CARBOXY-TERMINAL SEQUENCE OF RETINOL-BINDING PROTEIN FROM HUMAN PLASMA

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Received 1 August 1972

1. Introduction

Retinol-binding protein (RBP) has been isolated by several groups from human blood plasma [1–3] and from urine [4, 5] and its amino acid composition determined. The number of certain amino acids/mole protein (e.g. alanine, glycine, leucine and valine) obtained however by Peterson et al. [2, 4] mainly on urinary RBP were slightly but consistently less by at least one unit each than the values obtained by Kanai et al. [1] and by Kirby [6] in our Laboratory. In view of the ease by which native plasma RBP can be transformed during purification into derivatives with higher electrophoretic mobility, the possibility cannot be excluded that some terminal amino acids or a peptide might be lost in addition to the more easily removed amide groups. Again, in the case of RBP isolated from urine, some metabolic loss might have occurred during the passage of the plasma RBP through the kidney.

The amino-terminal sequence of human plasma RBP has been determined [7] and glutamate was identified as the N-terminal amino acid. This has been confirmed [5]. The authors also find the same N-terminal acid in their preparations, consequently the differences noted in overall composition do not occur at that end of the molecule. Rask et al. [5] following up a previous observation on the difference in amino acid composition between their holo-RBP molecule and an "apo"-RBP of similar molecular weight determined the carboxy-terminal amino acid sequences of each protein. They found arginine as the C-terminal acid in holo-RBP with lysine next to it, whereas lysine was the C-terminal acid in the "apo"-protein. Glutamic acid and serine were next in the sequence in both proteins. These findings are completely different from our findings on human plasma RBP. The carboxy-terminal sequence of the latter has now been re-examined on several preparations and the results below indicate that RBP obtained from urine has possibly lost a peptide compared with plasma RBP either in passage through the kidney or artifactually during isolation and purification.

2. Experimental

2.1. *Materials*

RBP was isolated from human plasma by a similar procedure to that previously described [1] in which the chromatography stage on DEAE-Sephadex was replaced by that on arginine-Sepharose [3]. The purified RBP moved as a single zone in the analytical ultracentrifuge and on a Sephadex G-100 column corresponding to a protein with M.W. approx. 22,000. Disc-gel electrophoresis of the preparation in Tris buffer on 6.5% polyacrylamide at pH 8.5 showed that the preparation contained about 96% holo-RBP and the remainder apo-RBP. This was confirmed by spectroscopic analysis which showed that the ratio of the extinction values for retinol chromophore at 329 nm to protein at 278 nm was 0.92. This corresponds closely to a ratio of 1 mole retinol to 1 mole protein.

Carboxypeptidases A (EC 3.4.2.1) and B (EC 3.4.2.2) (Coded COADFP and COBC, respectively) were obtained from Worthington. All other chemicals were of AnalaR quality. Double distilled water was used in preparing all solutions.

Table 1
Long term digestions of RBP with carboxypeptidases A and B.

Exp.	I			
Time (hr):	0.5	1	4	8
Amino acid	(Moles amino acid /mole RBP)			
Asp	<0.05	0.21	0.21	0.23
Thr	<0.05	0.11	0.10	0.10
Ser	0.90	0.96	1.00	1.00
Gly	0.49	0.42	0.40	0.39
Ala	0.34	0.34	0.32	0.55
Val	0.15	0.17	0.17	0.23
Ile	0.09	0.14	0.12	–
Leu	0.92	0.93	0.92	0.94
Tyr	0.24	0.22	0.18	–
Phe	<0.05	0.10	0.05	
Lys	0.13	0.14	–	0.29
Arg	0.10	0.17	–	0.37

RBP (2.0×10^{-8} M soln.) was incubated at 37° with a mixture of carboxypeptidase A (0.05 μmole/μmole RBP) and carboxypeptidase B (5 EC units/μmole RBP) in 0.2 M *N*-ethylmorpholine acetate containing 6 M urea at pH 8.6.

2.2. *Methods*

5.5 mg samples (0.25 μmole) of RBP were alkylated at pH 8.6 following reduction of the dithio-links with a 100-fold excess of mercaptoethanol over 24 hr in the presence of 6 M guanidine HCl. The liberated thiol groups were then treated with iodoacetamide (equimolar to mercaptoethanol) for 5 hr in the dark. Enzymic digestions were carried out according to the procedure described by Ambler [8] using either mixtures of both enzymes or carboxypeptidase A alone at 37° for various intervals up to 8 hr. The levels of enzyme used varied from 1/40 to 1/20 the molar concentration of RBP in the case of carboxypeptidase A and from 5–10 EC units/μmole RBP for carboxypeptidase B. The digestions were stopped by acidifying with acetic acid to pH 3.5 and freezing the samples in acetone–solid CO_2 mixture prior to concentrating by freeze drying. The free amino acids were determined on a dual column Eel 193 Amino acid analyser by the standard procedure [9].

3. Results and discussion

In exp. 1, RBP was incubated with a mixture of both enzymes for periods up to 8 hr and the amounts of amino acids released in moles/mole protein are given in table 1. Leucine and serine are liberated in greatest amounts at all stages, followed by glycine, alanine and tyrosine in decreasing order. Then at slightly lower concentrations there is another group containing lysine, arginine, valine and aspartic acid. Finally, other amino

Table 2
Short term digestions of RBP with carboxypeptidases A and B.

Exp.	II			III		IV	
Time (min):	5	15	30	10	30	24	40
Amino acid	(Moles amino acid/mole RBP)						
Ser	0.61	0.91	0.96	0.23	0.30	0.24	0.36
Gly	0.19	0.46	0.43	0.20	0.32	0.25	0.21
Ala	0.17	0.30	0.32	0.15	0.29	0.10	0.20
Val	0.15	0.20	0.25	n.d.*	n.d.	n.d.	n.d.
Leu	0.57	0.88	0.90	1.00	1.00	0.92	0.88
Tyr	0.21	0.29	0.29	tr	tr	tr	–
Lys	0.15	0.30	–	n.d.	n.d.	n.d.	n.d.
Arg	0.13	0.32	0.33	n.d.	n.d.	n.d.	n.d.

Identical conditions to those described in table 1 were used in exp. II. In exp. III, 2.7×10^{-8} M RBP was incubated with CPA (0.05 μmole/μmole RBP) and CPB (10 EC units/μmole RBP) and in exp. IV, 1.5×10^{-8} M RBP was used with half the concentrations of enzymes as those used in exp. I and II. * n.d.: not detected.

acids released in smallest amounts are threonine, isoleucine and phenylalanine.

Additional experiments were then carried out using shorter incubation periods so that the relative positions of the more rapidly released amino acids could be determined. The results obtained in exps. II–IV are set out in table 2 and clearly show that leucine occupies the COOH-terminal position with serine next to it. The remaining acids in decreasing order of the amounts detected appear to be glycine, alanine and tyrosine as in exp. I. The basic amino acids arginine and lysine are found in comparable amounts with tyrosine and all slightly higher than valine. Tyrosine, however, is placed nearer the C-terminal end of RBP than arginine and lysine because the amount released is always greater at the shortest time intervals of incubation. The lower levels of tyrosine at the longer time intervals probably reflect some oxidative destruction of this labile amino acid during the run.

Thus, both the long and short term incubation results indicate that the COOH-terminal sequence of the above preparations of RBP is: NH_2-Glu- - - -Lys–Arg–Tyr–Ala–Gly–Ser–Leu-COOH. The order of the remaining amino acids is difficult to determine on account of experimental errors of the technique but valine, threonine, phenylalanine, isoleucine and aspartic acid seem to be among the next group to be released. The significantly higher content of serine than that of leucine after several hours incubation in exp. I also indicates that a second serine was being released in these later stages. In two further experiments V and VI, 1.5×10^{-8} M RBP solution was incubated with carboxypeptidase A alone. Again, leucine was found to be the COOH-terminal acid with serine, glycine, alanine and tyrosine detected in decreasing order of amounts released, so confirming the earlier observations. These experiments also distinguish the above preparations of RBP from those isolated by Rask et al. [5] which are not attacked by this enzyme alone because they terminate in the basic amino acid, arginine [8].

The marked difference between the latter preparations and ours is difficult to understand except on the basis that possibly the RBP from urine may have lost a peptide by metabolism in passing through the kidney. However, the amino acid composition of the RBP obtained by Peterson from plasma [2] is similar to that for their RBP from urine, so an alternative explanation is more likely. It is perhaps possible that a peptide is lost artifactually during isolation and purification. It is noteworthy that the method used by Peterson and Berggard [4] for isolating RBP from urine involves several stages of ultrafiltration through Visking dialysis tubing. Now this type of procedure has been shown by Di Prisco and Strecker [10] to inactivate enzymes by causing some degradation of the peptide chain even when the Visking tubing is carefully washed with chelating agents. The reason for this loss of amino acids from proteins has not yet been determined but it was found to be more severe during ultrafiltration than in dialysis. Perhaps a similar phenomenon occurred during the isolation of urinary RBP [4, 5].

In our isolation procedure, Visking tubing is not used except for a preliminary short term dialysis of whole plasma. However, in order to determine if dialysis in Visking had any effect on the COOH-terminal amino acid of RBP, a pure preparation was dialysed for 91 hr in Visking tubing before examination. The same terminal sequence commencing with leucine was found as obtained formerly, indicating that dialysis had not affected the peptide bonds.

It is difficult to assess without fuller information on the amino acid sequence whether arginine and lysine at positions n-5 and n-6 respectively in our preparation are the same residues which appear at n and n-1 respectively in the RBP isolated from urine [5]. The difference in the estimated total number of residues in the latter preparation 177 to 183 [4, 5] compared with the total for plasma RBP of 189–197 [1] of 193–197 [6] seems greater than can be explained by the loss of only 5 residues.

Acknowledgements

The authors thank the Medical Research Council for a generous grant in aid of the work and G.H.W. thanks the Science Research Council for a Research Studentship.

References

[1] M. Kanai, A. Raz and D.S. Goodman, J. Clin. Invest. 47 (1968) 2025.
[2] P.A. Peterson, J. Biol. Chem. 246 (1971) 34.

[3] W. Kirby, G.H. White and J. Glover, Biochem. J. 123 (1971) 31P.
[4] P.A. Peterson and I. Berggard, J. Biol. Chem. 246 (1971) 25.
[5] L. Rask, A. Vahlquist and P.A. Peterson, J. Biol. Chem. 246 (1971) 6638.
[6] W. Kirby, Ph.D. Thesis, University of Liverpool (1971).
[7] F.J. Morgan, R.E. Canfield and D.S. Goodman, Biochim. Biophys. Acta 236 (1971) 798.
[8] R.P. Ambler, Methods in Enzymology 11 (1967) 155.
[9] D.H.W. Spackman, W.H. Stein and S. Moore, Anal. Chem. 30 (1959) 1190.
[10] G. Di Prisco and Strecker, H.J. FEBS Letters 6 (1970) 89.