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The Complete Amino Acid Sequence of Human Serum Retinol-binding Protein

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ABSTRACT

The complete amino acid sequence of human serum Retinol-binding protein (RBP) including the distribution of its three disulfide bridges, has been determined. The protein consists of 182 amino acid residues, the order of which was determined following the isolation of five CNBr-fragments. Direct amino acid sequence analysis in an automatic liquid phase sequencer provided almost the entire sequences of the five CNBr-fragments. Several sets of enzymatically derived peptides of RBP were also used to elucidate the primary structure. RBP displays significant homology to bovine β-lactoglobulin, human α_1-microglobulin and rat α_1-microglobulin. RBP contains an internal homology. Thus, residues 36 to 83 display statistically significant homology with residues 96 to 141.

INTRODUCTION

From its site of synthesis in the liver (28,36,48) the Retinol-binding protein (RBP)[1] carries one molecule of retinol (20,33,34) to vitamin A requiring cells. While transporting retinol in plasma, RBP forms a stable complex with thyroxine-binding prealbumin (20,33). This complex formation prevents RBP, which has a molecular weight of 21 000, to pass the kidney glomeruli (34). Cells requiring vitamin A express a receptor for RBP on their cell membranes (19,41). On recognizing RBP the receptor takes up the vitamin. Simultaneously, RBP undergoes a conformational change, the nature of which is presently unknown. This conformational change does not allow a sustained binding between RBP and prealbumin (19,43). Due to the abolished protein-protein interaction the free RBP molecule becomes degraded in the

[1]Abbreviations used are:

RBP	- Retinol-binding protein
dansyl	- 1-dimethyl-aminoaphthalene-5-sulphonyl chloride
EDTA	- ethylenediaminotetraacetate
CM-cysteine	- carboxymethylcysteine
cys-A	- cysteic acid

kidney following glomerular filtration and reabsorption in the tubuli cells (34). To understand how RBP interacts with retinol, prealbumin and the cell-surface receptor and how these interactions may become modulated, it appeared of importance to establish the amino acid sequence of RBP. The primary structure of RBP was also a prerequisite for the interpretation of high-resolution X-ray crystallographic data.

In this communication we describe the complete amino acid sequence of human RBP. Part of this information has appeared in preliminary form (40). A partial primary structure of human RBP has also been reported by Kanda and Goodman (21). Recently, a cDNA clone encoding human RBP has been analysed (5).

MATERIALS AND METHODS

Isolation of RBP - The RBP used in the sequence studies was isolated from human serum (34) and urine (35). The purity of the RBP preparations was assessed as described (34,35).

Peptide nomenclature - The peptides obtained after cyanogen bromide cleavage are designated A, B, and C followed in some instances of a number and a letter, indicating the order of emergence of a particular peptide during fractionation. H denotes a peptide obtained after acid cleavage. Peptides isolated after digestion of RBP by trypsin, chymotrypsin, thermolysin, and clostripain are symbolized by R, RC, RT and C1, respectively. Peptides obtained from CNBr-fragment A3b after digestion with clostripain are designated A3b and those from tryptic and chymotryptic digestions of CNBr fragment C, C and CT, respectively. Peptides isolated from CNBr fragment A1 after digestion with Staphylococcus aureus protease V8 are called SA and SB, with chymotrypsin AC, with thermolysin AT, with pepsin AP, with subtilisin AS and with clostripain AC1, respectively. Peptides obtained after cleavage of unreduced RBP with acid, trypsin and pepsin are denoted S, T and P, respectively. The numbers that follow the symbols indicate the order of emergence of a particular peptide during fractionation.

Reduction, alkylation, CNBr-fragmention and acid cleavage - These procedures were carried out as described (51).

Enzymatic digestion of RBP and RBP fragments - Trypsin digestions were performed on samples (0.1 to 3 μmoles) in 0.2 M NH_4HCO_3, pH 8.0, at protein to enzyme ratios of 100:1 to 50:1. The protein or peptide concentration was usually between 5 and 10 mg/ml. Digestions were carried out at 37° for 3 hours and were terminated by lyophilization. α-Chymotrypsin and subtilisin digestions were performed similarly. Also thermolysin digestions were

conducted in the same fashion but the buffer was 0.2 M NH_4HCO_3, pH 8.0, containing 5 mM $CaCl_2$ and the reaction was terminated after 2 hours. Pepsin digestions were carried out at an enzyme to substrate ratio of 1:50 (w/w) at 37° for 3 hours in 5% (v/v) formic acid. Digestions with clostripain were performed at pH 7.8 in 0.1 M NH_4HCO_3, containing 2 mM DTT and 1 mM $CaCl_2$. After 2 to 3 hours at 37° the reaction mixture was lyophilized. The substrate to enzyme ratio was 50:1. Staphylococcus aureus protease V8 was used at a protein to enzyme ratio of 50:1. The substrate was dissolved in 0.2 M NH_4HCO_3, pH 8.0, containing 1 mM EDTA and after 2 hours at 37° the digestion mixture was lyophilized.

To investigate the distribution of the disulfide bridges of RBP, the CNBr-fragments A1, A2, A3a and A3b, which were held together by disulfide bonds (fraction A of Fig. 1A), were digested with trypsin and pepsin. The CNBr-fragment mixture, at a concentration of about 10 mg/ml in 0.2 M Tris-acetate buffer, pH 6.0, was digested for 8 hours at 37° with trypsin at an enzyme to substrate ratio of 1:50. The same amount of CNBr-fragments in 0.2 M sodium acetate buffer, pH 5.0, was digested with pepsin (final concentration 0.2 mg/ml) for 8 hours at 37°. Carboxypeptidase A and B digestions were carried out as described (51).

Peptide fractionation - Large peptides of RBP were usually separated by gel chromatography on columns of Sephadex G-100 and G-50 equilibrated with 0.05 M sodium acetate buffer, pH 5.0, containing 6M guanidine-HCl or with 10% propanol - 0.025% ammonia in water. Smaller peptides were purified by high voltage electrophoresis in pyridine-acetate buffer, pH 6.5 (pyridine:acetic acid:water, 100:3:897 v/v) and pH 3.5 (pyridine:acetic acid:water, 1:10:189 v/v). The electrophoreses were carried out on 60 to 100 cm long Whatman No 3MM papers at 40 V/cm for 60 to 100 min. Further purification of impure peptide fractions was accomplished by descending paper chromatography developed with butanol:acetic acid:water:pyridine (15:3:12:10 v/v). Localization of peptides was accomplished by staining the papers with fluorescamine. Purified peptides were eluted from papers with 0.1% ammonia.

Some peptides were purified by ion exchange chromotography on DEAE-Sepharose columns equilibrated with 0.02 M NH_4HCO_3. The applied sample was usually eluted with a 250 ml linear gradient of NH_4HCO_3 from 0.02 to 0.2 M. The occurrence of peptides in the effluent was monitored by measuring the absorbance at 220 nm or at 280 nm. Occasionally aliquots were withdrawn for ninhydrin analysis (see below).

Peptide digests were also separated on a modified JEOL-5 AH amino acid analyzer (18). The column (11x0.5 cm), maintained at a temperature of 50°,

contained the JEOL type AR-15 sulfonated resin. The applied material, usually between 15 and 30 mg of peptide mixture, was eluted as described (18). The flow rate was 1.85 ml/min and fractions of 3.0 ml were collected. For the separation of some peptides advantage was taken of column zone electrophoresis (37). The column (86x1 cm), packed with water-pyridine-extracted cellulose and cooled by running water, was equilibrated with a pH 1.9 buffer composed of acetic acid:formic acid:water (78:25:897 v/v). After application the samples were usually displaced downward to an appropriate starting point and runs were conducted at 1000 V for 8 to 12 hours. After electrophoresis the column, which had a total free liquid volume of about 60 ml, was eluted at a flow rate of 12 ml/hour (13).

High pressure liquid chromatography was also used to purify some peptides. Two model 110A pumps (Altex, Berkley, California), an Altex model 400 solvent programmer and a micro-Bondapak C_{18} column (300x3.9mm, Waters Associates Inc., Milford, Mass.) were used. The column was equilibrated with 2 mM ammonia adjusted to pH 2.4 with trifluoroacetic acid and 5% methanol. Elution was accomplished with a linear gradient of methanol from 5% to 55% followed by 30 ml of 55% methanol in the ammonia trifluroacetic acid buffer. Peptides in the effluent were detected by measuring the absorbance at 206 nm. The flow rate was 24 ml/h and fractions of 0.4 ml were collected.

Alkaline hydrolysis and ninhydrin analysis - For alkaline hydrolysis appropriate aliquots were evaporated to dryness at 110°. 0.5 ml of 2.5 M NaOH was added. The hydrolysis was carried out at 110° for 3 hours. Following neutralization with 1.0 ml of 1.5 M acetic acid, 1.0 ml of the ninhydrin reagent was added. After 15 min in a boiling water bath each fraction was diluted with 2 ml of 50% ethanol and the absorbance at 570 nm was estimated.

Amino acid analyses - Amino acid analyses were carried out as described (51).

Amino acid sequence determinations - Automatic amino acid sequence determinations were carried out as described (51). Amino acid sequence determinations were also accomplished with the dansyl end group method in conjunction with the Edman technique (17). Dansyl amino acids were identified by two-dimensional chromatography on 5x5 cm polyamide thin layer sheets. The solvent systems used were those of Woods and Wang (56).

Statistical analyses for relatedness of RBP sequence to other proteins -The RBP sequence was compared to a data file containing sequences of other proteins (6-9), with the SEARCH program (9). The program ALIGN (29) was used to analyse the alignment of homologous sequences. The matrix bias parameter and the break penalty parameter were set to 2 and 6, respectively. These values are appropriate when comparisons are made between distantly related sequences.

RESULTS

Isolation and NH_2-Terminal Amino Acid Sequence Determination of Human RBP CNBr-Fragments -RBP was cleaved with CNBr and the resulting fragments purified by repeated gel chromatographies (Fig. 1 and 2). The amino acid compositions and the yields of the fragments are summarized in Table 1. Since human RBP contains four methionines (43) five CNBr-fragments were expected. However, six fragments were isolated (Table 1). This result together with the observations that the amino acid composition of fragment A2 is almost identical to the combined composition of fragments B and A3b, that A2 contains more homoserine residues than anyone of the other fragments and that the yields of fragments A2, B and A3b vary somewhat from preparation to preparation, strongly suggested that fragment A2 was a product of incomplete CNBr-cleavage. Fragment A1 was the only one lacking homoserine (Table 1).

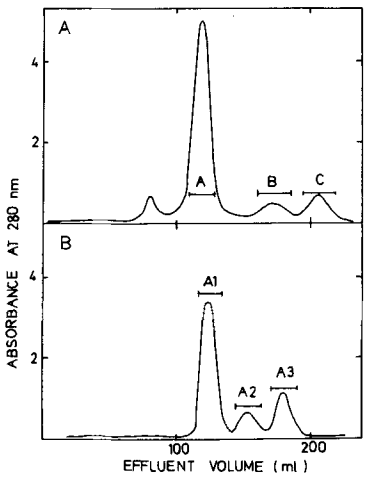

Fig. 1.
Gel chromatography on a column (142x1.5 cm) of Sephadex G-100 equilibrated with 0.05 M sodium acetate buffer, pH 5.0, containing 6M guanidine-HCl.
A. The sample, 80 mg of CNBr-cleaved RBP, was eluted at a flow rate of 4 ml/h and fractions of 1.5 ml were collected. The bars denote materials which were pooled, desalted and lyophilized.
B. Fraction A Fig. 1. A was dissolved in 3 ml of 1 M Tris-Cl buffer,pH 8.0, containing 6 M guanidine-HCl and 50 mM EDTA. After the addition of dithiothreitol to a final concentration of 10 mM the sample was incubated for 30 min at room temperature. Iodoacetic acid to a final concentration of 25mM was then added and after another 15 min in the dark the sample was exhaustively dialyzed against the equilibrating buffer of the column. The sample was then chromatographed on the Sephadex G-100 column under conditions identical to those described above. The bars denote materials which were used in subsequent analyses.

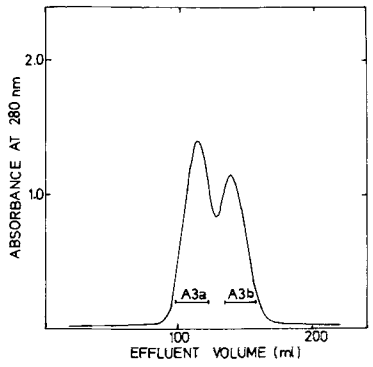

Fig. 2.
Gel chromatography on a column (125 x 1.5 cm) of Sephadex G-50 equilibrated with 0.05 M sodium acetate buffer, pH 5.0, containing 6 M guanidine-HCl. The material subjected to fractionation was fraction A3 of Fig. 1B. The column was operated with a flow rate of 6 ml/h and 1.5 ml fractions were collected. Material denoted by bars were pooled, desalted and lyophilized.

Table 1. Amino acid compositions of cyanogen bromide fragments of human RBP[a] The integral values in parantheses are based on the sequence.

Residue	CNBr-A1 89-182	CNBr-A2 28-73	CNBr-A3a 1-27	CNBr-A3b 54-73	CNBr-B 28-53	CNBr-C 74-88	RBP[d] 1-182
Lysine	3.41(3)	2.75(3)	2.00(2)	1.00(1)	1.86(2)	1.82(2)	10.19(10)
Histidine	1.88(2)	0.26					2.00(2)
Arginine	7.88(8)	2.36(2)	3.68(4)	2.09(2)	0.30		14.02(14)
CM-Cysteine	4.12(4)	1.33(1)	1.21(1)	0.72(1)			4.91(6)
Aspartic acid	14.01(14)	6.90(8)	3.36(3)	3.54(4)	4.00(4)	2.00(2)	26.03(27)
Threonine[b]	3.45(3)	1.90(2)	1.13(1)	0.95(1)	1.25(1)	2.60(3)	9.35(9)
Serine[b]	5.67(6)	2.65(2)	3.15(3)	1.23(1)	1.35(1)	0.38	10.89(11)
Homoserine	0.05	0.56(2)	0.39(1)	0.42(1)	0.40(1)	0.48(1)	
Glutamic acid	10.30(10)	5.94(5)	2.30(2)	0.43	4.38(5)	1.41(1)	18.28(18)
Proline	3.36(3)	1.36(1)			1.10(1)	1.18(1)	6.84(5)
Glycine	6.23(6)	2.87(3)	1.17(1)	1.20(1)	2.00(1)	1.41(1)	11.21(11)
Alanine	5.35(5)	4.08(5)	2.36(2)	2.57(3)	2.01(2)	1.12(1)	13.90(13)
Valine[c]	5.95(6)	3.69(4)	2.05(2)	1.97(2)	1.89(2)	1.00(1)	12.20(13)
Methionine							3.84(4)
Isoleucine[c]	3.07(3)	0.78(1)			0.68(1)		3.96(4)
Leucine	9.00(9)	3.89(4)		1.65(2)	1.95(2)	0.36	12.78)13)
Tyrosine	6.75(7)	0.41	0.93(1)		0.37		8.06(8)
Phenylalanine	3.29(3)	2.24(2)	2.64(3)	0.34	1.81(2)	1.80(2)	10.14(10)
Tryptophan	1.48(2)	0.61(1)	0.67(1)	0.71(1)			5.22(4)
Yield[f] (%)	80	40	82	45	40	85	-

[a]Except where noted all figures are average values of one 24 h and one 72 hydrolysis
[b]Values obtained by extrapolation to 0 h hydrolysis.
[c]72 h hydrolysis value.
[d]Data taken from ref. 40.
[e]Determined spectrophotometrically.
[f]These data are based on one preparation from 6μmoles of RBP. Other preparations gave similar values although the yields of fragments A2, A3b and B somewhat varied.

Intact, reduced and carboxymethylated RBP and the six CNBr-fragments were separately subjected to NH_2-terminal amino acid sequence determination in an automatic liquid phase sequencer. By this procedure almost the entire primary structure of fragments A3a, B, A3b and C could be elucidated (Table 2 and Fig. 3). The NH_2-terminal amino acid sequences of fragments A2 and B were identical confirming that fragment A2 is the result of incomplete CNBr-cleavage of RBP.

The NH_2-terminal amino acid sequence of intact RBP provided unambiguous information for 40 residues (Table 2 and Fig. 3). This information was sufficient to establish that fragment A3a is the NH_2-terminal CNBr-fragment and that it is followed by fragment B(A2) in the sequence.

Alignment of the CNBr-fragments of human RBP - In order to establish the order of the CNBr-fragments of RBP the intact protein was digested separately with several enzymes and during fractionation methionine-containing peptides were particularly looked for. During the course of this work a number of other

peptides were also obtained, some of which were important for establishing the primary structure of RBP. Thus, in this section such peptides will also be described.

Reduced and carboxymethylated RBP was digested with trypsin, chymotrypsin and thermolysin, respectively. After lyophilization each digest was separately subjected to gel chromatography on a column of Sephadex G-25 (Fig. 4). NH_2-terminal amino acid sequence determinations demonstrated that all peptide fractions obtained were impure. Further purification was accomplished by combining high-voltage paper electrophoresis with paper chromatography. The amino acid compositions, the purification procedure and the amino acid sequence of each peptide are presented in Table 3A. Peptides R5, R10, RC2, RC3, RC8 and RT1 contained methionine residues. The amino acid sequences of peptides R5, R10 and RC2 (Table 3B) established that CNBr-fragments A3a and B were juxtaposed (see above). Peptide RT1 connected CNBr-fragments B and A3b. Since CNBr-fragment A1 lacked homoserine it had to be the COOH-terminal fragment. Consequently, the only remaining fragment, C, had to be positioned in between fragments A3b and A1. This notion was supported by the observation that peptide RC8 connected C with A1 (Table 3).

Further support for the order of the CNBr-fragments was obtained from analyses of two other peptides. After cleavage of intact, reduced and carboxymethylated RBP with clostripain, two peptides were isolated following gel chromatography on a column of Sephadex G-100 (Fig.5). After rechromatography on the same column of the peaks denoted C1 I and C1 II (Fig. 5B and C) amino acid analyses demonstrated that C1 I comprised 59 and C1 II 41 amino acid residues (Table 4). NH_2-terminal amino acid sequence determination in the automatic sequencer of fragment C1 I provided almost its entire structure (Table 4). This result definitely showed that CNBr-fragment A3a was followed by B and A3b. The NH_2-terminal 15 residues of fragment C1 I were determined (Table 4) which connected fragment A3b with C. Thus, the order of the CNBr-fragments of RBP is A3a-B-A3b-C-A1. CNBr-fragment A2 occurs as a consequence of the incomplete cleavage of the Met-Ser bond joining CNBr-fragment B with A3b (see Fig. 6).

Fig. 3. (Next page)

The yields of PTH-amino acids in each degradation cycle. The materials subjected to amino acid sequence determination contained A) intact, reduced and alkylated RBP; 120 nmoles. B) CNBr-fragment A3a; 90 nmoles. C) CNBr-fragment A2; 210 nmoles. D) CNBr-fragment B; 110 nmoles. E) CNBr-fragment A3b; 80 nmoles. F) CNBr-fragment C; 120 nmoles.G) CNBr-fragment A1; 240 nmoles and H) acid cleavage fragment H-2; 220 nmoles. The initial yields, which in the figure are normalized to 100% were A) 74 nmoles. B) 52 nmoles. C) 130 nmoles. D) 61 nmoles. E) 49 nmoles. F) 67 nmoles. G) 138 nmoles and H) 113 nmoles, respectively.

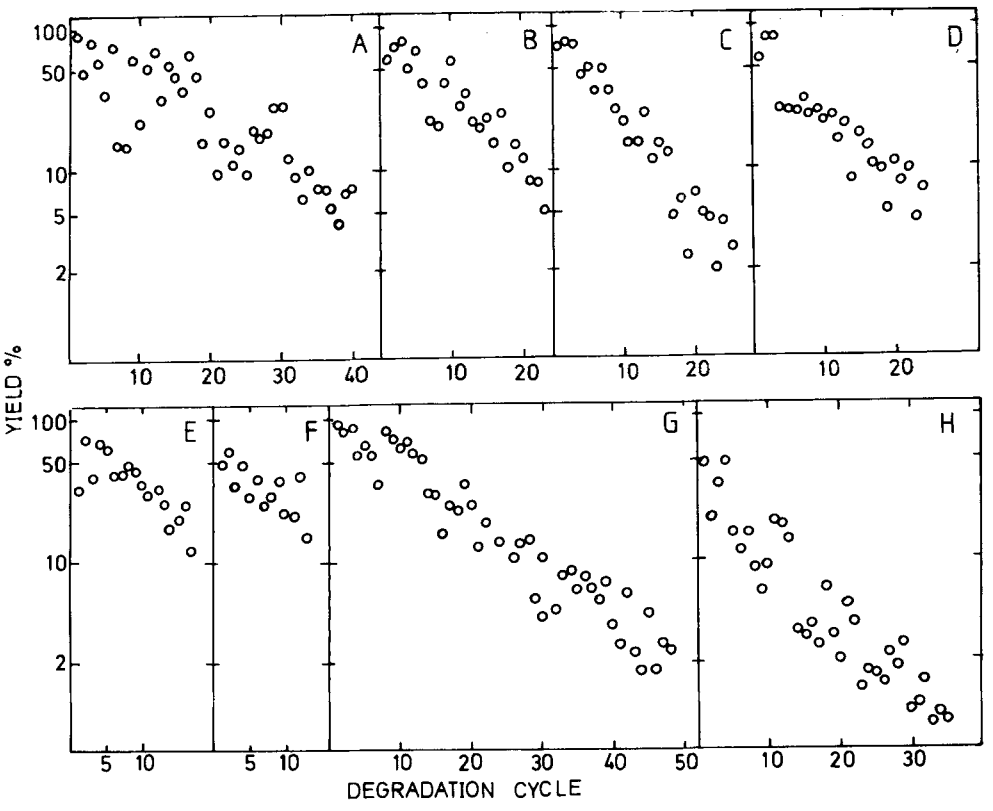

Table 2. NH_2-terminal amino acid sequence determination of CNBr-fragments of RBP (A) and of intact RBP (B).

Material	Amino acid sequence
A. A1	Lys-Tyr-Trp-Gly-Val-Ala-Ser-Phe-Leu-Gln-Lys-Gly-Asn--Asp-Asp-His-Trp-Ile-Val-Asp-Thr-Asp-Tyr-Asp-Thr-Tyr--Ala-Val-Gln-Tyr-Ser-CMCys-Arg-Leu-Leu-Asn-Leu-Asp--Gly-Thr-CMCys-Ala-Asp-Ser-Tyr-Ser-Phe-Val-
A2	Ala-Lys-Lys-Asp-Pro-Glu-Gly-Leu-Phe-Leu-Gln-Asp-Asn--Ile-Val-Ala-Glu-Phe-Ser-Val-Asp-Glu-Thr-Gly-Gln-
A3a	Glu-Arg-Asp-CMCys-Arg-Val-Ser-Ser-Phe-Arg-Val-Lys-Glu--Asn-Phe-Asp-Lys-Ala-Arg-Phe-Ser-Gly-Thr-
A3b	Ser-Ala-Thr-Ala-Lys-Gly-Arg-Val-Arg-Leu-Leu-Asn-Asn--Trp-Asp-Val-CMCys-
B	Ala-Lys-Lys-Asp-Pro-Glu-Gly-Leu-Phe-Leu-Gln-Asp-Asn--Ile-Val-Ala-Glu-Phe-Ser-Val-Asp-Glu-Thr-Gly-
C	Val-Gly-Thr-Phe-Thr-Asp-Thr-Glu-Asp-Pro-Ala-Lys-Phe-
B. RBP	Glu-Arg-Asp-CMCys-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--Asn-

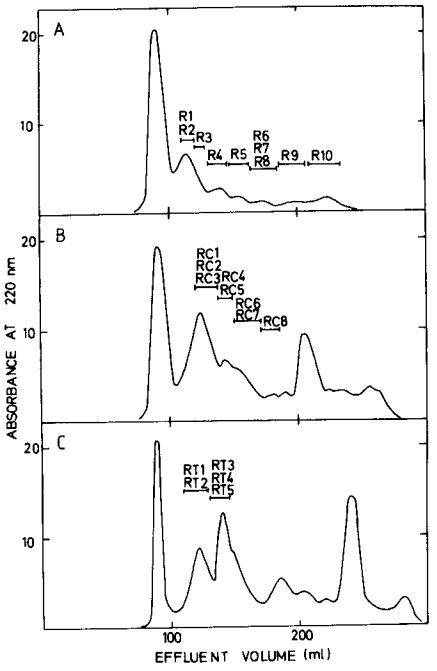

Fig. 4.
Gel chromatography of tryptic (A), chymotryptic (B) and thermolytic (C) digests of RBP. In each case 2 μmoles of reduced and carboxymethylated RBP were digested. The columns (152x1.5cm) of Sephadex G-25 were equilibrated with 0.025% ammonia and 10% n-propanol in water. Fractions of 2.0 ml were collected at 10-min intervals. The bars indicate fractions pooled and lyophilized. The peptides have the same designations as in Table 3.

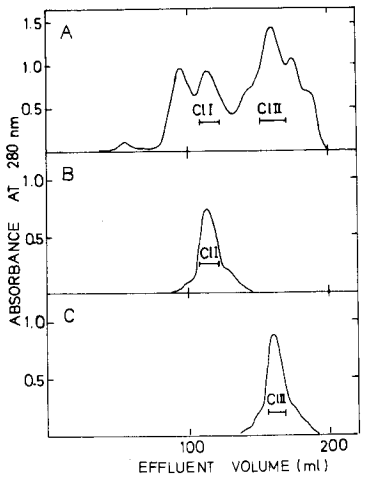

Fig. 5.
Gel chromatography on a column (175x1.2 cm) of Sephadex G-100 equilibrated with 0.05 M sodium acetate buffer, pH 5.0, containing 6 M guanidine-HCl of a clostripain digest of 3.1 μmoles of carboxymethylated RBP (A). Fractions denoted by the bars (Cl I and Cl II) in A were pooled, desalted, lyophilized and resub-jected to the same Sephadex G-100 column (B and .C). The flow rate of the column was 2.8 ml/h and fractions were collected every 25 min. Fractions denoted by the bars in B and C were pooled, desalted and subjected to amino acid analysis and sequential degradation.

The complete Amino Acid Sequence of CNBr-Fragment A3a -The complete amino acid sequence of CNBr-fragment A3a was obtained by automatic amino acid sequence determination of intact RBP (Table 2B). This sequence was corroborated by direct automatic sequencing of fragment A3a (Table 2A), which

provided information for 23 out of the 27 residues. Additional information was obtained from the amino acid sequences of peptides R3, R4, R6, R10, RC2, RC4, RC6, RC7, RT5 (Table 3) and clostripain fragment C1 II (Table 4) as summarized in Fig. 6.

Table 3. Amino acid composition[a] and amino acid sequence[b] of some tryptic, chymotryptic and thermolytic peptides of carboxymethylated RBP

Peptide Designation	Amino Acid Composition	Yield[c]		Purification[d] Procedure	
A.		nmoles	%		residue
Tryptic					
R1	$K_{0.8}D_{2.2}E_{1.8}P_{3.0}G_{1.0}A_{1.1}L_{1.2}$	184	9.2	AB	140-150
R2	$C_{0.9}D_{3.0}T_{0.9}S_{0.8}G_{1.0}A_{1.1}L_{3.0}Y_{0.9}$	385	19.5	AB	122-133
R3	$K_{2.0}D_{2.1}E_{1.1}V_{0.8}F_{1.0}$	231	11.7	ABD	11-17
R4	$R_{2.0}C_{0.8}D_{1.0}E_{0.7}$	180	9.1	ABC	1-5
R5	$K_{2.4}A_{2.0}M_{0.9}$	92	4.6	AB	26-30
R6	$R_{1.3}S_{1.9}V_{0.9}F_{1.0}$	274	13.7	AB	6-10
R7	$R_{1.0}V_{0.9}$	233	11.7	ABD	61-62
R8	$R_{1.0}G_{0.7}$	319	16.0	AB	59-60
R9	$K_{1.0}F_{1.0}$	57	2.9	AB	86-87
R10	$K_{1.4}T_{0.9}S_{1.0}G_{1.0}A_{1.8}M_{0.9}Y_{0.8}F_{1.0}$	75	3.7	AB	20-29
Chymotryptic					
RC1	$K_{1.7}D_{0.9}E_{1.1}P_{1.0}G_{1.0}A_{0.9}L_{0.9}F_{0.9}$	233	11.7	ABCD	28-36
RC2	$K_{2.3}D_{1.1}E_{1.0}P_{1.3}G_{1.4}A_{2.1}M_{1.0}F_{1.0}$	114	3.8	ABCD	26-36
RC3	$D_{1.3}T_{1.2}S_{0.9}E_{2.0}G_{1.0}V_{1.2}M_{0.8}$	435	21.7	ABC	46-53
RC4	$R_{2.0}C_{0.7}D_{0.9}S_{1.7}E_{1.4}V_{1.0}F_{0.9}$	87	4.3	ABD	1-9
RC5	$R_{0.7}K_{1.2}T_{1.0}S_{0.9}G_{1.1}A_{1.9}V_{1.1}$	55	2.8	AB	54-61
RC6	$K_{1.2}R_{1.2}D_{0.7}A_{0.8}F_{0.8}$	473	23.7	ABDC	16-20
RC7	$K_{1.3}R_{1.3}D_{0.9}E_{0.9}V_{1.0}F_{1.1}$	194	9.7	ABDC	10-15
RC8	$K_{2.0}M_{0.9}Y_{1.0}$	35	1.8	AB	87-90
Thermolytic					
RT1	$D_{1.2}T_{1.0}S_{1.0}E_{2.1}G_{1.1}V_{1.0}M_{0.8}$	48	2.4	ABD	47-54
RT2	$K_{0.9}D_{1.9}T_{1.7}E_{1.1}P_{1.0}A_{1.0}F_{0.8}$	356	17.8	ABD	77-85
RT3	$D_{1.8}E_{1.0}L_{0.8}$	215	10.8	ABC	37-40
RT4	$E_{1.0}A_{0.8}V_{0.4}I_{0.4}$	200	10.0	ABC	41-44
RT5	$K_{0.8}D_{1.0}E_{1.2}V_{0.8}$	293	14.7	ABD	11-14

	Amino Acid Sequence
B.	
Tryptic	
R1	Asx-Pro-Asx-Gly-Leu-Pro-Pro-Glx-Ala-Glx-
R2	Leu-Leu-Asx-Leu-Asx-Gly-Thr-CMCys-Ala-
R3	Val-Lys-Glx-Asx-Phe-Asx-
R4	Glx-Arg-Asx-CMCys-Arg
R5	Ala-Met-Ala-Lys-
R6	Val-Ser-Ser-Phe-Arg
R7	Val-Arg
R8	Gly-Arg
R9	Phe-Lys
R10	Phe-Ser-Gly-Thr-(-)-Tyr-Ala-Met

Chymotryptic

RC1 Ala-Lys-Lys-Asx-Pro-Glx-Gly-Leu-Phe
RC2 Ala-Met-Ala-Lys-Lys-Asx-Pro-Glx-Gly-Leu-Phe
RC3 Ser-Val-Asx-Glx-Thr-Gly-Glx-Met
RC4 Glx-Arg-Asx-CMCys-Arg-Val-Ser-Ser-Phe
RC5 Ser-Ala-Thr-Ala-Lys-Gly-
RC6 Asx-Lys-Ala-Arg-Phe
RC7 Arg-Val-Lys-Glx-Asx-Phe
RC8 Lys-Met-Lys-Tyr

Thermolytic

RT1 Val-Asx-Glx-Thr-Gly-Glx-Met-Ser
RT2 Phe-Thr-Asx-Thr-Glx-Asx-Pro-Ala-
RT3 Leu-Glx-Asx-Asx
RT4 Ile-Val-Ala-Glx
RT5 Val-Lys-Glx-Asx

a) All analyses are 24h hydrolysis values.
b) All sequence analyses were carried out by the dansyl-Edman technique.
c) Yields are not corrected for material taken for analyses during the course of the purification procedure.
d) The letters have the following meaning: A, gel chromatography on Sephadex G-25; B, high voltage paper electrophoresis at pH6.5; C, high voltage paper electrophoresis at pH 3.5; D, paper chromatography.

Table 4A. Amino acid composition of two clostripain peptides obtained from carboxymethylated RBP[a]

Amino Acid	Cl I residue 63-121 Found	To nearest integer	Sequence[d]	Cl II residue 20-60 Found	To nearest integer	Sequence[d]
		mole/mole			mole/mole	
Lysine	3.8	4	4	2.7	3	3
Histidine	1.0	1	1	0.3		
Arginine	1.3	1	1	1.2	1	1
CM-cysteine	1.7	2	2	0.1		
Aspartic acid	10.7	11	12	4.1	4	4
Threonine[b]	4.4	4	5	2.7	3	3
Serine[b]	2.0	2	2	3.3	3	3
Glutamic acid	3.1	3	3	5.1	5	5
Proline	1.4	1	1	1.0	1	1
Glycine	3.4	3	3	3.7	4	4
Alanine	4.0	4	4	4.8	5	5
Valine[c]	4.6	5	5	2.1	2	2
Methionine	1.6	2	2	1.6	2	2
Isoleucine[c]	1.3	1	1	1.0	1	1
Leucine	3.4	3	3	2.0	2	2
Tyrosine	3.4	3	4	1.0	1	1
Phenyl-alanine	2.8	3	3	2.8	3	3
Tryptophan	2.6	3	3	0.6	1	1
Yield (%)		45			68	

[a]Except where noted all figures are average values of one 24 h and one 72 h hydrolysis.
[b]Values obtained by extrapolation to 0 h hydrolysis.
[c]72 h hydrolysis value.
[d]Calculated from Fig. 6.

Table 4B. Amino acid sequence of clostripain fragments C1I and C1II.

Peptide Designation	Amino Acid Sequence
C1 I	Leu-Leu-Asn-Asn-Trp-Asp-Val-CMCys-Ala-Asp-Met-Val-Gly-Thr-Phe-
C1 II	Phe-Ser-Gly-Thr-Trp-Tyr-Ala-Met-Ala-Lys-Lys-Asp-Pro-Glu-Gly-Leu-Phe-Leu-Gln-Asp-Asn-Ile-Val-Ala-Glu-Phe-Ser-Val Asp-Glu-Thr-Gly-Gln-Met-Ser-Ala-Thr-Ala-

[a]For both peptides 120 nanomoles were subjected to automatic sequence determination in the liquid phase sequencer. The repetetive yield for C1 II was 92% and for C1 I 84%.

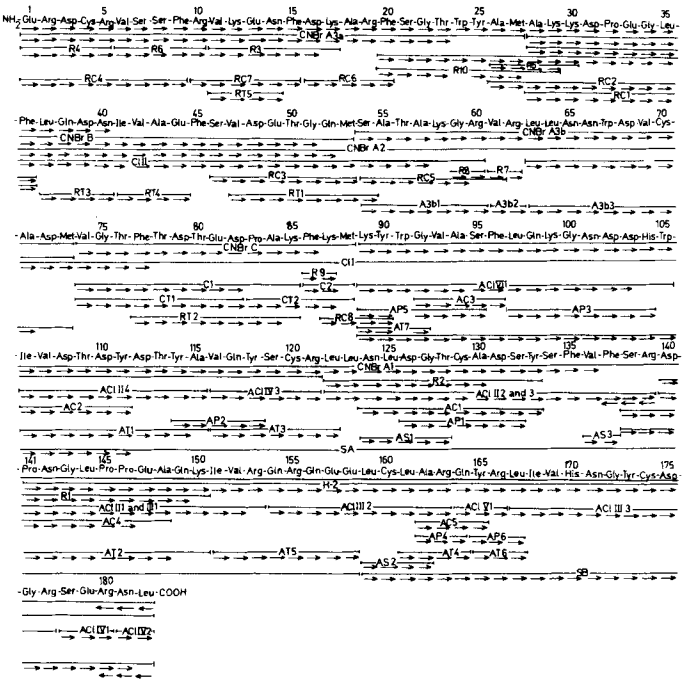

Fig. 6.
Amino acid sequences of peptides used to establish the primary structure of RBP. For designations of peptides, see text. ⟶ denotes NH_2-terminal amino acid sequencing by means of liquid phase sequencer or by the manual dansyl-Edman procedure. ⟵ denotes amino acid residues released after carboxypeptidase digestion as quantitated by amino acid analysis.

The complete Amino Acid Sequence of CNBr-fragment B - Thirteen out of the 26 amino acid residues of CNBr-fragment B were obtained by automatic amino acid sequence analysis of intact RBP (Table 2B). By the same procedure fragments B and A2 provided 24 and 25 residues, respectively. Corroborative information was obtained from peptides R10, RC1, RC2, RC3, RT1, RT3, RT4 (Table 3) and CI II (Table 4).

The Complete Amino Acid Sequence of CNBr-fragment A3b - Automatic amino acid sequence analysis of CNBr-fragment A3b provided information for 17 out of the 20 positions (Table 2A). Clostripain peptide C1 II (Table 4) corroborated the NH_2-terminal sequence of the fragment and peptide C1 I (Table 4) established the COOH-terminal region of A3b (Fig.6). Other peptides like R7, R8 and RC5 (Table 3) supported the established sequence. However, since the amino acid sequence of the COOH-terminal half of fragment A3b relied only on

analyses performed on rather large peptides, A3b was digested with clostripain. The peptide mixture was fractionated by DEAE-Sepharose ion exchange chromatography. Three peptides were obtained (Fig. 7). The combined amino acid composition of peptides A3b1, A3b2 and A3b3 was identical to that of fragment A3b (Table 5) and with the amino acid sequence of the three peptides (Table 5) the sequence of CNBr-fragment A3b (Fig.6) was ascertained.

The complete Amino Acid Sequence of CNBr-fragment C - The amino acid sequence of the CNBr-fragment C, comprised of 15 amino acid residues (Table 1), was elucidated by automatic sequencing of the intact fragment, which yielded information in 13 positions (Table 2), and by sequence analysis of peptides R9and RC8 (Table 3). Corroborative information was obtained from peptide C1 I (Table 4) and from tryptic and chymotryptic peptides of fragment C. These peptides, C1, C2, CT1 and CT2 were isolated by high voltage paper electrophoresis and paper chromatography (Table 4) and their sequences established the primary structure of CNBr-fragment C (Table 6 and Fig. 6).

Table 5. Amino acid composition and amino acid sequence of clostripain peptides derived from 0.8 μmole of CNBr-fragment A3b

Peptide Designation	Amino Acid Composition[a]	Yield		Residue
		nmoles	%	
A. A3b1	$K_{1.0}$ $R_{0.9}$ $T_{0.9}$ $S_{0.9}$ $G_{1.2}$ $A_{1.9}$	320	40	54-60
A3b2	$R_{1.0}$ $V_{0.9}$	290	36	61-62
A3b3	$C_{0.9}$ $D_{3.6}$ $A_{1.1}$ $V_{1.0}$ $M_{0.9}$[b] $L_{1.6}$	380	48	63-73
	Amino Acid Sequence[c]			
B. A3b1	Ser-Ala-Thr-Ala-Lys-Gly-Arg			
A3b2	Val-Arg			
A3b3	Leu-Leu-Asn-Asn-Trp-Asp-Val-CMCys-Ala			

[a]The amino compositions are based on 24 hydrolysis values only.
[b]Determined as homoserine.
[c]For A3b1 (68 nanomoles) and A3b3 (82 nanomoles) sequence determination was accomplished in an automatic liquid sequencer. Peptide A3b2 (32 nanomoles) was analyzed by the manual dansyl-Edman technique.

The Complete Amino Acid Sequence of CNBr-fragment A1 - CNBr-fragment A1 represents more than half of RBP. Automatic sequencing of the entire A1- fragment provided information for 48 out of its 94 residues (Table 2). Since

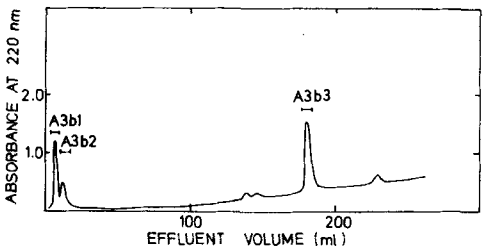

Fig. 7.
DEAE-Sepharose ion exchange chromatography of a clostripain digest of 0.8 μmoles of CNBr-fragment A3b. The column (12x1 cm) was equilibrated with 0.02 M NH_4HCO_3. The applied sample was eluted with a 250 ml linear gradient of NH_4HCO_3 from 0.02 to 0.2 M. The flow rate was 18ml/h and fractions of 2 ml were collected. The bars indicate material pooled, lyophilized and used in further analyses.

preliminary studies had shown that CNBr-fragment A1 contained a single aspartyl-prolyl bond, which is sensitive to acid proteolysis (23), fragment A1 was cleaved with formic acid. Two fragments of similar size,

Table 6. Amino acid composition and amino acid sequence of tryptic and chymotryptic peptides obtained from CNBr fragment C[a]

Peptide Designation	Amino Acid Composition[b]	Yield		Purification Procedure[c]	Residue
A		nmoles	%		
Tryptic					
C1	$K_{0.9}$ $D_{1.8}$ $T_{2.7}$ $E_{1.2}$ $P_{1.2}$ $G_{1.0}$ $A_{1.0}$ $V_{0.8}$ $F_{0.9}$	190	9.5	B D	74-85
C2	$K_{1.0}$ $M_{0.6}$[d] $F_{1.1}$	280	14	B	86-88
Chymotryptic					
CT1	$D_{2.1}$ $T_{2.8}$ $E_{1.0}$ $G_{1.1}$ $V_{1.0}$ $F_{0.9}$	180	9	B	74-82
CT2	$K_{1.8}$ $M_{0.5}$[d] $P_{0.9}$ $A_{1.2}$ $F_{1.0}$	150	7.5	B	83-88

	Amino Acid Sequence[e]
B.	
Tryptic	
C1	Val-Gly-Thr-Phe-Thr-Asx-Thr-Glx-Asx-
C2	Phe-Lys-
Chymotryptic	
CT1	Val-Gly-Thr-Phe-Thr-Asx-Thr-Glx-Asx
CT2	Pro-Ala-Lys-Phe-Lys-

[a]Two μmoles of fragment C were subjected to each enzymatic digestion.
[b]The amino acid compositions are based on 24 h hydrolysis values only.
[c]B denotes high voltage paper electrophoresis at pH 6.5 and D denotes paper chromatography.
[d]Determinated as homoserine
[e]All sequence determinations were carried out with the manual dansyl-Edman technique. Except for peptide C2 (50 nanomoles) 120 nanomoles of each peptide were subjected to manual degradation.

as shown by SDS-polyacrylamide gel electrophoresis, and charge, as evidenced by ion-exchange chromatography, were obtained in excellent yield. Since the properties of the two fragments precluded their separation, intact fragment A1 was succinylated prior to the acid cleavage. The cleavage mixture was added to the automatic sequencer without prior peptide separation. As expected, the only amino acid sequence obtained, was that of the COOH-terminal acid cleavage fragment H-2 (Fig.3,6). This information together with the NH_2-terminal automatic amino acid sequence analysis of intact fragment A1 gave almost all of the primary structure of A1 (see Fig.6).

Fragment A1 was digested with Staphylococcus Aureus protease V8 and the resulting peptide mixture was resolved by gel chromatography on a column of Sephadex G-50 (Fig.8). Two peptides, denoted SA and SB in Fig. 8A were further purified by column electrophoresis (Fig. 8B and C). Amino acid analysis and automatic sequencing of the two peptides demonstrated that SA represented the NH_2-terminal part of A1 (Table 7). The COOH-terminal fragment SB gave clear sequence information in 23 out of its 24 positions (Table 7). However, this information did not establish a connection between the NH_2-terminal region of fragment A1 and the COOH-terminal acid cleavage fragment H-" (see Fig. 6 and Fig. 3.)

To obtain further amino acid sequence information about CnBr-fragment A1, this fragment was separately digested with chymotrypsin, thermolysin, pepsin and subtilisin. All digests were subjected to ion-exchange chromatography (Fig. 9). Table 8 summarizes the amino acid compositions and sequences of the isolated peptides. Fragment A1 was also digested with clostripain and the digest was fractionated on a Sephadex G-50 column (Fig. 10). Fraction I contained aggregated material and fraction VI contained a single peptide. All other fractions were further purified by DEAE-Sepharose ion-exchange chromatography (Fig. 10). A total of twelve clostripain peptides were recovered and they made up the entire A1 fragment (Table 9). It should be noted that peptides AC1 II 1 and AC1 III 1 were identical and that peptides AC1 II 4, AC1 IV 1, AC1 IV 2 and AC1 IV 3 probably arose by thermolysin-like activity present in the clostripain preparation (see Table 9). The peptides obtained from the various digests (Table 8 and 9) together with the tryptic peptides R1 and R2 (Table 3) gave the entire sequence of CNBr-fragment A1. The gap between the NH_2-terminal amino acid sequence (residues 89-136) and the sequence of the acid cleavage fragment H-2 (residues 141-175) was bridged by clostripain peptide AC1 II 2 (table 9), the chymotryptic peptide AC4 (Table 8) and the thermolysin peptide AT2 (Table 8). To firmly establish the sequence of peptide

AC1 II 2 it was subjected to carboxpeptidase digestion (Fig. 6) in addition to NH_2-terminal sequencing. Thus, the information obtained was sufficient to establish the primary structure of CNBr-fragment A1.

Table 7A. Amino acid composition of two Staphylococcus Aureus protease V8 peptides derived from CNBr-fragment A1[a,b]

Amino acid	SA residue 89-158			SB residue 159-182		
	Found	To nearest integer	Sequence[c]	Found	To nearest integer	Sequence[c]
	mole/mole			mole/mole		
Lysine	2.8	3	3	0.3		
Histidine	1.0	1	1	0.9	1	1
Arginine	3.6	4	4	3.6	4	4
CM-Cysteine	2.4	2	2	2.0	2	2
Aspartic Acid	10.6	11	11	2.8	3	3
Threonine[d]	3.3	3	3			
Serine[d]	4.7	5	5	1.1	1	1
Glumatic Acid	8.1	8	8	2.3	2	2
Proline	2.9	3	3			
Glycine	4.0	4	4	1.9	2	2
Alanine	3.9	4	4	1.2	1	1
Valine[e]	4.6	5		5	0.9	1
Methionine						
Isoleucine[e]	1.6	2	2	0.7	1	1
Leucine	5.2	5	5	3.8	4	4
Tyrosine	4.8	5	5	1.7	2	2
Phenylalanine	3.0	3	3			
Tryptophan	1.7	2	2			
Yield	76%			48%		

[a]Two µmoles of CNBr-fragment A1 were digested with the enzyme.
[b]Except where noted all values are average values of one 24 h and one 72 h hydrolysis.
[c]Obtained from the sequence shown in Fig. 6.
[d]Values obtained by extrapolation to 0 h hydrolysis.
[e]72 h hydrolysis value

Table 7B. Amino acid sequence analyses of Staphylococcus Aureus protease V8 peptides obtained from CNBr-fragment A1[a]

Peptide Designation	Amino Acid Sequence
SA	Lys-Tyr-Trp-Gly-Val-Ala-Ser-Phe-Leu-Gln-Lys-Gly- -Asn-Asp-Asp-His-Trp-Ile-Val-Asp-Thr-Asp-Tyr-
SB	-Leu-CMCys-Leu-Ala-Arg-Gln-Tyr-Arg-Leu-Ile-Val- -His-Asn-Gly-Tyr-CMCys-Asp-Gly-Arg-Ser-Glu-Arg- -Asn-

[b]Both peptides were degraded in the automatic liquid phase sequencer. The overall repetitive yield was 89% for peptide SA and 91% for peptide SB. In each case 210 nanomoles were subjected to analysis.

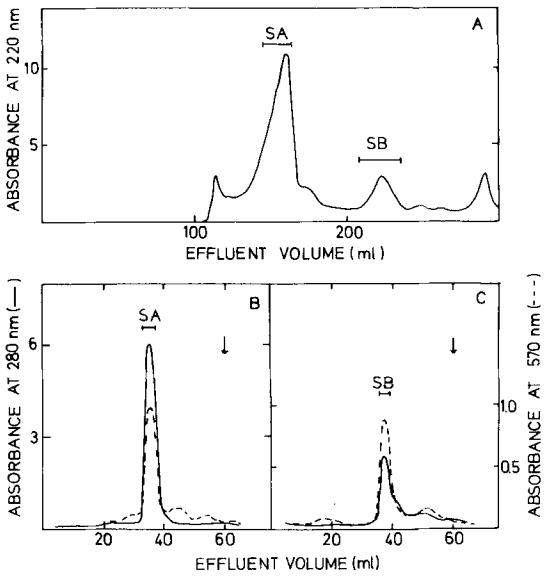

Fig.8.
Purification of Staphylococcus aureus, protease V8 peptides obtained from digestion of 2 µmoles of CNBr-fragment A1. The digest was applied onto a Sephadex G-50 column (110x2 cm) equilibrated with 0.025% ammonia - 10% propanol (A). Fractions of 2.0 ml were collected at 9-min intervals. The bars denote materials (SA and SB) which were further purified by column electrophoresis at pH 1.9 (B and C). After completed electrophoretic separation the columns were eluted at a flow rate of 12 ml/hour. Fractions of 1.0 ml were collected. The occurrence of peptides in the effluent was monitored by measuring the absorbance at 280 nm. In addition 25 µl-aliquots from each fraction were subjected to alkaline hydrolysis and the ninhydrin reaction. The color developed was measured at 570 nm. Fractions indicated by the bars were pooled and lyophilized.

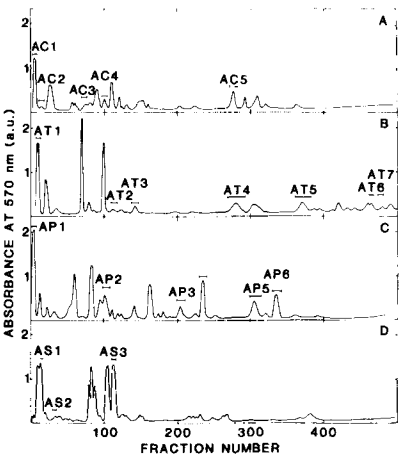

Fig. 9.
Ion exchange chromatography on a polystyrene resin (Jeol AR-15) of peptides derived from CNBr-fragment A1 after digestion with with chymotrypsin (A), thermolysin (B), pepsin (C) and subtilisin (D). The digests representing 0.8 µmoles (A and D) and 1.3 µmoles (B and C) of fragment A1 were separately applied onto the column. Fractions of 3.0 ml of 3.0 ml were collected at a flow rate of 110 ml/h. The bars denote highly purified peptides used in subsequent analyses (see Table VIII). Further experimental details are given under Methods. The ninhydrin color at 570 nm is expressed in arbitrary units (a.u.).

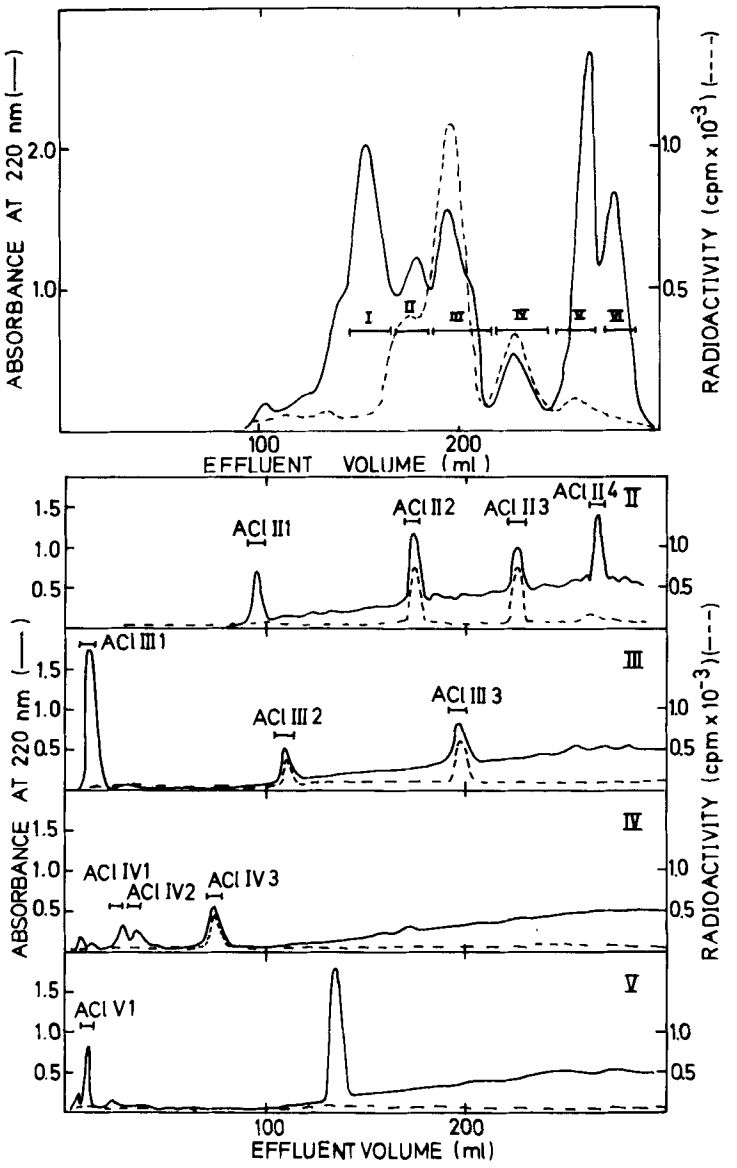

Fig.10.
Gel chromatography (upper) on a column (146x1.5 cm) of Sephadex G-50 equilibrated with 0.025% ammonia - 10% n-propanol of 0.75 µmole of [^{14}C] carboxmethylated CNBr-fragment A1 digested with clostripain. The flow rate of the column was 12 ml/h and 2.0 ml fractions were collected. The fractions denoted by the bars were pooled, lyophilized and, except for fraction VI, separately subjected to ion exchange chromatography (lower, II-V) on columns (12x1 cm) of DEAE-Sepharose equilibrated with 0.02 NH_4HCO_3. Elution was carried out with 300-ml linear gradients of NH_4HCO_3 from 0.02 to 0.3 M at flow rates of 20 ml/h. Fractions of 2.0 ml were collected. The occurence of peptides in the effluents was monitored by the absorbance at 220 nm. Cysteine-containing peptides were detected by measuring the radioactivity of 25 µl aliquots withdrawn from each fraction. Peptide-containing fractions were pooled as indicated by the bars. The designation of the peptides refers to Table 9.

Table 8. Amino acid composition[a] and amino acid sequence[b] of some chymotryptic, thermolytic, peptic and subtilisin peptides derived from CNBr-fragment A1

Peptide Designation	Amino Acid Composition	Yield[c] nmoles	Yield[c] %	Residue
A.				
Chymotryptic[d]				
AC1	$C_{1.0}$ $D_{2.7}$ $T_{0.8}$ $S_{0.9}$ $G_{1.0}$ $A_{0.6}$ $L_{0.9}$ $Y_{0.8}$	190	24	124-133
AC2	$D_{1.7}$ $T_{1.1}$ $V_{0.3}$ $I_{0.3}$ $Y_{1.0}$	400	50	106-111
AC3	$D_{0.3}$ $S_{1.0}$ $E_{0.3}$ $G_{1.1}$ $A_{1.1}$ $V_{0.9}$ $F_{1.0}$	90	11	92-96
AC4	$R_{0.9}$ $D_{2.2}$ $S_{0.9}$ $E_{1.0}$ $P_{2.9}$ $G_{1.1}$ $A_{0.8}$ $L_{1.0}$	210	26	138-148
AC5	$R_{1.0}$ $E_{1.1}$ $A_{0.9}$ $Y_{1.0}$	220	28	162-165
Thermolytic				
AT1	$D_{3.0}$ $T_{2.1}$ $S_{0.3}$ $A_{0.8}$ $V_{0.6}$ $I_{0.4}$ $L_{0.1}$ $Y_{1.8}$	660	51	106-115
AT2	$K_{1.0}$ $R_{1.1}$ $D_{2.4}$ $S_{1.1}$ $E_{2.2}$ $P_{2.8}$ $G_{1.3}$ $A_{1.1}$ $L_{1.1}$	180	14	138-150
AT3	$R_{1.0}$ $C_{0.9}$ $S_{1.1}$ $E_{1.3}$ $V_{0.9}$ $L_{1.0}$ $Y_{0.9}$	150	12	116-122
AT4	$R_{1.1}$ $E_{1.2}$ $A_{0.9}$ $L_{1.0}$	600	46	161-164
AT5	$R_{2.0}$ $E_{4.2}$ $V_{0.4}$ $I_{0.4}$	530	41	151-158
AT6	$R_{1.0}$ $L_{1.0}$ $Y_{0.9}$	320	25	165-167
AT7	$K_{0.8}$ $G_{1.0}$ $Y_{1.0}$	330	25	89-92
Peptic[e]				
AP1	$C_{1.1}$ $D_{2.2}$ $T_{1.2}$ $S_{0.9}$ $G_{1.0}$ $A_{1.0}$ $Y_{0.3}$	680	52	126-133
AP2	$D_{0.3}$ $E_{1.2}$ $A_{0.9}$ $V_{1.0}$ $Y_{2.0}$	220	17	114-118
AP3	$K_{1.0}$ $H_{0.9}$ $D_{3.0}$ $E_{1.1}$ $G_{1.0}$ $L_{1.1}$	420	32	97-104
AP4	$R_{1.0}$ $E_{1.2}$ $A_{0.9}$	710	55	162-164
AP5	$K_{1.0}$ $S_{0.9}$ $E_{0.3}$ $G_{1.0}$ $A_{0.9}$ $V_{1.1}$ $Y_{1.0}$	340	26	89-95
AP6	$R_{1.1}$ $L_{1.0}$ $Y_{0.9}$	730	56	165-167
Subtilisin[d]				
AS1	$D_{2.0}$ $T_{0.9}$ $G_{0.9}$ $L_{0.9}$	370	46	124-128
AS2	$C_{0.9}$ $A_{1.1}$ $L_{2.0}$	80	10	159-162
AS3	$V_{1.0}$ $F_{1.0}$	90	11	136-137

	Amino Acid Sequence
B.	
Chymotryptic	
AC1	Asx-Leu-Asx-Gly-Thr-CMCys-Ala-Asx-Ser-Tyr
AC2	Ile-Val-Asx-Thr-Asx-Tyr
AC3	Gly-Val-Ala-Ser-Phe
AC4	Ser-Arg-Asx-Pro-Asx-Gly-Leu-Pro-Pro-
AC5	Ala-Arg-Glx-Tyr
Thermolytic	
AT1	Ile-Val-Asx-Thr-Asx-Tyr-Asx-Thr-Tyr-
AT2	Ser-Arg-Asx-Pro-Asx-Gly-Leu-Pro-Pro-Glx-Ala-
AT3	Val-Glx-Tyr-Ser-CMCys-Arg-Leu
AT4	Leu-Ala-Arg-Glx
AT5	Ile-Val-Arg-Glx-Arg-Glx-Glx-Glx
AT6	Tyr-Arg-Leu
AT7	Lys-Tyr-(-)-Gly
Peptic	
AP1	Asx-Gly-Thr-CMCys-Ala-Asx-Ser-
AP2	Tyr-Ala-Val-Glx-Tyr
AP3	Leu-Glx-Lys-Gly-Asx-Asx-Asx-His
AP4	Ala-Arg-Glx
AP5	Lys-Tyr-(-)-Gly-Val-Ala-Ser
AP6	Tyr-Arg-Leu
Subtilisin	
AS1	Asx-Leu-Asx-Gly-Thr
AS2	Leu-CMCys-Leu-Ala
AS3	Val-Phe

[a]All analyses are 24 h hydrolysis values.
[b]All sequence analyses were carried out by the manual dansyl-Edman technique.
[c]Yields are not corrected for material taken for analyses during the course of the purification procedure.
[d]The enzymatic digestions were carried out on 0.8 μmoles of fragment A1.
[e]The enzymatic digestion was carried out on 1.3 moles of fragment A1.

Table 9. Amino acid composition[a] and amino acid sequence[b] of clostripain peptides obtained after digestion of 0.75 μmole of [^{14}C] carboxymethylated CNBr-fragment A1

Peptide Designation	Amino Acid Composition	Yield nmoles	Yield %	Residue
A.				
A C1 II1	$K_{1.1}$ $R_{1.0}$ $D_{2.2}$ $E_{2.1}$ $P_{3.1}$ $G_{1.2}$ $A_{1.1}$ $V_{0.7}$ $I_{0.5}$ $L_{1.0}$	250	33	140-153
A C1 II2	$R_{1.3}$ $C_{1.2}$ $D_{3.1}$ $T_{1.0}$ $S_{2.9}$ $G_{1.1}$ $A_{1.4}$ $V_{1.0}$ $L_{2.2}$ $Y_{1.0}$ $F_{2.0}$	150	20	122-139
A C1 II3	$C_{1.3}$ $D_{3.0}$ $T_{1.0}$ $S_{2.0}$ $G_{1.1}$ $A_{1.0}$ $L_{3.0}$ $Y_{1.0}$	240	32	122-134
A C1 II4	$D_{3.0}$ $T_{1.6}$ $A_{1.0}$ $V_{0.8}$ $I_{0.5}$ $L_{0.2}$ $Y_{1.7}$	80	11	106-115
A C1 III1	$K_{1.3}$ $R_{1.0}$ $D_{2.1}$ $S_{0.4}$ $E_{2.2}$ $P_{3.0}$ $G_{1.4}$ $A_{1.3}$ $V_{0.9}$ $I_{0.7}$ $L_{1.2}$	80	11	140-153
A C1 III2	$R_{2.0}$ $C_{1.1}$ $D_{0.4}$ $E_{4.0}$ $G_{0.3}$ $A_{1.2}$ $V_{0.3}$ $L_{2.3}$	230	31	154-163
A C1 III3	$H_{0.8}$ $R_{1.0}$ $C_{1.2}$ $D_{2.0}$ $E_{0.4}$ $G_{1.7}$ $V_{0.7}$ $I_{0.5}$ $L_{1.1}$ $Y_{0.8}$	280	37	167-177
A C1 IV1	$R_{1.0}$ $S_{1.1}$ $E_{1.2}$	70	9	178-180
A C1 IV2	$D_{1.0}$ $L_{0.9}$	60	8	181-182
A C1 IV3	$R_{1.0}$ $C_{1.2}$ $S_{1.0}$ $E_{1.2}$ $V_{1.0}$ $Y_{1.0}$	300	40	116-121
A C1 V1	$R_{1.0}$ $E_{1.2}$ $Y_{1.0}$	340	45	164-166
A C1 VI1	$K_{1.9}$ $H_{0.8}$ $D_{3.0}$ $S_{0.9}$ $E_{1.2}$ $G_{2.1}$ $A_{1.0}$ $V_{0.9}$ $L_{1.0}$ $Y_{0.9}$ $F_{1.0}$	530	71	89-105

	Amino acid sequence
B.	
A C1 II1	Asp-Pro-Asn-Gly-Leu-Pro-Pro-Glu-Ala-Gln-Lys-Ile-
A C1 II2	Leu-Leu-Asn-Leu-Asp-Gly-Thr-CMCys-Ala-Asp-Ser-Tyr-Ser-Phe-Val-Phe-Ser-Arg-
A C1 II3	Leu-Leu-Asn-Leu-Asp-Gly-Thr-CMCys-Ala-Asp-Ser-Tyr-
A C1 II4	Ile-Val-Asp-Thr-Asp-Tyr-Asp-Thr-Tyr-Ala
A C1 III1	Asp-Pro-Asn-Gly-Leu-Pro-Pro-Gln-Ala-Gln-Lys-Ile-Val-
A C1 III2	Gln-Arg-Gln-Glu-Glu-Leu-CMCys-Leu-Ala-Arg
A C1 III3	Leu-Ile-Val-His-Asn-Gly-Tyr-CMCys-Asp-
A C1 IV1	Ser-Glx-Arg
A C1 IV2	Asx-Leu
A C1 IV3	Val-Glx-Tyr-Ser-CMCys-Arg
A C1 V1	Glx-Tyr-Arg
A C1 VI1	Lys-Tyr-Trp-Gly-Val-Ala-Ser-Phe-Leu-Gln-Lys-Gly-Asn-Asp-Asp-

[a]All analyses are 24 h hydrolysis values.
[b]The peptides designated A C1 II, A C1 III and A C1 VI were all subjected to automatic sequence analysis in the liquid phase sequencer. Between 50 to 110 nanomoles of peptide were used in these analyses. The repetitive yield varied between 91 and 94%. Peptides designated A C1 IV and A C1 V were degraded manually with use of the dansyl-Edman technique. Between 30 and 70 nanomoles of peptide were used.
[c]Yields have not been corrected for material taken for analyses during the purification procedure.

The COOH-Terminal Amino Acid Sequence of RBP - We have previously suggested that the COOH-terminus of RBP is involved in the regulation of the catabolism of the protein and we obtained data that the COOH-terminal residue of RBP is

arginine (43). Other authors have obtained other COOH-terminal sequences for RBP (12,55). It was, therefore, of importance to clarify this discrepancy. Fig. 6 shows that amino acid sequence determinations of peptides SB (Table 7) and AC1 IV 1 (Table 9) provided the sequence Arg-Asn-Leu. To corroborate this information, intact, reduced and carboxmethylated RBP as well as CNBr-fragment A1 and peptide SB were separately subjected to carboxypeptidase A and B digestions. Fig. 11 summarizes the results obtained with fragments A1 and peptide SB. Both types of materials clearly showed that the COOH-terminal sequence is Asn-Leu. The digestions of peptide SB also provided strong support for the established sequence (Fig.6), i.e. that arginine preceeds the asparagine. The arginine was not as evident when carboxypeptidase B digestions of fragment A1 (Fig.11A) and intact RBP (not shown) were carried out. The reason for this was that several amino acid residues were released almost simultaneously on the addition of carboxypeptidase B. However, the carboxypeptidase digestions together with the amino acid sequence information summarized in Fig.6 establish the COOH-terminal sequence of RBP.

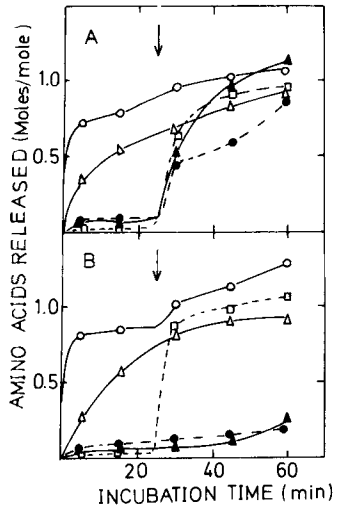

Fig.11.
Carboxypeptidase digestions of CNBr-fragment A1 (A) and Staphylococcus aureus protease V8 peptide SB (B). The samples, each comprising 50 nanomoles of peptide were mixed with carboxypeptidase A (40µg).After 30 min of incubation carboxypeptidase B (25µg) was added (arrow). Samples were withdrawn at the indicated times and amino acids released were identified and quantitated by amino acid analysis. The values given in the figure have been corrected for the presence of free amino acids in the carboxypeptidase preparations and in the peptide fractions. The symbols in the figure are : o---o, leucine; △—△, asparagine; □- - -□, arginine; ●---●, alanine; ▲—▲, tyrosine.

Localization of Disulfide Bridges in RBP - Intact RBP was subjected to acid cleavage and gel chromatography on a column of Sephadex G-100. Fig. 12 depicts the chromatogram. Fraction SI was subjected to amino acid analysis and NH_2-terminal amino acid sequence determination (Table 10) which clearly showed that SI corresponds to residues 83 to 140 (Fig.6.). Thus, half-cystines 120 and 129 must form a disulfide bridge as RBP does not contain any free sulfhydryl groups.

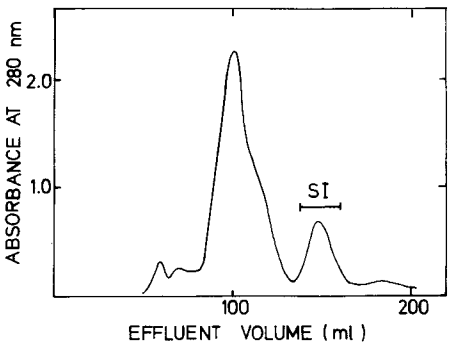

Fig.12
Gel chromatography of 3 µmoles of acid-cleavaged RBP on a column (160x2cm) of Sephadex G-100 equilibrated with 0.05 M sodium acetate, pH 5.0, containing 6M guanidine-HCl. The acid cleavage was obtained by incubating the protein in 70% (v/v) formic acid at 37° for 24 h. After this period of time the formic acid was diluted with H_2O and the protein was lyophilized. The column had a flow rate of 3.4 ml/h and fractions of 2.0 ml were collected. Material denoted by the bar was pooled, dialyzed and lyophilized.

Table 10 A. Amino acid composition of acid cleavage fragment SI derived from intact RBP[a,b]

Amino acid	SI residue 83-140		
	Found	To nearest integer mole/mole	Sequence[c]
Lysine	3.7	4	4
Histidine	1.0	1	1
Arginine	2.0	2	2
CM-cysteine[d]	2.1	2	2
Aspartic acid	9.6	10	10
Threonine[e]	3.1	3	3
Serine[e]	4.7	5	5
Glutamic acid	2.3	2	2
Proline	1.3	1	1
Glycine	3.2	3	3
Alanine	4.0	4	4
Valine[f]	3.8	4	4
Methionine	1.0	1	1
Isoleucine[f]	1.1	1	1
Leucine	4.3	4	4
Tyrosine	5.1	5	5
Phenylalanine	4.0	4	4
Tryptophan	1.9	2	2
Yield	27%		

[a]Three µmoles of RBP were subjected to acid cleavage.
[b]Except where noted all values are average values of one 24 h and one 72 h hydrolysis.
[c]Calculated from the sequence shown in Fig.6 (residues 83-140).
[d]The acid cleavage fragment was reduced and carboxymethylated after the gel chromatography separation (see Fig.12).
[e]Values obtained by extrapolation to 0 h hydrolysis.
[f]72 h hydrolysis value.

Table 10 B. Amino acid sequence analysis of acid cleavage fragment SI[a]

Pro-Ala-Lys-Phe-Lys-Met-Lys-Tyr-Trp-Gly-Val-Ala-Ser-Phe-Leu-Gln-
Lys-Gly-Asn-Asp-Asp-His-Trp-Ile-Val-Asp-Thr-Asp-Tyr-Asp-Thr-Tyr-
Ala-Val-Gln-Tyr-Ser-CMCys-Arg-Leu-Leu-Asn-

[a]Degradation was accomplished on 190 nanomoles of peptide in the automatic sequencer. The repetitive yield was 93%.

Material corresponding to fraction A of Fig. 1A which comprised the half-cystine-containing CNBr-fragments of RBP, was separately subjected to trypsin digestion at pH 6.0 and pepsin digestion at pH 5.0. The digests were separately subjected to Sephadex G-50 gel chromatography in dilute acetic acid (Fig.13). Fractions denoted T and P in Fig. 13 contained half-cystine as monitored by amino acid analysis. These two fractions were pooled and lyophilized. Fraction T was further purified by column electrophoresis (Fig.14) and the pooled fraction T1 was subjected to performic acid oxidation and re-electrophoresed (Fig. 14) to yield peptides T1A and T1B. Both the amino acid composition and the sequence establish that peptide T1A corresponds to residues 167 to 177 and T1B to residues 63 to 73 of RBP (Table 11 and Fig. 6).Thus, the half-cystines in positions 70 and 174 of RBP form a disulfide bridge. Fraction P Fig. 13 was further purified by column electrophoresis (Fig. 14). Both peptide P1 and P2 contained half-cystine. After performic acid oxidation and re-electrophoresis peptide P1 appeared as a single homogenous peak (not shown). Amino acid analysis and sequence determination (Table 11) demonstrated that peptide P1 corresponded to residues 118 to 135, which corroborates the previously established disulfide bond between half-cystines 120 and 129 (see above).

Peptide P2 was further purified by high pressure liquid chromtography (Fig. 15) to yield fraction P2A. After performic acid oxidation this material was re-subjected to high pressure liquid chromatography. Fig. 15B demonstrates that three peptides, P2A1, P2A2 and P2A3, could be isolated. Table 11 ascertains that P2A1 and P2A2 represent residues 1 to 9 of RBP and that P2A3 corresponds to residues 159 to 161. Thus, the third disulfide bond engages the half-cystines in positions 4 and 160. The complete amino acid sequence of human RBP, including the disulfide bridges, is depicted in Fig. 16.

DISCUSSION

Prior to the analysis of the RBP sequence reported here, two laboratories presented partial NH_2-terminal sequence information (27,38). Their information is in full agreement with the sequence elucidated here. After completion of this study Kanda and Goodman (21) reported the primary structure for the NH_2-terminal 121 positions of human RBP. Although their data are generally in good agreement with those described here, some noteable differences occur in positions 50 to 53 (our numbering), 58 to 60, 111 to 114 and 119 to 120. In most of these positions Kanda and Goodman assigned amino acid residues from data obtained by COOH-terminal digestions or from the amino acid composition of

Fig.13.
Gel chromatography of trypsin (A) and pepsin (B) digested disulfide-linked CNBr-fragments A1,A2, A3a and A3b (cf. fraction A of Fig.1A) on a column (100x2 cm) of Sephadex G-50 equilibrated with 10% (v/v) acetic acid. Five μmoles of RBP cleaved with CNBr were subjected to gel chromatography as described in the legend of Fig.1A. Material corresponding to fraction A of Fig.1A was pooled, desalted, lyophilized and divided into two equal parts. One part was dissolved in 4 ml of Tris-acetate buffer, pH 6.0, and the other part was dissolved in 4 ml of 0.2 M sodium acetate buffer, pH 5.0. Each enzyme (0.8 mg) was added to one aliquot and the digestions were allowed to proceed for 8 hours at 37°. The samples were then immediately applied onto the columns, which were eluted at a flow rate of 6.0 ml/hour. Fractions of 2.0 ml were collected. Aliquots (50 μl) from every third fraction were subjected to amino acid analysis. Fractions denoted by the bars contained cysteine and accordingly were pooled and lyophilized.

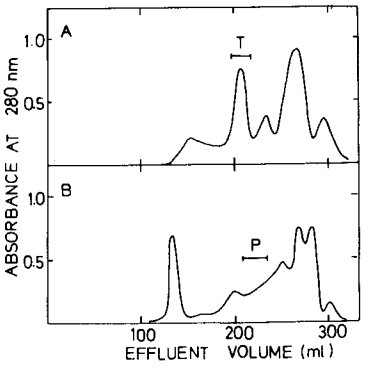

Fig. 14.
Fraction T of Fig. 13A was subjected to electrophoresis on a column of cellulose at pH 1.9 (A).Fractions denoted T1 in A, which contained cysteine as monitored by amino acid analyses on aliquots from alternate fractions, were pooled, lyophilized, subjected to performic acid oxidation and re-electrophoresed under identical conditions (B). Fractions denoted by the bars were pooled and lyophilized. Material in fraction P of Fig. 12B was also subjected to electrophoresis at pH 1.9 (C). Material denoted by the bars contained cysteine as revealed by amino acid analysis performed on aliquots withdrawn from every second fraction. Consequently, those fractions were pooled and lyophilized. The experimental details were the same as in Fig. 8B and C.

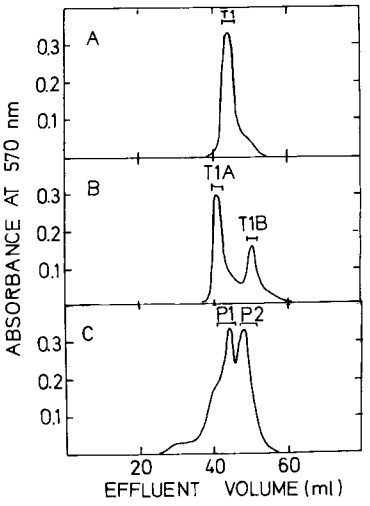

Fig.15.(next page)
High pressure liquid chromatography of the material designated P2 in Fig.14C (A). The C_{18} reversed phase column was equilibrated with 2 mM ammonia, adjusted to pH 2.4 with trifluoroacetic acid, and 5% methanol. The applied material was eluted with an 80-ml linear gradient of methanol from 5 to 55% followed by 30 ml of 55% methanol. The flow rate of the column was 24 ml/h and fractions were collected at 1 min intervals. All absorbance peaks were separately pooled and aliquots from each were subjected to amino acid analysis. Only the fraction denoted P2A contained significant amounts of cysteine. Therefore, this fraction was subjected to performic acid oxidation and re-chromatographed on the C_{18} column (B) under conditions identical to those described above. Fractions denoted by the bars were pooled and lyophilized.

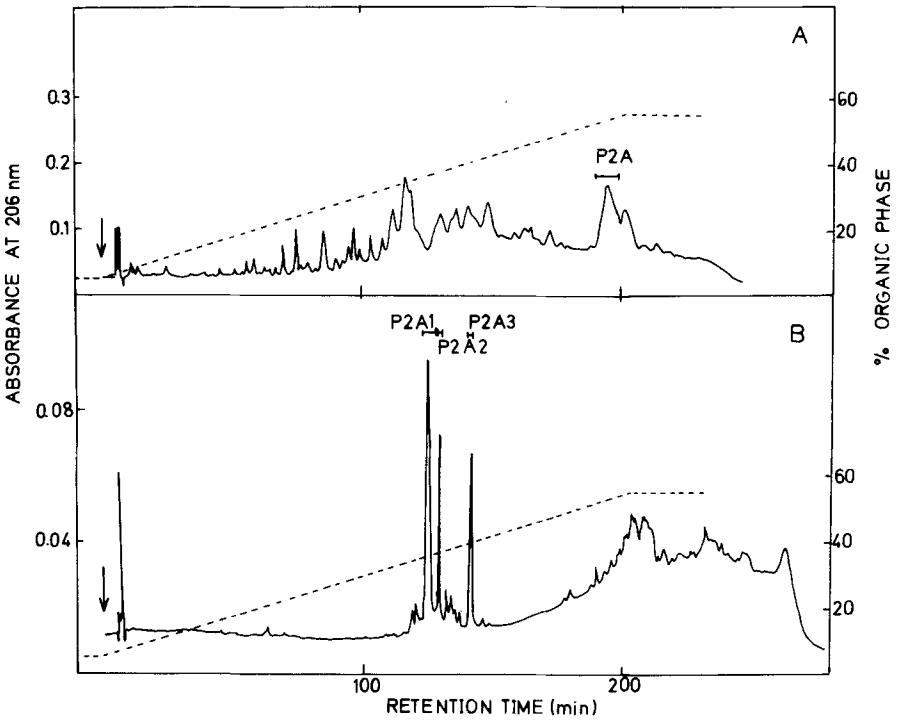

Fig.15.

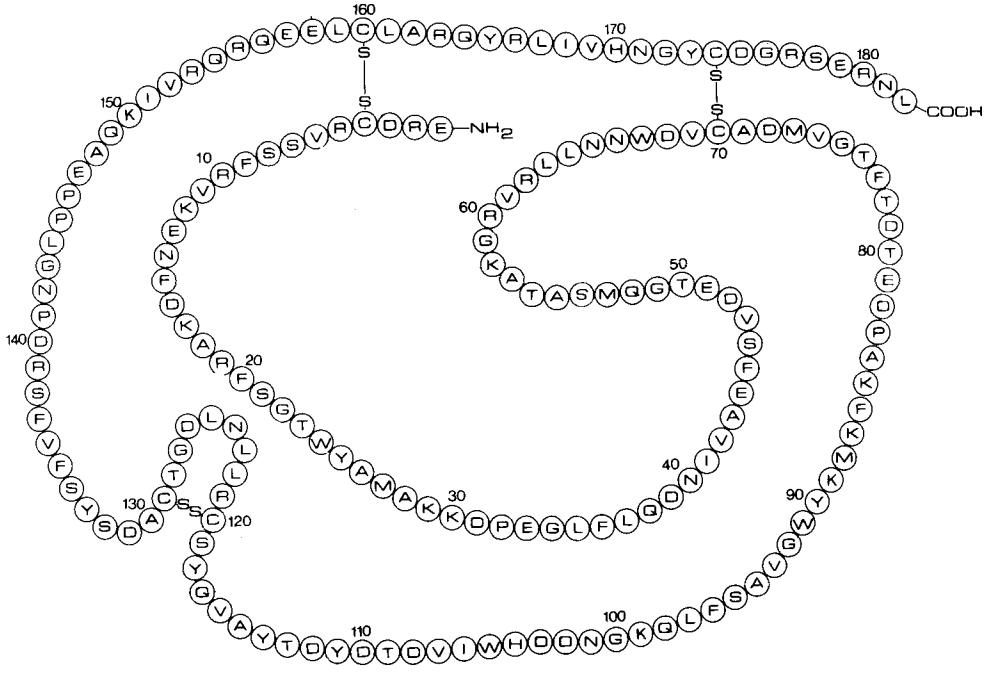

Fig.16.
The complete amino acid sequence of human RBP depicting the distribution of the three disulfide bonds.

Table 11. Amino acid composition[a] and amino acid sequence[b] of tryptic and peptic cysteine-containing RBP peptides

Peptide Designation	Amino acid composition	Yield[c] nmoles	Yield[c] %	Residue
A.				
Tryptic				
T1	$H_{1.0}$ $R_{1.3}$ $D_{5.6}$ $M_{0.6}$ [d] $G_{2.1}$ $A_{1.2}$ $C_{1.1}$ $V_{1.8}$ $I_{0.8}$ $L_{2.3}$ $Y_{1.0}$	520	21	
T1A	$H_{1.2}$ [f] $R_{1.2}$ $C_{1.1}$ [d] $D_{2.3}$ $G_{2.2}$ $V_{0.9}$ $I_{0.9}$ $L_{0.8}$ $Y_{0.8}$	250	10	167-177
T1B	$C_{1.0}$ [f] $D_{4.0}$ $M_{0.7}$ [d] $A_{1.2}$ $V_{1.0}$ $L_{2.2}$	350	14	63- 73
Peptic				
P1	$R_{1.0}$ $C_{1.8}$ [f] $D_{2.9}$ $T_{1.0}$ $S_{2.9}$ $G_{1.1}$ $A_{1.0}$ $L_{3.0}$ $Y_{1.5}$ $F_{0.7}$	390	16	118-135
P2	$R_{2.1}$ $C_{1.2}$ [e] $D_{1.2}$ $S_{1.9}$ $E_{1.0}$ $V_{1.0}$ $L_{2.2}$ $F_{1.1}$	440	18	
P2A1	$R_{1.9}$ $C_{1.0}$ [f] $D_{1.0}$ $S_{1.8}$ $E_{0.9}$ $V_{1.1}$ $F_{0.7}$	150	6	1-9
P2A2	$R_{2.1}$ $C_{0.9}$ [f] $D_{1.1}$ $S_{2.0}$ $E_{1.0}$ $V_{1.1}$ $F_{0.6}$	130	5	1-9
P2A3	$C_{1.0}$ $L_{1.9}$	200	8	159-161

	Amino acid Sequence
B.	
T1A	Leu-Ile-Val-His-Asn-Gly-Tyr-Tyr-CysA-Asp-Gly-
T1B	Leu-Leu-Asn-Asn-Trp-Asp-Val-CysA-Ala-
P1	Tyr-Ser-CysA-Arg-Leu-Leu-Asn-Leu-Asp-Gly-Thr-CysA-Ala-Asp-Ser-
P2A1	Glu-Arg-Asp-CysA-Arg-Val-Ser-
P2A2	Glu-Arg-Asp-CysA-Arg-Val-
P2A3	Leu-CysA-Leu

[a] All analyses are 24 h hydrolysis values. Tryptophan was not determined.
[b] All peptides except P2A3 were degraded in the automatic sequencer. Peptide P2A3 was analyzed by the manual dansyl-Edman method. Between 70 and 160 nanomoles of each peptide were subjected to the automatic sequencer. The repetitive yield varied between 87 and 94% for the different peptides. Of peptide P2A3 50 nanomoles were used for the amino acid sequence determination.
[c] Yields were not corrected for material taken for analyses during the isolation procedure.
[d] Determined as homoserine
[e] Determined as cysteine
[f] Determined as cysteic acid after performic acid oxidation

peptides. In contrast, these positions are in our sequence analyzed by NH_2-terminal degradation of several peptides. In all other positions identical residues were found although Kanda and Goodman could not unequivocally assign amides and acids in few positions. The sequence of human RBP predicted from a cDNA clone (5) agrees completely with the one described here, except in the COOH-terminus (see below).

The COOH-terminus of RBP has received particular attention in as much as two forms of RBP exist physiologically (34). They differ in their ability to interact with prealbumin. The non-bound form contains very little retinol, has a changed conformation (42) and is more acidic than the prealbumin-binding species (43). We previously reported that the more acidic form lacked arginine in its COOH-terminus (43). This erroneous information, which was obtained by carboxypeptidase B digestion, probably arose from the occurrence of trypsin-like activity present in the carboxypeptidase preparation. Two other laborato-

ries have also attempted to establish the COOH-terminal sequence of human RBP (12,55). In both cases data were obtained which do not agree with the present results. However, in the present study the COOH-terminal sequence was established not only by carboxypeptidase digestions but also by NH_2-terminal sequencing of peptidose whose amino acid compositions corroborated the results obtained.

Nevertheless, the amino acid sequence predicted from a cDNA clone encoding human RBP is one residue longer in the COOH-terminus than the determined protein sequence (5). Analysis of a cDNA clone for rat RBP also predicted an additional amino acid residue compared to the determined protein sequence of human and rabbit RBP (51). Neither the data on the rabbit nor on the human sequence support the presence of an additional residue of leucine as the COOH-terminus, although admittedly it is difficult with available sequencing techniques to distinguish the sequence -Asn-Leu-COOH from Asn-Leu-Leu. However, since the COOH-terminus of RBP is located at the surface and seems to be quite flexible (30), it is possible that the additional COOH-terminal leucine residue encoded by the RBP gene might be removed in a post-translation event.

The amino acid sequence of RBP was subjected to a computer search to investigate whether any of the previously sequenced proteins would display any structural homology to RBP (6,9). Three proteins, β-lactoglobulin (3), human α_1-microglobulin (11,25) and rat α_2-microglobulin (54) were found. The sequences of these proteins, which are of similar sizes, were aligned to that of human RBP by the computer program ALIGN (29). The alignment scores are shown in Table 12. As all values above 3 are regarded as significant this analysis clearly shows that all four protein sequences are related to each other. The same conclusion has been reached by two other laboratories (15,32). A closer look at the aligned sequences (Fig.17) shows that in eight instances only one type of amino acid residue occupies the same position in all four sequences. In another 20 positions only two alternative amino acids exist. It can accordingly be inferred that the four proteins belong to the same protein superfamily.

Three of the four proteins, RBP (28,36), the rodent α_2-microglobulin (44), and most probably human α_1-microglobulin (1) are produced in liver cells. Rodent α_2-microglobulin and, to a certain extent, RBP are under androgen control (10,45,49). The same might also hold true for human α_1-microglobulin (53). The synthesis of RBP and of rodent α_2-microglobulin is also influenced by glucocorticoids (2,4). Whether any physiological similarities exist between these three liver-produced proteins remains to be established as the molecular functions of rodent α_2-microglobulin and of human α_1-microglobulin are still unknown.

Table 12. Alignment scores for comparisons of human RBP with bovine β-lactoglobulin, human α_1-microglobulin and rat α_2-microglobulin

	Bovine β-lacto-globulin	Human α_1 micro-globulin	Rat α_2-micro-globulin
Human RBP (1-182)	5.97	8.21	5.18
Bovine β-lactoglobulin (1-162)		7.53	10.69
Human α_1-microglobulin (1-167)			9.35

The alignment scores, which were obtained with the use of the program ALIGN, represent the number of standard deviations of the real score above the random score. Numbers in parentheses: residue numbers compared. The sequnces of bovine β-lactoglobulin, human α_1-microglobulin, and rat α_2-microglobulin were taken from ref. 3, 25 and 54, respectively.

Fig.17.
Comparison of the amino acid sequence of human RBP with the sequence of bovine β-lactoglobulin (3), human α_1-microglobulin (1,44), and rat α_2-microglobulin (45). The alignment was obtained by maximizing the homology using the computer program ALIGN. Boxes: residues shared by the RBP sequence and any of the other sequences. Arrows: positions with a single amino acid residue shared by all the four proteins. Stars: positions with two amino acidresidue alternatives for the four sequences.

Bovine β-lactoglobulin has been reported to bind retinol in a similar way to RBP (14). Indeed, β-lactoglobulin has been suggested to function in binding, protecting and facilitating the uptake of retinol in the intestine of suckling young animals (16,32). The four proteins of the RBP superfamily might also have similar tertiary structures. Disulfide bonds homologous to that between

residues 75 and 180 in RBP are also found in β-lactoglobulin (26) and in human α-microglobulin (44) and a disulfide bound homologous to that between residues 125 and 134 in RBP is present in β-lactoglobulin. Moreover, recently the three-dimensional structures of both RBP (30) and β-lactoglobulin (46) were reported. The polypeptides folds of the two proteins are remarkably similar.

The computer analyses suggested that RBP might have arisen by an internal duplication of its primordial gene. Residues 36-83 and 96-141 of human RBP display statistically significant homology (6). A similar internal homology has been noted in β-lactoglobulin (22). This internal homology would suggest that the primordial gene for RBP once coded for a protein with a molecular weight of about 14,000. This is the molecular weight of the intracellular Retinol-binding protein (31) but the amino acid sequence of that protein is not homologous to that of serum RBP (39,50). However, piscine serum RBP which does not bind to prealbumin has a molecular weight of about 16,000 (47) and therefore the possibility was raised that the gene for serum RBP underwent a partial duplication after the divergence of fish and mammals. The three-dimensional structure of RBP is also consistent with a partial duplication event. However, the retinol binding site is formed by side-chains from both putative duplicated portions (30). It is therefore probable that the two homologous portions found in mammalian RBP also are present in piscine RBP, assuming that the site for retinol has been conserved. Moreover, the exon-intron organization and the nucleotide sequence of the rat RBP gene (24) did not show any obvious similarities between the portions of the gene encoding residues 36-83 and 96-141.

These data together with the similarities in three-dimensional structure between RBP and β-lactoglobulin suggest that if a partial duplication has been involved in the evolution of RBP, it occurred before the divergence of RBP from β-lactoglobulin and the other proteins in the RBP superfamily.

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