ISOLATION AND CHARACTERIZATION OF THE CYANOGEN BROMIDE FRAGMENTS FROM HUMAN LACTOTRANSFERRIN

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1. Introduction

Lactotransferrin (LTF) from human milk [1–5] is a glycoprotein (mol. wt. 76 700) which consists of a single polypeptide chain [6–7] to which two identical oligosaccharides are conjugated by *N*-glycosidic linkages [8]. We have undertaken the study of the structure of this glycoprotein in order, firstly, to localize the two glycans and the two iron binding sites on the polypeptide chain and secondly, to compare, both structures of sero- and lactotransferrins. In the present paper we describe the splitting of the lactotransferrin by CNBr treatment, the isolation, purification and some physico-chemical characterization of the CNBr-fragments. By these results the way to the determination of the primary structure of lactotransferrin can be considered as open.

2. Materials and methods

Pure iron free human lactotransferrin was prepared by methods previously described [5]. Cleavage at the methionyl residues of apolactotransferrin was effected by treating the glycoprotein with a 100-fold molar excess of CNBr in 70 p. 100 formic acid, for 24 hr at room temperature [9]. After incubation, the sample was diluted 10-fold with cold distilled water and the excess of reagents was removed by lyophilisation. The product obtained was applied to separation on a column (5 × 100 cm) of Bio-Gel P-100. The equilibration of the column as well as the elution of the peptides were carried out by 0.1 N formic acid. A second fractionation was performed with a DEAE-Sephadex A-50 column (2 × 30 cm) equilibrated with 4 M urea in 0.004 M Tris–HCl buffer pH 7.8. A continuous pH 7.8 gradient from 0.004 M to 0.3 M Tris–HCl in 4 M urea was applied and 0.5 M Tris–HCl pH 7.2 in 4 M urea was used for the final elution. Each polypeptide fraction collected from the DEAE-Sephadex A-50 column purified by gel filtration on Bio-Gel P-60 (2 × 120 cm) columns or on Sephadex G-50 columns (1.5 × 80 cm) equilibrated with 0.1 M ammonium bicarbonate buffer pH 8.2. Performic acid oxidation was carried out according to Hirs [10] and acylation procedure, involving maleic anhydride treatment, by the method of Itano and Gottlieb [11].

Amino acid composition of the polypeptides was determined in presence of 2-mercaptoethanol according to Keutmann et al. [12]. N-terminal amino acids were identified by dansylation [13,14] and C-terminal by hydrazinolysis [15]. Neutral sugars and hexosamines were estimated by gas-liquid chromatography after methanolysis and trifluoroacetylation [16]. Molecular weights were determined by three different methods: i) equilibrium sedimentation [17] in a 6 M guanidine hydrochloride–0.1 M Tris–HCl solution adjusted to pH 7 (partial specific volumes were calculated according to Cohn and Edsall [18] for the amino acids and according to Squire et al. [19] for the monosaccharides); ii) gel filtration of the maleylated polypeptides on Sephadex G-50 column (1 × 80 cm) equilibrated with 0.1 M ammonium bicarbonate buffer; iii) polyacrylamide gel electrophoresis [20].

3. Results

3.1. *Preparation of CNBr peptides*

The chromatography of the CNBr peptidic fragments on Bio-Gel P-100 gave four fractions (A, B, C and D) which were collected monitored by the extinction at E_{280} (fig. 1). In fractions A and B, aggregated and uncleaved materials were identified. Fraction D is a low molecular weight and homogenous CNBr-fragment of lactotransferrin (F-VII fragment). Fraction C was recovered and, after splitting of the disulphide bridges by performic acid oxidation, was subjected to chromatography on DEAE-Sephadex A-50 column. Three fractions (C-I, C-II and C-III) were separated (fig. 2) under the above conditions. Fraction C-I was purified, after maleylation, by Bio-Gel P-60 chromatography in order to eliminate aggregates and contamination of C-II. The pure fragment obtained is called F-III. Fraction C-II was submitted to gel filtration on Sephadex G-50 giving three subfractions ($C\text{-}II_a$; $C\text{-}II_b$; $C\text{-}II_c$). Subfraction $C\text{-}II_c$ is homogenous and called F-VI fragment. Subfractions $C\text{-}II_a$ and $C\text{-}II_b$ were re-chromatographied under the same conditions resulting in homogenous fragments F-VI and F-V, respectively. Fraction C-III was resolved by Bio-Gel P-60 chromatography in two subfractions which were rechromatographied leading to the homogeneous fragments F-I and F-II.

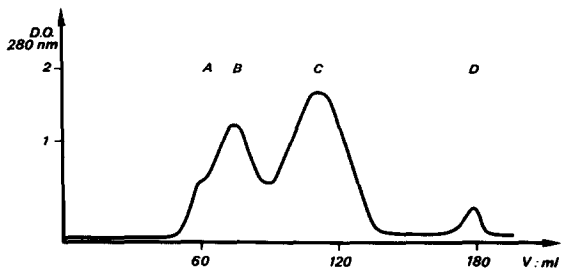

Fig. 1. Separation of CNBr fragments of human lactotransferrin on Bio-Gel P-100 (see Materials and methods).

3.2. *Characteristics of the CNBr peptides*

The amino acid and carbohydrate compositions of the seven fragments (F-I to F-VII) are described in table I. Carbohydrates were found only in the F-I and F-II fragments. The monosaccharide molecular ratios are in good agreement with those of native lactotransferrin except the loss of one sialic acid residue.

A single N-terminal group was found for each fragment from F-II to F-VII. F-I fragment, as the native lactotransferrin, failed to give N-terminal group. This result suggests that this fragment corresponds to the N-terminal sequence of the polypeptide chain of lactotransferrin. Homoserine and homoserine lactone were identified after hydrazinolysis in all CNBr-fragments except in the F-VI fragment which corresponds to the C-terminal sequence of lactotransferrin.

The molecular weights of the 7 CNBr-fragments are summarized in table I.

4. Discussion

Isolation and characterization of seven peptides as products of CNBr splitting of lactotransferrin agree

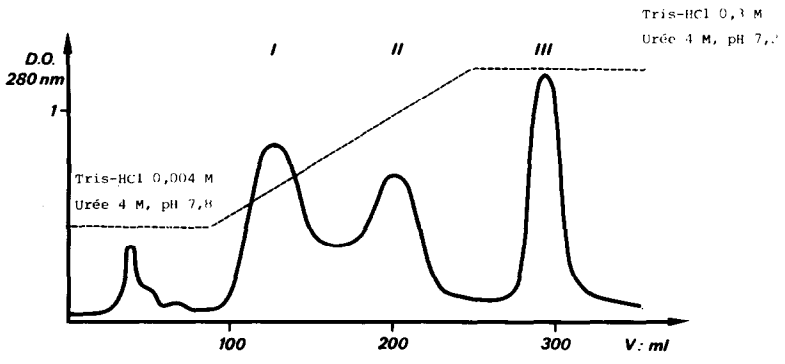

Fig. 2. Chromatography on DEAE-Sephadex A-50 of the C fraction (see fig. 1 and Materials and methods).

Table 1
Carbohydrate and amino acids molar compositions of the native and CNBr treated lactotransferrin (LTF) and of the 7 fragments isolated after CNBr treatment

Composition	Native LTF* [5]	CNBr-treated LTF	Fragments F-I	F-II	F-III	F-IV	F-V	F-VI	F-VII	Total
Amino acids										
$CySO_3H$	–	–	8	8	6	5	3	2		32
Asp	71	70	18	20	14	12	5	4	2	75
Thr	30	29	8	5	8	6	3	2	1	33
Ser	54	46	15	10	15	7	5	3	2	57
Glu	62	66	20	13	11	7	5	4	1	61
Pro	26	27	8	4	5	4	3	1	–	25
Gly	53	51	16	11	15	7	5	3	1	58
Ala	59	60	19	13	10	8	5	3	2	60
1/2 Cys	26	26	–	–	–	–	–	–	–	–
Val	37	35	9	5	3	7	2	1	1	28
Met	6	–	–	–	–	–	–	–	–	–
Ile	14	12	5	2	2	1	1	1	–	12
Leu	54	54	15	11	12	8	4	3	1	54
Tyr	21	20	4	3	3	3	1	1	–	15
Phe	32	31	12	8	7	3	2	2	–	34
Lys	41	41	12	8	8	7	3	2	1	41
His	8	8	2	1	3	1	0	1	1	9
Arg	40	41	13	8	5	5	2	1	1	36
HSer	–	6	1	1	1	1	1	–	1	6
Try	13**	ND***	ND	ND	ND	ND	ND	ND	ND	ND
Total	647	623	185	131	128	92	50	35	15	636
Carbohydrates										
Galactose	4		2	2	–	–	–	–	–	
Mannose	6		3	3	–	–	–	–	–	
Fucose	2		1	1	–	–	–	–	–	
N-acetyl-glucosamine	8		4	4	–	–	–	–	–	
N-acetyl-neuraminic acid	3		1	1	–	–	–	–	–	
N-terminal	None	None	None	Gly	Asp	Ser	Phe	Leu	Ala	
Mol. wt.										
Equilibrium sedimentation	76 700		24 000	17 000	–	10 000	–	–	–	
Disc electrophoresis	77 000		25 000	18 000	13 000	–	–	–	–	
Gel filtration	76 000		–	–	15 000	–	5 500	3 500	–	
Calculated	–		23 200	17 034	13 930	10 039	5 629	3 669	1 468	74 969

* The number of serine and threonine were extrapoled to zero time, while that of leucine and isoleucine were determined after 70 hr hydrolysis.
** Edelhoch method [21].
*** ND: not determined.

with the presence of six methionine residues. As shown in table I the sum of the molecular weights of these fragments coincides well with that of the native lactotransferrin. Since the presence of free homoserine or homoserine lactone was not detected after CNBr cleavage we could eliminate the possibility of Met–Met sequences.

The determination of N-terminal group as well as the disc electrophoresis of the 7 peptides refer to their homogeneity. Isolation of both glycopeptides F-I and F-II different in their amino acids composition confirms the presence in the lactotransferrin of 2 carbohydrate moieties [8]. F-I fragment comes from the N-terminal sequence of lactotransferrin. F-VI fragment is related to the C-terminal sequence.

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