Human protein HC displays variability in its carboxyl-terminal amino acid sequence

C. Lòpez, A. Grubb+ and E. Méndez

Servicio de Endocrinologia, Centro Ramon y Cajal, Carretera de Colmenar, Km 9100, Madrid 34, Spain and *Department of Clinical Chemistry, University of Lund, Malmo General Hospital, S-214 Ol Malmo, Sweden*

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C-Terminal CNBr fragment of protein HC

1. INTRODUCTION

Protein HC (human complex-forming glycoprotein, heterogeneous in charge) is a glycoprotein present in human biological fluids [1]. It carries yellow-brown chromophores and forms complexes with IgA and albumin to a considerable extent [1]. Although it gives a single band on SDS–polyacrylamide gel electrophoresis, it shows an extensive charge heterogeneity on isoelectric focussing and on agarose gel electrophoresis which does not decrease after desialylation [1]. The complete amino acid sequence of protein HC isolated from the urine of an individual was reported in [2]; no evidence for sequence variability of the single polypeptide chain of protein HC was found [3]. This work was undertaken to investigate if the polypeptide chain of protein HC isolated from a pool of urine from several individuals displayed any variability. The polypeptide chain of the protein HC (pool) showed a unique NH_2-terminal amino acid sequence up to residue 35, which is identical to the one reported for protein HC (single). However, two different C-terminal CNBr fragments were isolated from protein HC (pool). One had the same C-terminal amino acid sequence as reported for protein HC (single) and the other as reported for α_1-microglobulin [4], a protein closely related to protein HC.

2. MATERIALS AND METHODS

Protein HC was isolated as in [1] from the urine of an individual (J.L.) and from a pool of urine from several individuals. The 2 protein HC preparations are referred to as protein HC (single) and protein HC (pool), respectively. CN7 is the C-terminal CNBr fragment isolated from protein HC (single) as in [3]. Complete reduction and *S*-carboxymethylation of the protein preparations was performed in the presence of 6 M guanidinium–HCl [1]. Hydrolyses were carried out at 110°C for 20 h using 200 μl 5.7 M HCl with 0.02% 2-mercaptoethanol. The analyses were performed using a Beckman 121-MB analyzer. Automated amino acid sequence determinations by the procedure in [5] were performed on a Beckman 890 C Sequencer using the program in [6]. The phenylthiohydantoin amino acids were identified by high-performance liquid chromatography and in some instances by amino acid analysis after back hydrolysis [7]. Reduced and [^{14}C]alkylated protein HC was dissolved in 70% (v/v) formic acid to 20 mg/ml and treated with CNBr with a protein–CNBr ratio of 1:25 (w/w) for 24 h at room temperature [8]. Protein HC (150 μg) or 5 nmol CNBr fragments thereof was used for each of the following carboxypeptidase treatments. Digestion with carboxypeptidase A was carried out in 0.2 M *N*-methylmorpholine acetate buffer (pH 8.2), for 5 h at 37°C at an enzyme–substrate ratio of 1:10 (w/w) [9]. Digestion with carboxypeptidase B was performed in 0.2 M *N*-methylmorpholine acetate buffer (pH 8.2) for 16 h at 37°C at an enzyme

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–substrate ratio of 1:200 (w/w) [10]. Digestion with carboxypeptidase Y was carried out in 0.1 M pyridine acetate buffer (pH 5.5) for 1–3 min at 37°C at an enzyme–substrate ratio of 1:200 (w/w) [11]. Glucosamine and galactosamine were determined using the amino acid analyzer after hydrolysis of samples in 4 M HCl at 110°C for 2, 4, 6 and 20 h. Sialic acid was determined as in [1].

Table 1

N-terminal amino acid sequence of *S*-carboxymethylated protein HC (pool) (150 nmol)

Cycle	Residue	PTH (nmol)
1	G	60.1
2	P	37.6
3	V	76.5
4	P	38.5
5	T	11.4
6	P	47.2
7	P	50.2
8	D	23.7
9	N	17.9
10	I	16.9
11	Q	9.5
12	V	14.1
13	Q	13.1
14	E	15.7
15	N	12.2
16	F	14.6
17	N	2.4
18	I	16.5
19	S	6.2
20	R	9.3
21	I	12.6
22	Y	9.7
23	G	10.4
24	K	9.4
25	W	8.2
26	Y	6.9
27	N	5.4
28	L	5.8
29	A	5.3
30	I	4.9
31	G	4.8
32	S	1.2
33	T	1.4
34	C	1.6
35	P	0.6

3. RESULTS AND DISCUSSION

Thirty-five automatic Edman degradation cycles of the intact [^{14}C]carboxymethylated protein HC (pool) released one single amino acid residue at each step (table 1). Residue 34 was identified as carboxymethylcysteine, both by high-performance liquid chromatography and radioactivity measurements. The amino acid sequence was identical to the N-terminal one of protein HC (single) as in [2,3].

When protein HC (pool) was digested with carboxypeptidase B the only amino acid released was arginine (0.2 mol Arg/mol protein). But when protein HC (pool) was digested with carboxypeptidase A the only amino acid released was isoleucine (0.3 mol Ile/mol protein). On the other hand, when protein HC (single) was subjected to identical carboxypeptidase treatments the only amino acid released was arginine (0.4 mol Arg/mol protein), table 2.

To demonstrate a possible heterogeneity of the C-terminal end of protein HC (pool) its C-terminal CNBr fragments were isolated; 10 mg reduced and [^{14}C]-carboxymethylated protein HC (pool) was treated with CNBr and chromatographed on a column of Sephacryl S-200 (fig.1A). Pool 7 was re-

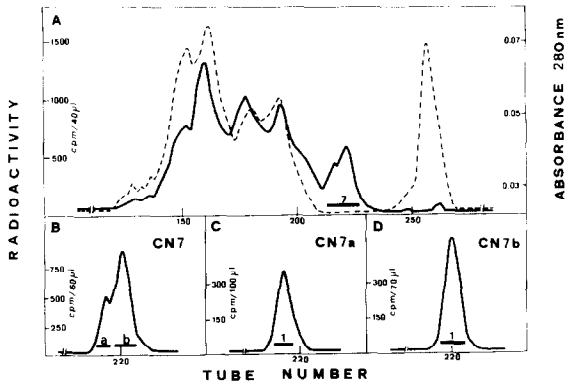

Fig.1. Isolation of the C-terminal CNBr fragments from protein HC (pool). (A) Fractionation of CNBr fragments from the reduced and [^{14}C]carboxymethylated protein on a column of Sephacryl S-200 (2 × 200 cm) in 10% (v/v) formic acid. The flow rate was 18 ml/h and 2.0 ml fractions were collected. (B) Rechromatography of pool CN7 on the same column. (C,D) Rechromatography of pools CN7a and CN7b on the same column.
(—) Radioactivity; (- - -) A_{280}.

Table 2

Amino acid and carbohydrate composition of protein HC (single[b] and pool[a]) and their C-terminal CNBr fragments

Amino acid	Protein HC[a]	CN7a1[a]	CN7b1[a]	Protein HC[b]	CN7[b,c]
Lys	10.0			10.0	
His	3.5			3.8	
Arg	8.6	1.5	0.8	9.1	1.6
CMCys	2.8	0.5	0.3	2.8	0.5
Asp	14.1	1.2	1.1	14.9	1.0
Thr	14.4			14.8	
Ser	9.2			10.5	
Glu	22.7	4.3	4.5	22.0	4.7
Pro	10.6	3.3	2.7	11.7	3.9
Gly	12.0	1.9	1.9	12.8	2.0
Ala	10.3	1.0	1.0	9.3	1.0
Val	10.6	1.0	1.0	10.1	1.3
Met	3.0			4.7	
Ile	10.7	1.6	1.0	12.7	1.5
Leu	11.3	1.0		11.9	1.0
Tyr	7.4			8.3	
Phe	6.1			7.8	
Trp	1.5			1.4	
CpA[d]	Ile	–	Ile	–	–
CpB[d]	Arg	Arg	–	Arg	Arg
Sialic acid (%)	4.3			4.5	
Hexoses (%)	12.0			11.0	

[c] Data was taken from [3]
[d] Amino acid released by carboxypeptidase A (CpA) and carboxypeptidase B (CpB)

chromatographed on the same column (fig.1B) and the radioactive peaks CN7a and CN7b were further purified on the same column (fig.1C,D). Fractions containing peaks CN7a1 and CN7b1 were pooled and lyophilized. Amino acid analysis of these 2 peptides showed that they were completely devoid of homoserine (table 2). The composition of CN7a1, is identical to the C-terminal CNBr fragment CN7, from protein HC (single) (table 2). Both fragments CN7a1 and CN7b1 were also devoid of carbohydrates.

The peptide CN7a1 had alanine as its N-terminal residue and its C-terminal amino acid was arginine, as deduced from digestions with carboxypeptidase B (0.4 mol Arg/mol peptide) table 2. The C-terminal sequence of CN7a1 was not clearly determined by CPY digestion, due to the rapid release of the last 4 residues, which could not be controlled by decreasing the enzyme concentration or by shortening the incubation time. The same residues were released simultaneously when the C-terminal CNBr fragment CN7, from protein HC (single) was treated with carboxypeptidase B (table 2) and with carboxypeptidase Y (table 3). CN7b1 had alanine as N-terminal residue and the C-terminal amino acid sequence –Pro–Ile as deduced from digestions with carboxypeptidase A (0.3 mol Ile/mol peptide) and Y (tables 2,3). The 2 isolated different CNBr

Table 3

Amino acids released from CN7al and CN7bl (pool) and CN7 (single) by carboxypeptidase Y

	1 min	3 min
CN7bl	Ile (1.0) Pro (0.5)[a]	Ile (1.0) Pro (0.7)
CN7al	Arg (1.0) Pro (1.0) Ile (1.2) Leu (1.0)	Arg (1.0) Pro (1.5) Ile (1.9) Leu (1.0)
CN7	Arg (1.0) Pro (1.0) Ile (1.1) Leu (1.0)	Arg (1.0) Pro (1.6) Ile (2.0) Leu (1.0)

[a]mol/mol peptide

fragments show by their N-terminal amino acids, their compositions and their C-terminal amino acids that they correspond to different regions of the C-terminal part of the polypeptide chain of protein HC isolated from the urine of a single individual [2,3]. Peptide CN7bl corresponds to residues 162–177 of protein HC (single) and CN7al corresponds to residues 162–181 which represents the C-terminal end of protein HC (single) (fig.2).

The present observations demonstrate that protein HC isolated from a pool of urine from several individuals contains ≥2 molecules with different C-terminal amino acid sequences (fig.2). Although it can be questioned whether they differ in other areas too, these observations are not conclusive but argue against this possibility, since no heterogeneity in the N-terminal amino acid sequence was found and since the amino acid composition (table 2) of protein HC (pool) was very similar to the one of protein HC (single) in which the sequence shows no variability [2].

The molar concentration ratio of the 2 protein HC types from pooled urine can be estimated to ~35:65 (molecule with C-terminal tetrapeptide: molecule without) from radioactivity measurements of the 2 isolated CNBr fragments. Variations in this ratio could explain some of the reported small differences in amino acid composition of protein HC isolated from various pools of urines and from urines of healthy and diseased persons [1, 12–16]. α_1-Microglobulin and α_1-microglycoprotein are 2 human proteins which are closely related to protein HC [13–16]. The amino acid sequence of α_1-microglobulin isolated from pooled urine [4] showed extensive sequence identities with protein HC isolated from an individual but also differences [2]. One of the main differences was the lack in α_1-microglobulin of 29–36 octapeptide present in protein HC (single) [2–4]. We demonstrate here that these residues, including the cysteine at residue 34 are also present in protein HC (pool) table 1 and fig.3. Another difference was the lack in α_1-microglobulin of the C-terminal tetrapeptide of protein HC [2–4]. It is therefore of interest that the C-terminal CNBr fragment of α_1-microglobulin probably is identical with the shorter of the 2 different C-terminal CNBr fragments isolated in this work from protein HC (pool) (fig.2). Whether the 2 types of protein HC described here and the sequenced α_1-microglobulin preparation constitute 3

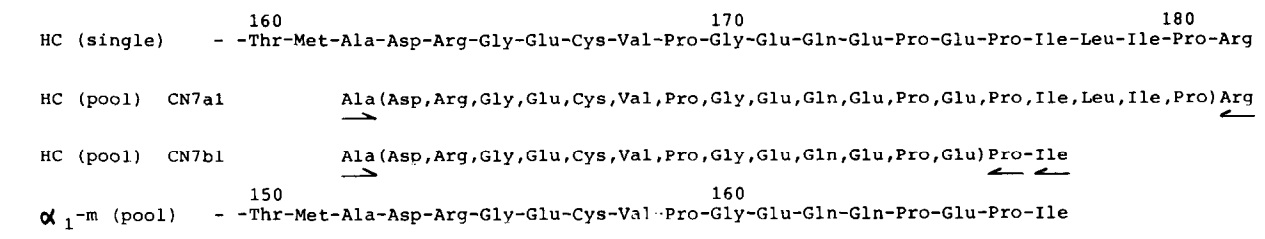

Fig.2. Localization of CN7al and CN7bl from protein HC (pool) in the C-terminal region of protein HC (single) and α_1-microglobulin.

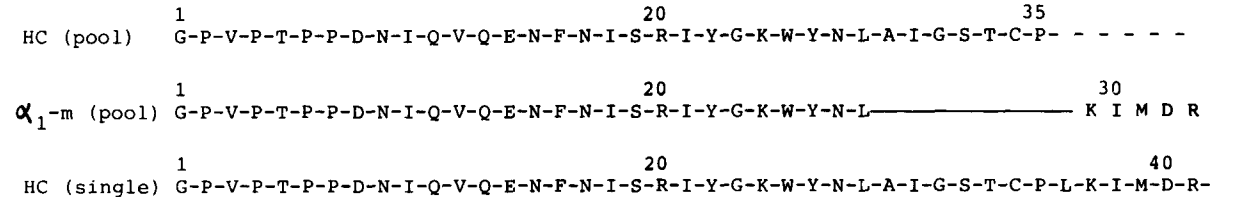

Fig.3. Comparison between the N-terminal amino acid sequence of protein HC (pool) and that reported for protein HC (single) [2] and for α_1-microglobulin, α_1-m (pool) [4].

individual molecular species of a greater population of closely related molecules remains to be investigated. So does the question of whether the 2 forms of protein HC are both secreted from protein HC-producing cells or whether one is formed by extracellular proteolysis of the other.

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