acetic acid (New England Nuclear Corp., Boston, MA) to give a final specific activity of 0.05 μCi/μmol. Urea (ultra-pure) was purchased from Schwartz/Mann, Orangeburg, NY and guanidine HCl from Heico, Inc., Delaware Water Gap, PA. The reagents and solvents for the automated Edman degradation (21) were obtained from Pierce Chemical Co., Rockford, IL. Diethyl ether was USP grade for anesthesia (E.R. Squibb and Co., Princeton, NJ) and was tested for the absence of peroxides before use. Fluorescamine was obtained from Hoffmann-La Roche, Inc., Nutley, NJ. All other chemicals were reagent grade.

Modification reactions

Reduction and *S*-carboxymethylation of RBP were performed as described previously (17). Maleylation was performed by published procedures (22). RCM-RBP (10 mg/ml) was dissolved in 8 M urea–0.1 M borate, pH 8.8, and solid maleic anhydride (0.15 M final concentration) was added in 10 portions during a 15-min period. The pH was maintained above 8.5 by addition of 1 N NaOH as needed, and the reaction was allowed to proceed at room temperature for 30 min. The reaction mixture was desalted on a column of Sephadex G-50. Subsequent removal of maleyl groups was accomplished by incubation of the maleylated protein or peptide in 5% formic acid at 45°C for 24 hr.

Cyanogen bromide cleavage

RCM-RBP, 5 mg/ml, was incubated with a 100-fold molar excess of CNBr over methionine in 70% formic acid at room temperature. After 24 hr the reaction mixture was diluted 10-fold with water and lyophilized to remove the excess reagents. The mixture of CNBr fragments was maleylated, as described above for RCM-RBP, before fractionation by gel filtration.

Digestion with proteolytic enzymes

Conditions for digestion of various substrates by proteolytic enzymes are shown in **Table 1.** In all cases

TABLE 1. Conditions for digestion of RBP derivatives with various proteolytic enzymes[a]

Enzyme[b]	Substrate	Ratio Enzyme to Substrate	Substrate Concentration	Time	Buffer[c]	pH
		w/w	*% (w/v)*	*hr*		
Trypsin	Maleyl RCM-RBP	1:50	2.00	2	0.1 M NH_4HCO_3	8.4
Trypsin	MCB-III	1:15	0.58	2	0.1 M NH_4HCO_3	8.4
Trypsin	MCB-V	1:44	1.00	2	0.1 M NH_4HCO_3	8.4
Chymotrypsin	TM6	1:20	1.12	3	0.1 M NH_4HCO_3	8.4
Chymotrypsin	TM8	1:30	1.50	3	0.1 M NH_4HCO_3	8.4
Chymotrypsin	MCB-V	1:40	1.58	3	0.1 M NH_4HCO_3	8.4
Carboxypeptidase A	MCB-II	1:2.7	0.088	0–2	0.2 M EMA	8.5
Carboxypeptidase B	TM6-C4	1:25	0.015	0–2	0.2 M EMA	8.5

[a] At 24°C except for carboxypeptidase reactions which were at 37°C.
[b] Chymotrypsin was used together with soybean trypsin inhibitor, in an amount 1/10th that of chymotrypsin (w/w).
[c] EMA, ethyl morpholine-acetate buffer.

the products of the proteolytic digestions were completely soluble and were applied directly to columns under the conditions described under Results.

Separation of peptides

The following scheme was followed for isolation of the peptides produced by CNBr cleavage and proteolytic digestions of RBP. The peptide mixtures were subjected to gel filtration, usually followed by ion-exchange chromatography of the major peaks obtained, or directly to ion-exchange chromatography. Peptide fractions obtained from these columns were assessed for purity by two-dimensional thin-layer chromatography–electrophoresis, using chloroform–methanol–NH_4OH 2:2:1 (v/v) for chromatography and 600 volts in pyridine–acetic acid–water 10:1:489 (v/v) for electrophoresis. When peptides were found to be impure by these methods, they were further purified by gel filtration on Sephadex G-25, ion-exchange chromatography, or preparative paper electrophoresis.

Fluorescamine assays

This procedure was used to assay column fractions for their content of protein or peptide (23, 24). After evaporation of the solvent, the sample (usually ca. 50 ng of peptide) was dissolved in 1 ml of 0.2 M sodium borate buffer, pH 7, 100 μl of fluorescamine solution (10 mg/100 ml in acetone) was added, and fluorescence was assayed after 5 min at 390 nm excitation and 475 nm emission.

Amino acid analysis

Proteins or peptides were generally hydrolyzed in 6 N HCl in vacuo for 24 hr at 110°C. For tryptophan determinations, proteins or peptides were hydrolyzed in 4 N methane sulfonic acid containing 0.2% 3-(2-aminoethyl)indole hydrochloride at 110°C for 24 hr (25). PTH amino acids were hydrolyzed in 50% HI in vacuo for 6 hr at 150°C. Amino acid analysis was performed using the general conditions of Spackman, Stein, and Moore (26), with a JEOL amino acid analyzer model JLC 6AH (JEOL Inc., Cranford, NJ).

Sequence analysis

The phenylisothiocyanate degradation procedure (27) together with direct identification of the PTH-amino acid was used throughout. Both automated and manual versions were employed. Automated PITC degradation was performed by the method of Edman and Begg (21), using a JEOL protein peptide sequencer model JAS-47K. Quadrol was usually used as the buffer for the automated sequence assays; for short peptides, dimethylallylamine trifluoroacetic acid buffer was used instead. The manual PITC degradation procedure (28) was modified, as described previously (29), to permit smaller quantities of peptide to be sequenced. The resulting phenylthiocarbamyl peptide was cleaved in trifluoroacetic acid and the thiazolinone was extracted with diethyl ether, as described previously (29). The PTH derivatives formed by conversion from the thiazolinone in 1 N HCl at 80°C for 10 min were identified by thin-layer chromatography in Systems D, E, and H (28). In some cases identification was confirmed by gas–liquid chromatography (30) and, in some cases, the identifications of the PTH derivatives derived from the automated sequencer experiments were also confirmed quantitatively by amino acid analysis after HI hydrolysis. In a few instances this latter method was also used to confirm the identities of PTH derivatives obtained by the manual PITC degradation procedure. Detection of ^{14}C with a low background planchet counter was used to confirm the presence of *S*-carboxymethylcysteine-PTH.

Nomenclature

Peptides are designated according to the methods used for cleavage as follows: CB, CNBr cleavage; MCB, maleylated peptide after CNBr cleavage; TM, tryptic peptide from maleyl RCM-RBP; T, trypsin cleavage; and C, chymotrypsin cleavage. Peptides are numbered in order of their position in the partial sequence proposal presented.

RESULTS

Isolation of cyanogen bromide fragments

RBP is a single polypeptide chain containing four methionine residues. In order to study the primary structure of RBP, CNBr was used to cleave the protein at its methionines into five fragments. In initial studies, significant amounts of aggregation and/or precipitation of the fragments occurred after CNBr cleavage. Accordingly, in these initial studies, we were unable to isolate all five fragments for amino acid composition and sequence analyses. In subsequent experiments, however, we found that aggregation and precipitation of the CNBr fragments could be prevented by maleylation of the unfractionated mixture of CNBr fragments followed by the dissolving of the maleylated fragments in buffer containing 6 M urea. This approach was then used to successfully isolate and study the five CNBr fragments.

Previous studies in this laboratory (3) have shown that reductive alkylation of the disulfide bonds of RBP

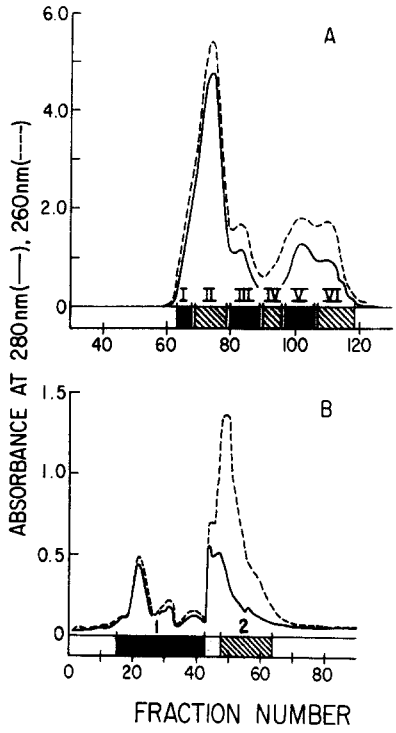

Fig. 1. Separation of the CNBr peptides of RCM-RBP. *A*. Gel filtration of the maleylated CNBr fragments of 200 mg of RCM-RBP on Sephadex G-50. Column size, 2.5 × 145 cm; eluent, 6 M urea in 0.1 M Tris buffer, pH 8.5; fraction size, 4 ml; flow rate, 60 ml/hr; temperature, 5°C. Absorbance was assayed at 280 and 260 nm; absorbance at 260 nm largely reflects the maleyl groups added to the protein. Column fractions were combined into pools (numbered I through VI) as indicated by the solid or cross-hatched bars. *B*. Chromatography of pool VI (Fig. 1*A*) on DEAE-cellulose. Column size, 0.6 × 12 cm. Elution was conducted with a linear gradient from 0.01 M to 0.3 M NH_4HCO_3, pH 8.4, at 5°C. Fraction size, 2.5 ml. Column fractions were combined into two pools (1 and 2) as indicated by the solid and cross-hatched bars.

leads to a complete loss of protein-bound retinol, apparently reflecting a loss of the protein's affinity for retinol. These observations were confirmed in the present studies, where it was noted that reduction and *S*-carboxymethylation of RBP (prior to CNBr cleavage) led to a loss of all protein-bound retinol, as assayed fluorometrically. From these observations it can be anticipated that none of the individual CNBr fragments of RBP would show any binding affinity for retinol, although this question was not examined directly in the present studies.

The results of gel filtration of the maleylated CNBr fragments from 200 mg of RCM-RBP are shown in **Fig. 1A.** The experiment illustrated in Fig. 1 has been carried out three times, at intervals of several months, with similar results obtained each time. The six peptide pools (Fig. 1*A*) were each desalted by gel filtration on Sephadex G-50 with 0.05 M NH_4HCO_3, pH 8.4, and were lyophilized. Each pool was then subjected to ion-exchange chromatography on DEAE-cellulose (DE-52). After DEAE-cellulose chromatography, pools II, IV, and V (Fig. 1*A*) each yielded one major pool which contained a single peptide, as determined by Edman degradation. These peptides represented, respectively, CB-V, CB-II, and CB-I (see **Table 2**), from pools II, IV, and V. Pure and useful peptide samples were not obtained from pools I and III (of Fig. 1*A*). Pool VI yielded two major pools (1 and 2 in Fig. 1*B*) after DEAE-cellulose chromatography. Each of these pools was found, by Edman degradation, to contain a single peptide representing, respectively, CB-III and CB-IV (see Table 2).

Each of the peptide pools obtained after DEAE-cellulose chromatography was lyophilized. Five peptides were isolated and their total amino acid composition accounted for that of the whole RBP molecule (Table 2).

Strategy for alignment of the cyanogen bromide fragments

Automated Edman degradation of RBP allowed the direct identification of the NH_2-terminal 41 residues of RBP. From this sequence, one of the CNBr peptides (CB-I) was identified as representing the NH_2-terminal portion of the RBP molecule, and another CNBr peptide (CB-II) could be positioned as the adjacent, second fragment. The largest of the CNBr fragments did not contain homoserine (whereas the other four fragments did, see Table 2). Accordingly, this peptide was identified as the carboxy-terminal one (CB-V). The order of alignment of the remaining two CNBr peptides was determined from the study of the amino acid sequences of some of the TM peptides of RCM-RBP.

Isolation of tryptic peptides of peptide MCB-III

Before removal of maleyl groups, peptide MCB-III was subjected to trypsin digestion. The tryptic digest was fractionated by ion-exchange chromatography on DEAE-cellulose (**Fig. 2**). Three pools were obtained and lyophilized. Each pool contained a single peptide, with peptides MCB-III-T1, -T2, and -T3 recovered, respectively, in pools 2, 1, and 3 from the DEAE-cellulose column (Fig. 2). The total composition of the three isolated peptides accounted for that of peptide CB-III (**Table 3**).

Isolation of tryptic peptides of maleyl RCM-RBP

RBP contains 14 arginine residues; thus, trypsin digestion of maleyl RCM-RBP would be expected to result in the production of 15 peptides. The experiments summarized in **Fig. 3** and in Tables 4 and 5 were designed to produce and isolate these TM pep-

TABLE 2. Amino acid composition of the cyanogen bromide fragments of RCM-RBP[a]

Amino Acid	CB-I	CB-II	CB-III	CB-IV	CB-V	Sum CB-I–V	RCM-RBP[d]
Lys	1.81 (2)	2.00 (2)	1.00 (1)	2.00 (2)	3.54	10.35	10.00 (10)
His					2.00	2.00	2.29 (2)
Arg	4.00 (4)		1.74 (2)		7.53	13.27	13.67 (14)
Asp	2.93 (3)	3.79 (4)	3.02 (4)	2.10 (2)	14.28	26.12	25.49 (26)
Thr	0.81 (1)	1.10 (1)	0.78 (1)	2.70 (3)	3.06	8.45	9.45 (9)
Ser	2.14 (3)	1.01 (1)	0.85 (1)		6.28	10.28	9.60 (10)
Glu[b]	1.74 (2)	4.03 (4)		1.03 (1)	10.81	17.61	19.87 (20)
Pro		1.48 (1)		0.77 (1)	4.16	6.41	5.37 (6)
Gly	1.04 (1)	2.09 (2)	0.91 (1)	0.99 (1)	7.36	12.39	13.13 (13)
Ala	2.10 (2)	2.23 (2)	2.91 (3)	1.16 (1)	6.40	14.80	14.19 (14)
Val	1.85 (2)	1.56 (2)	1.69 (2)	1.08 (1)	5.39	11.57	10.27 (11)
Met[c]	1.01 (1)	1.07 (1)	0.90 (1)	1.00 (1)		3.98	3.78 (4)
Ile		0.53 (1)			2.40	2.93	2.82 (3)
Leu		1.82 (2)	1.74 (2)		8.58	12.14	11.80 (12)
Tyr	0.98 (1)				7.66	8.64	9.11 (9)
Phe	2.89 (3)	1.77 (2)		2.15 (2)	3.22	10.03	11.41 (11)
½ Cys (CM)	1.09 (1)		0.85 (1)		5.02	6.96	8.05 (8)
Trp	0.75 (1)		0.71 (1)		1.76	3.22	4.25 (4)
TOTAL	(27)	(25)	(20)	(15)	99.5	(186–187)	184.6 (186)
Yield[e] (%)	53	32	32	56	58		

[a] The values listed for CB-I through CB-V represent the relative amounts of each amino acid residue found on analysis of each peptide. The integral numbers in parentheses for CB-I–IV are the values determined from the sequence analyses. All samples (including RCM-RBP) were hydrolyzed with 4 N methane sulfonic acid for 24 hr. Analyses were made on 0.05–0.1 μmol samples of each peptide. Values less than 0.20 are not listed.

[b] Homoserine, present in CB-I through CB-IV, strongly overlapped glutamic acid on amino acid analysis under the conditions used. Accordingly, the observed values for Glu (+ Hse) in each of these peptides were one residue greater than the number of Glu residues actually present. The observed values were corrected by subtraction of 1.00 to provide the values of Glu shown here.

[c] Met was determined as homoserine on separate analysis.

[d] These are the results of amino acid analysis of a portion of the RCM-RBP sample that was used for CNBr digestion. The data are in good agreement with other published (1, 6) and unpublished results from this laboratory. The numbers in parentheses represent the integer number of each residue assumed, from the analysis, to be present. The data in this column agree closely with those in the preceding column (sum of CB-I–V), with the differences present being well within the limitations of the methods employed.

[e] Yield (in this and subsequent tables) refers to the amount of each peptide isolated, compared to the amount of starting material that was used in the experiment from which it was derived.

tides. Most but not all of the TM peptides were isolated and used for further study.

Maleyl RCM-RBP was digested with trypsin, and the resulting peptide mixture was applied to a column of Sephadex G-50 (Fig. 3*A*). The major peptide pools obtained from this gel filtration step were further fractionated in several ways. Pool I was fractionated on DEAE-cellulose (Fig. 3*B*). After demaleylation of the peptides in pool II, some of the peptide material was soluble in pyridine acetate buffer, pH 3.1, and some of the material was not. The pyridine acetate-soluble material was chromatographed on Dowex 50 X 4, providing two major pools, each containing a single peptide. The acid (pyridine acetate)-insoluble material of pool II was dissolved in 0.01 M NH_4HCO_3, pH 8.4, and chromatographed on DEAE-cellulose to yield one major pool containing a single peptide. Pool III was fractionated by a combination of gel filtration on Sephadex G-25 (Fig. 3*C*) and high voltage paper electrophoresis. Pool IV was subjected directly to high voltage paper electrophoresis, yielding four major spots, each representing a single peptide. The procedures used for the isolation of the TM peptides are summarized in **Table 4,** and the amino acid compositions of the isolated peptides are summarized in **Table 5.**

Methionine residues were present in each of the two largest TM peptides isolated (TM6 and TM8, see Table 5). Accordingly, these peptides were able to provide the overlapping sequence information needed for alignment of the CNBr fragments. In addition, sequence analysis indicated that peptide TM6 was produced by an anomalous tryptic cleavage between Tyr-25 and Ala-26. Replicate experiments with other samples of maleyl RCM-RBP demonstrated that this anomalous cleavage and the production of peptide TM6 were consistently observed under the conditions employed.

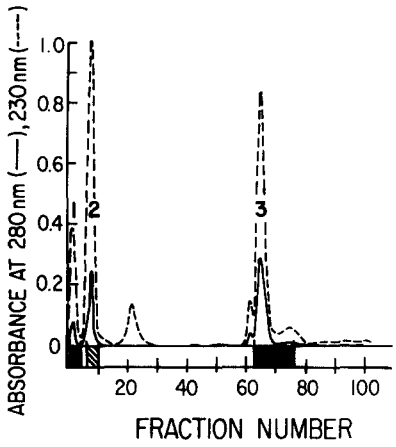

Fig. 2. Separation of the tryptic peptides of maleyl CB-III. A 2.92 mg portion of MCB-III was digested with trypsin, and the resulting tryptic digest was chromatographed on a DEAE-cellulose (DE-52) column, size 0.6 × 12 cm. Elution was conducted with a linear gradient from 0.01 to 0.3 M NH_4HCO_3, pH 8.4, at 5°C. Fraction size 2.5 ml. The fractions were combined into three pools (1, 2, and 3) as indicated by the solid or cross-hatched bars.

Isolation of chymotryptic peptides of peptide TM6

Peptide TM6 was digested with chymotrypsin, and the chymotryptic digest was fractionated by ion-exchange chromatography on DEAE-cellulose (**Fig. 4**). Peptides TM6-C1, -C2, -C3, and -C4 were recovered, respectively, in pools 6, 4, 5, and 1 from the DEAE-cellulose column (Fig. 4). The total composition of the four isolated peptides accounted for that of peptide TM6 (**Table 6**).

Isolation of chymotryptic peptides of peptide TM8

Peptide TM8 was digested with chymotrypsin, and the chymotryptic digest was fractionated by gel filtration on Biogel P4 (**Fig. 5*A***). Five peptide pools were obtained. Two of these (pools 4 and 5) each contained a single peptide (TM8-C1 and -C5, respectively). Pool 1 was fractionated further on DEAE-cellulose (Fig. 5*B*) to yield two peptides (TM8-C3 and -C6). Pool 2 was subjected to DEAE-cellulose column chromatography to yield one major peak (and one peptide, TM8-C4). Pool 3 was subjected directly to high voltage paper electrophoresis, yielding two major spots, each representing a single peptide. The procedures used for the isolation of the TM8-C peptides, and the amino acid compositions of the isolated peptides, are summarized in **Table 7.**

Seven TM8-C peptides were isolated and analyzed. The sum of these seven peptides accounted for 48 of the 58 amino acid residues of TM8. The composition (and sequence) of the remaining 10 residues (comprising peptide TM8-C2, see Table 7) could, however, be unambiguously inferred from the results

TABLE 3. Amino acid composition of the tryptic peptides of maleyl CB-III[a]

Amino Acid	MCB-III-T1	MCB-III-T2	MCB-III-T3	Total Residues (Sum MCB-III-T1–T3)	CB-III[c]
Lys	0.92 (1)			0.92 (1)	1.00 (1)
His					
Arg	1.00 (1)	1.00 (1)		2.00 (2)	1.74 (2)
Asp			3.30 (4)	3.30 (4)	3.02 (4)
Thr	0.87 (1)			0.87 (1)	0.78 (1)
Ser	0.87 (1)			0.87 (1)	0.85 (1)
Glu					
Pro					
Gly	0.92 (1)			0.92 (1)	0.91 (1)
Ala	1.92 (2)		0.92 (1)	2.84 (3)	2.91 (3)
Val		0.78 (1)	0.88 (1)	1.66 (2)	1.69 (2)
Met[b]			0.83 (1)	0.83 (1)	0.90 (1)
Ile					
Leu			2.00 (2)	2.00 (2)	1.74 (2)
Tyr					
Phe					
½ Cys (CM)			1.25 (1)	1.25 (1)	0.85 (1)
Trp			0.71 (1)	0.71 (1)	0.71 (1)
TOTAL	(7)	(2)	(11)	(20)	(20)
Yield (%)	79	89	29		

[a] The values listed represent the relative amounts of each amino acid residue found on analysis of each peptide. The integral numbers in parentheses are the values determined from the sequence analysis. See footnote *a* of Table 2 for further information.
[b] Determined as homoserine.
[c] From Table 2.

of direct automated Edman degradation of peptide TM8.

Studies with peptide CB-V

Samples of maleyl CB-V (peptide MCB-V) were subjected to trypsin or to chymotrypsin digestion (see Table 1) and the resulting digests were fractionated by gel filtration (on Biogel P-6 for the tryptic and on Biogel P-4 for the chymotryptic digests, respectively). Subsequent fractionation of the resulting peptide pools led to the isolation of some of the tryptic and some of the chymotryptic peptides of CB-V. Problems were encountered, however, in that some of the peptides resulting from the proteolytic cleavage of CB-V apparently aggregated and/or became insoluble, so that recovery of all of the product peptides was not achieved. As yet, this difficult problem has not been resolved, so we have not yet been able to establish the amino acid sequence of CB-V. Future studies will be needed to achieve this goal.

One of the tryptic peptides recovered from MCB-V had the composition glu-arg, identical with peptide TM1. Accordingly, in Table 5 this peptide (glu-arg) has been listed twice, as TM1 and as TM9.

Three of the chymotryptic peptides recovered from MCB-V had amino acid compositions identical with those of TM8-C6, TM8-C7, and TM8-C8. These MCB-V-C peptides were recovered in good yield and were used for manual sequence analyses that confirmed the results of the sequence analyses carried out on the respective TM8-C peptides.

Amino acid sequence of peptides derived from RCM-RBP

The extent of the amino acid sequence determinations of the CNBr and the TM peptides of RCM-RBP is shown in **Fig. 6.** The PTH derivatives were identified directly, as indicated by the arrows. In many instances, adequate sequence information was obtained without the need for further peptide fragmentation.

Peptide CB-II (28–52)

The sequence of the NH_2-terminal 14 residues was determined by direct automated Edman degradation. The sequence of the COOH-terminal three residues was determined by carboxypeptidase A hydrolysis. The remainder of the sequence of CB-II was determined from the sequence analyses carried out with peptide TM6.

Peptide TM6 (26–59)

The sequence of the NH_2-terminal 22 residues was determined by direct automated Edman degradation.

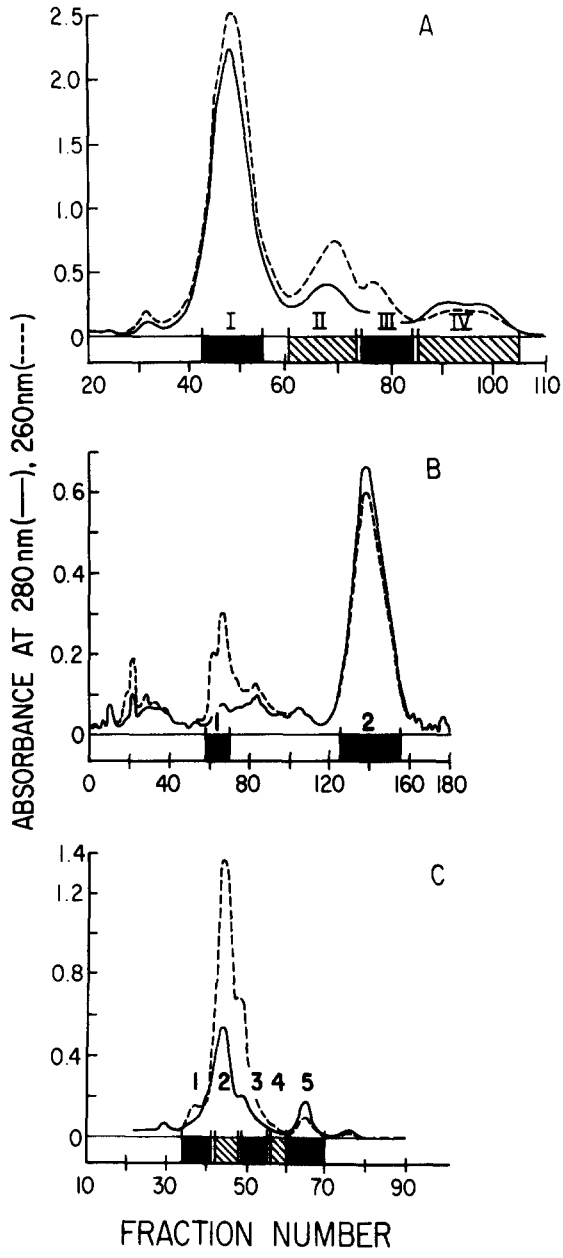

Fig. 3. Separation of the tryptic peptides of maleyl RCM-RBP. *A*. Gel filtration of the tryptic digest of 200 mg of maleyl RCM-RBP on Sephadex G-50 (fine). Column size, 2.5 × 145 cm; eluent, 0.1 M NH_4HCO_3, pH 8.4; fraction size, 7 ml; temperature, 5°C. *B*. Chromatography of pool I (Fig. 3*A*) on DEAE-cellulose (DE-52). Column size, 1.4 × 17 cm; elution was conducted with a linear gradient from 0.01 to 0.6 M NH_4HCO_3, pH 8.4, at 5°C. Fraction size, 2.5 ml. *C*. Gel filtration of pool III (Fig. 3*A*) on Sephadex G-25 (fine). Column size, 1.2 × 110 cm; eluent, 0.1 M NH_4HCO_3, pH 8.4; fraction size, 7 ml; temperature, 5°C. Column fractions were combined into pools as indicated by the solid or cross-hatched bars and numbers beneath the elution profiles.

The peptide was digested with chymotrypsin and the resulting peptides were separated on DEAE-cellulose (Fig. 4). The extent of sequence determinations on peptides TM6-C3 and TM6-C4 is shown in Fig. 6.

TABLE 4. Summary of isolation of tryptic peptides of maleyl RCM-RBP

Peptide	Sephadex G-50 (Fig. 3A)	DEAE-Cellulose		Sephadex G-25 (Fig. 3C)	Other Procedure[a]	Yield
		Fig.	Pool			
	Pool			*Pool*		%
TM1	IV				HVE	4
TM3	III			3	HVE	14
TM4	II				Dowex	10
TM5	IV				HVE	10
TM6	I	3B	1		DEAE	19
TM7	IV				HVE	22
TM8	I	3B	2			15
TM9	IV				HVE	4
TM10	II	NS[b]	1			21
TM11	III[c]			5		13
TM12	II				Dowex	29

[a] HVE, high voltage electrophoresis on paper at pH 3.55; Dowex, chromatography on a column of Dowex 50 X 4 in pyridine acetate buffer, with a linear gradient from pH 3.1 (0.2 M) to 5.1 (2.0 M) at 50°C; DEAE, repeat chromatography on DEAE-cellulose under conditions similar to those for Fig. 3*B*.

[b] NS, figure not shown.

[c] TM11 (Glu-Tyr-Arg) was also recovered as a major spot after HVE of the material in pool IV.

Peptide CM-III (53–72)

The sequence of the NH_2-terminal two residues was determined by manual Edman degradation. The peptide was digested with trypsin and the resulting peptides were separated on DEAE-cellulose (Fig. 2). The extent of sequence determination of peptides MCB-III-T1, -T2, and -T3 is shown in Fig. 6.

Peptide TM8 (62–119)

The sequence of the NH_2-terminal 39 residues was determined by directed automated Edman degradation. The initial part of this sequence was also confirmed by manual Edman degradation, which allowed the assignment of Cys at 69 and Asp at 71. The peptide was digested with chymotrypsin and the resulting pep-

TABLE 5. Amino acid composition of tryptic peptides of maleyl RCM-RBP[a]

Amino Acid	TM1	TM2[b]	TM3	TM4	TM5	TM6	TM7	TM8	TM9[c]	TM10	TM11	TM12
Lys				2.00 (2)		3.22 (3)		4.44 (4)				
His								1.00 (1)				
Arg	1.00 (1)	(1)	1.00 (1)	1.00 (1)		1.00 (1)	1.00 (1)	0.89 (1)	1.00 (1)	1.00 (1)	1.00 (1)	
Asp		(1)		2.04 (2)		3.72 (4)		11.3 (11)		2.51 (3)		1.70 (2)
Thr					0.84 (1)	1.92 (2)		5.00 (5)		0.83 (1)		
Ser			1.72 (2)		0.70 (1)	1.74 (2)		2.09 (2)		2.25 (2)		
Glu	1.07 (1)			1.00 (1)		4.41 (4)		3.09 (3)	1.07 (1)		1.04 (1)	2.24 (2)
Pro						1.41 (1)		1.09 (1)				3.25 (3)
Gly					0.90 (1)	2.86 (3)		3.51 (3)		1.02 (1)		1.17 (1)
Ala				0.85 (1)		4.39 (5)		3.80 (4)		1.00 (1)		1.06 (1)
Val			1.06 (1)	1.15 (1)		1.93 (2)	1.16 (1)	4.44 (5)		1.01 (1)		
Met						1.63 (2)		1.42 (2)				
Ile						0.83 (1)		0.84 (1)				
Leu						2.14 (2)		3.03 (3)		2.67 (3)		
Tyr					1.00 (1)			3.91 (4)		0.95 (1)	1.06 (1)	1.00 (1)
Phe			1.12 (1)	1.03 (1)	1.22 (1)	2.08 (2)		3.23 (3)		1.92 (2)		
½ Cys (CM)		(1)						2.48 (2)		1.21 (1)		
Trp					1.00 (1)			3.07 (3)				
TOTAL	(2)	(3)	(5)	(9)	(6)	(34)	(2)	(58)	(2)	(17)	(3)	(10)

[a] See footnote *a*, Table 3. For peptides TM1 through TM8, the integral numbers in parentheses are the values determined from the sequence analysis; for peptides TM9–12, these numbers represent the integer number of each residue assumed, from the analysis, to be present. For peptides TM1 through TM8, the order in which they are numbered corresponds to their position in the partial sequence proposal presented. Peptide TM12 lacks Arg, and hence was tentatively assumed to represent the COOH-terminus of RBP. The relative positions of TM9–11 were not defined.

[b] Peptide TM2 was not isolated, but its composition was inferred from the direct NH_2-terminal sequence analysis of RCM-RBP.

[c] Peptide TM9 is identical with TM1. It is listed separately because a tryptic peptide of this composition was isolated from MCB-V (see later text).

tides were separated by various procedures, including gel filtration, ion-exchange chromatography, and high voltage paper electrophoresis (Table 7). The extent of sequence determinations of peptides TM8-C5, -C6, -C7, and -C8 is shown in Fig. 6.

Peptide CB-V (88-COOH-terminus)

The sequence of the NH_2-terminal 20 residues was determined by direct automated Edman degradation.

Alignment of CNBr peptides of RBP and proposal for partial amino acid sequence

Automated Edman degradation of RCM-RBP permitted the direct alignment of peptides CB-I and CB-II and of peptides TM1 through TM6, and established the sequence from residues 1 through 41. The composition data and manual Edman results on TM1, TM3, TM4, and TM5, and the automated Edman degradation results on CB-II, confirmed most of this proposed sequence.

Peptide TM6 contained within it the sequence of peptide CB-II, and showed overlap with peptides CB-I and CB-III, thus establishing the placement of CB-III. Automated Edman degradation of TM6 confirmed the sequence 26 to 41 and established the sequence to 47. Three of the chymotryptic peptides of TM6 could be aligned by the result of automated Edman degradation of TM6. The remaining TM6-C peptide (TM6-C4) contained arginine, confirming its assignment as the COOH-terminal peptide of TM6. The manual Edman degradation and composition data on TM6-C3, together with the carboxypeptidase A assays of CB-II, provided the sequence to 52. Manual Edman degradation of TM6-C4 established the sequence to 54.

Manual Edman degradation of CB-III confirmed the sequence at 53 and 54, and permitted the place-

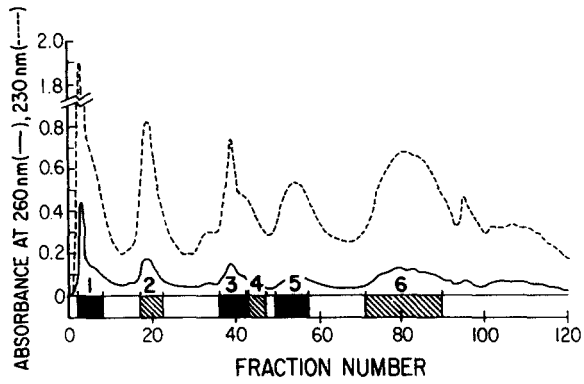

Fig. 4. Separation of the chymotryptic peptides of peptide TM6. A 9.32 mg portion of TM6 was digested with chymotrypsin and the resulting digest was chromatographed on a DEAE-cellulose (DE-52) column, size 0.6 × 12 cm. Elution was conducted with a linear gradient from 0.01 to 0.6 M NH_4HCO_3, pH 8.4; fraction size, 2.5 ml. Column fractions were combined into pools as indicated by the solid or cross-hatched bars beneath the elution profiles. Pools 2 and 3 appeared to be heterogeneous and were not used further. The chymotryptic peptides were isolated from the other four pools shown.

TABLE 6. Amino acid composition of the chymotryptic peptides of peptide TM6[a]

Amino Acid	TM6-C1	TM6-C2	TM6-C3	TM6-C4	Total Residues (Sum TM6-C1–C4)	TM6[b]
Lys	2.11 (2)			0.89 (1)	3.00 (3)	3.22 (3)
His						
Arg				0.85 (1)	0.85 (1)	1.00 (1)
Asp	1.25 (1)	2.00 (2)	1.00 (1)		4.25 (4)	3.72 (4)
Thr			0.90 (1)	1.27 (1)	2.17 (2)	1.92 (2)
Ser			0.92 (1)	1.25 (1)	2.17 (2)	1.74 (2)
Glu	1.19 (1)	2.08 (2)	1.49 (1)		4.76 (4)	4.41 (4)
Pro	1.42 (1)				1.42 (1)	1.41 (1)
Gly	0.93 (1)		0.96 (1)	0.87 (1)	2.76 (3)	2.86 (3)
Ala	1.80 (2)	0.92 (1)		1.65 (2)	4.37 (5)	4.39 (5)
Val		0.69 (1)	1.00 (1)		1.69 (2)	1.93 (2)
Met	1.00 (1)		0.95 (1)		1.95 (2)	1.63 (2)
Ile		0.59 (1)			0.59 (1)	0.83 (1)
Leu	1.01 (1)	1.00 (1)			2.01 (2)	2.14 (2)
Tyr						
Phe	1.10 (1)	0.96 (1)			2.06 (2)	2.08 (2)
½ Cys (CM)						
Trp						
TOTAL	(11)	(9)	(7)	(7)	(34)	(34)
Yield (%)	72	52	20	43		

[a] See footnote *a* of Table 3.
[b] From Table 5.

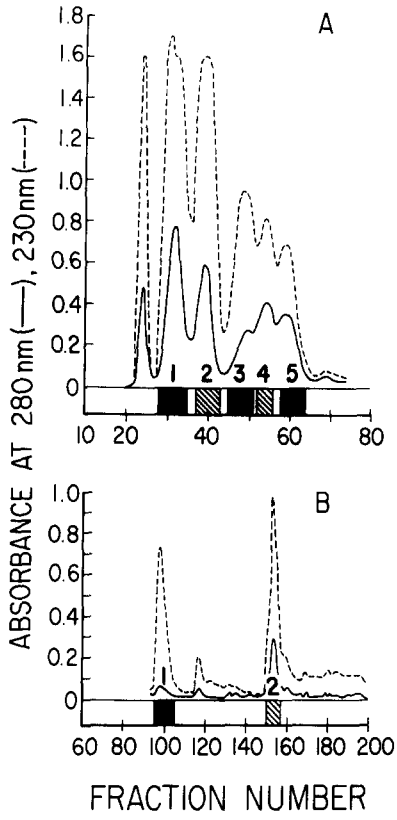

Fig. 5. Separation of the chymotryptic peptides of peptide TM8. *A*. Gel filtration of the chymotryptic digest of 15 mg of TM8 on BioGel P4. Column size, 1.2 × 115 cm; eluent, 0.1 M NH_4HCO_3, pH 8.4; fraction size, 2 ml; temperature, 5°C. *B*. Chromatography of pool 1 (Fig. 5*A*) on DEAE-cellulose (DE-52). Column size, 0.6 × 10 cm; elution was conducted with a linear gradient from 0.01 to 0.6 M NH_4HCO_3, pH 8.4, at 5°C; fraction size, 2.5 ml. Column fractions were combined into pools as indicated by the solid or cross-hatched bars and numbers beneath the elution profiles.

ment of MCB-III-T1 at the NH_2-terminus of MCB-III. MCB-III-T3 was placed at the COOH-terminus of MCB-III, since it contained homoserine (derived from methionine), thus establishing the alignment of the three MCB-III-T peptides. The amino acid compositions and the manual Edman results with peptides TM6-C4 and MCB-III-T1 were identical, confirming the overlap between TM6 and CB-III. The compositions and manual Edman results on MCB-III-T1, -T2, and -T3 provided the sequence to 72.

Peptide CB-V did not contain homoserine and could therefore be placed at the COOH-terminus of RBP. Since peptides CB-I, CB-II, and CB-III were already aligned, this allowed the placement of CB-IV between CB-III and -V.

Automated Edman degradation of TM8 provided a sequence (of 39 residues) that contained within it the entire sequence of CB-IV and that showed overlap with CB-III and CB-V. These data thus confirmed the alignment of peptides CB-III, CB-IV, and CB-V, confirmed the sequence from 62 to 72, and established the sequence to 100.

The automated Edman data with TM8 established the alignment of peptides TM8-C1 through TM8-C6. Peptide TM8-C8 was assigned to the COOH-terminus of TM8, since it possessed arginine, thus fixing the placement of TM8-C7. The compositions and manual Edman results of TM8-C5 and -C6 confirmed the sequence from 90 to 100, and provided the sequence to 109. Carboxypeptidase assays on TM8-C6, together with the compositions and manual Edman results on TM8-C7 and TM8-C8, then established the sequence to 119.

Automated Edman degradation of CB-V showed overlap with TM8, confirmed the placement of CB-V, and confirmed the sequence from 88 to 107. The remainder of the sequence of CB-V, and hence of RBP, beyond 119 will require future studies for its determination. Future studies will also be required to establish whether acid or amide groups are present in the five residues shown as Asx or Glx in Fig. 6.

DISCUSSION

The RBP molecule is of considerable interest as a specialized lipid binding and lipid transport protein. The binding of all-*trans* retinol to RBP is highly but not absolutely specific. A number of isomers of retinol and of retinaldehyde, as well as retinoic acid and retinyl acetate, can bind to apo-RBP with varying degrees of effectiveness (31–36). A number of other retinyl derivatives, particularly some Schiff base retinylideneamines (34), can also bind to some extent to apo-RBP. Compounds unrelated to vitamin A in structure (e.g., cholesterol, phytol) bind minimally to RBP or not at all.

Circular dichroic studies have provided information about the secondary structure of RBP (37, 38). RBP appears to have a relatively high content of unordered conformation, a significant but small complement of β-conformation, and little or no α-helix.

The interaction of RBP with prealbumin is very sensitive to ionic strength, with dissociation of the protein–protein complex occurring at low ionic strength (39, 40). The protein–protein interaction is also strongly pH dependent, with maximal binding occurring near physiological pH (40).

A great deal of detailed information is now available about the structure of prealbumin. In addition to its role in vitamin A transport, prealbumin plays a role in the binding and plasma transport of thyroid hormones (41). The prealbumin molecule is a stable and symmetrical tetramer, composed of four identical subunits, with a molecular weight of 54,980 (29). The

TABLE 7. Amino Acid Composition of the Chymotryptic Peptides of Peptide TM8[a]

Amino Acid	TM8-C1	TM8-C2[b]	TM8-C3	TM8-C4	TM8-C5	TM8-C6	TM8-C7	CM8-C8	Total Residues	TM8[c]
Lys			1.05 (1)	1.83 (2)		0.97 (1)			(4)	4.44 (4)
His						1.00 (1)			(1)	1.00 (1)
Arg								1.03 (1)	(1)	0.89 (1)
Asp	1.63 (2)	(2)	2.37 (2)			5.60 (5)			(11)	11.3 (11)
Thr		(1)	1.85 (2)			1.80 (2)			(5)	5.00 (5)
Ser					0.60 (1)			0.94 (1)	(2)	2.09 (2)
Glu			0.93 (1)			1.13 (1)	0.96 (1)		(3)	3.09 (3)
Pro			0.87 (1)						(1)	1.09 (1)
Gly		(1)			0.91 (1)	1.27 (1)			(3)	3.51 (3)
Ala		(1)	1.07 (1)		0.92 (1)		1.00 (1)		(4)	3.80 (4)
Val		(2)			0.89 (1)	0.70 (1)	1.04 (1)		(5)	4.44 (5)
Met		(1)		1.15 (1)					(2)	1.42 (2)
Ile						0.47 (1)			(1)	0.84 (1)
Leu	2.04 (2)					1.20 (1)			(3)	3.03 (3)
Tyr				1.00 (1)		1.87 (2)	1.00 (1)		(4)	3.91 (4)
Phe		(1)	1.00 (1)		0.77 (1)				(3)	3.23 (3)
½ Cys (CM)		(1)						1.00 (1)	(2)	2.48 (2)
Trp	1.02 (1)				1.00 (1)	1.17 (1)			(3)	3.07 (3)
TOTAL	(5)	(10)	(9)	(4)	(6)	(17)	(4)	(3)	(58)	(58)
BioGel Pool[d]	4		1	2	5	1	3[f]	3[f]		
DEAE Pool[e]			1	1		2				
Yield (%)	70		70	45	66	66	39	45		

[a] See footnote *a* of Table 3.
[b] This peptide was not isolated, but its composition (and sequence) was inferred from the direct automated sequence analysis of peptide TM8.
[c] From Table 5.
[d] From Fig. 5*A*.
[e] From Fig. 5*B*, for TM8-C3 and -C6; DEAE-cellulose chromatography of BioGel Pool 2 (from Fig. 5*A*) gave one major peptide, TM8-C4.
[f] These peptides were separated from BioGel pool 3 by high voltage paper electrophoresis.

complete amino acid sequence of human prealbumin has been reported from our laboratory (29). Crystalline prealbumin has been studied at 6 Å (42), at 2.5 Å (43), and at 1.8 Å (44) resolution. These studies have shown that the subunits have extensive β-sheet structure and are linked into stable dimers, each comprising two of the four subunits. A channel runs through the center of the prealbumin molecule, in which are located two symmetry-related binding sites for iodothyronine molecules. Only one molecule of thyroxine binds to prealbumin with high affinity, however, because of negative cooperativity (43, 45).

Recent high-resolution X-ray crystallographic studies have shown that the prealbumin molecule contains two surface sites with structural complementarity to double-helical DNA (46). Although the binding of prealbumin to DNA has not been reported, it has been suggested that the prealbumin molecule may serve as a model for the kind of structure that may be involved in hormone receptors with nuclear effects on DNA transcription (46). Human prealbumin has been reported to contain four binding sites for human RBP (40, 47). It seems reasonable to assume that each prealbumin subunit might contain one binding site for RBP, although direct evidence for this suggestion is not available.

Much less detailed structural information is available about RBP. RBP has been crystallized (48), although the crystals produced were not suitable for detailed X-ray study. The studies reported here were aimed at obtaining information about the primary structure of the RBP molecule. These studies involved the cyanogen bromide cleavage of RBP into five unique fragments and the isolation, determination of composition, and alignment of the five fragments. The amino acid sequences of four of the five CNBr fragments were determined, and a sequence proposal for almost two-thirds of the RBP molecule is presented. Examination of this sequence reveals no apparent relationship to the amino acid sequence of human prealbumin.

Information was sought concerning the possible existence of sequence homologies between the RBP partial sequence reported here and the amino acid sequences of other proteins whose primary structures are known. A search for such sequence similarities between RBP and all known sequenced proteins tabulated in the Atlas of Protein Sequence and Struc-

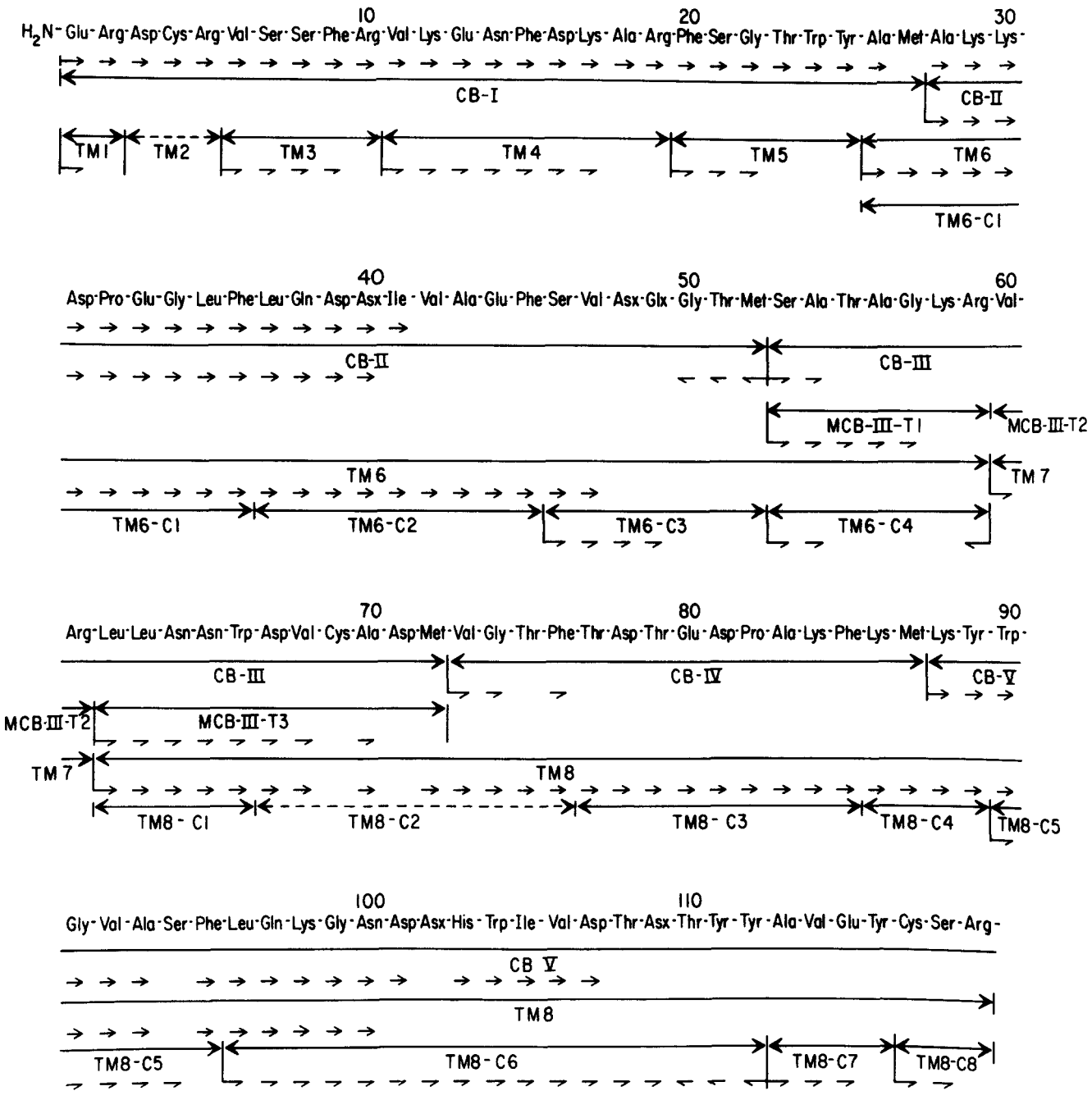

Fig. 6. Reconstruction of the partial amino acid sequence of human RBP from the tryptic peptides and CNBr fragments and the smaller peptides obtained from them. Symbols: arrows pointing to the right, sequences determined with an automated sequenator; half arrows pointing to the right, sequences determined by the manual Edman degradation; half arrows pointing to the left, sequences determined by the use of carboxypeptidase A. The nomenclature of the peptides is described in the text. The amounts (in μmol) of peptide (or protein) used for the sequence determinations summarized in this figure were as follows: *A*. Automated sequence analysis: (*1*) RCM-RBP, 0.43; (*2*) CB-II, 0.16; (3) CB-V, 0.12; (*4*) TM6, 0.73; (*5*) TM8, 0.48. *B*. Manual Edman degradation: (*1*) TM6-C3, 0.05; (*2*) TM6-C4, 0.05; (*3*) TM8-C5, 0.27; (*4*) TM8-C6, 0.37; (*5*) TM8-C7, 0.35; (*6*) MCB-III-T1, 0.05; (*7*) MCB-III-T3, 0.35.

tures, Volume 5 and Supplements 1, 2, and 3, was conducted by the National Biomedical Research Foundation, Georgetown University Medical Center, Washington, DC. This search employed computer programs developed for this general purpose. No homologies in sequence were observed between RBP and any other known sequenced protein.

Future studies will be needed to establish the amino acid sequence of the fifth DNBr fragment (CB-V) and of the complete RBP molecule. In the meantime, the work reported here may be of value as a basis for chemical studies aimed at exploring the characteristics of the binding sites on RBP for retinol and for prealbumin. Comparison of the structure of plasma RBP with those of the intracellular cytosol-binding proteins for retinol (15, 16) would also be of considerable interest.

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