Discovery of a γ-carboxyglutamic acid-containing protein in human spermatozoa

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Here we describe the identification of a γ-carboxyglutamic acid-containing protein in human spermatozoa. After thermal decarboxylation the protein is a good substrate for vitamin K-dependent carboxylase from various origins. A quick purification procedure for the decarboxylated protein is presented and in a preliminary characterization we have established its M_r (28000–30000) and its amino acid composition.

Vitamin K *Carboxylase* *Warfarin* *Sperm* *γ-Carboxyglutamic acid*

1. INTRODUCTION

Vitamin K-dependent carboxylase has been demonstrated in liver as well as in a number of non-hepatic tissues [1,2]. The enzyme system is involved in the conversion of glutamic acid (Glu) residues into γ-carboxyglutamic acid (Gla) residues and hence carboxylase-containing tissues are expected to be involved in the synthesis of Gla-containing proteins. Whereas the hepatic Gla-containing proteins have been well identified [3], the nature of the non-hepatic ones is less clear. Since relatively high amounts of carboxylase were found in testes, it seemed plausible that one or more Gla-containing proteins are present in sperm. Indeed we have found such a protein and its purification and preliminary characterization are reported in this paper.

2. MATERIALS AND METHODS

2.1. *Chemicals*

Vitamin K_1 was obtained from Hoffmann-La Roche and vitamin K hydroquinone was prepared as described [4]. Dithiothreitol benzamidine and 3-([3-cholamidopropyl]dimethylammonio)-1-propanesulfonate (CHAPS) were from Sigma and the pentapeptide Phe-Leu-Glu-Glu-Leu (FLEEL) was from Vega. $NaH^{14}CO_3$ (40–60 Ci/mol) was purchased from Amersham. QAE-Sephadex and the molecular mass marker proteins were from Pharmacia. All other chemicals were from Merck.

2.2. *Preparation of crude sperm Gla-protein*

Fresh human sperm was collected from healthy donors and diluted 10-fold with a buffer containing 0.25 M sucrose, 0.10 M NaCl and 0.05 M Tris-HCl, pH 8.0. Sperm count and sperm quality were checked routinely and they were within the normal range. The spermatozoa were sedimented by centrifugation for 15 min at 600 × *g* and 0°C and homogenized in a buffer containing 10 mM benzamidine, 0.5 M KCl and 50 mM EDTA, pH 8.0. The suspension was subsequently centrifuged for 1 h at 100000 × *g* and the supernatant was removed, diluted 5-fold with distilled water and extracted batchwise with QAE-Sephadex (1 ml slurry per 100 ml diluted supernatant). The Sephadex was washed with 0.1 M NaCl in 0.01 M Tris-HCl (pH 7.4) and the sperm Gla-protein was eluted

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with 1 M NaCl. The preparation was subsequently desalted by size exclusion chromatography on a Sephadex G-25 column in water, brought to pH 2.0 with 1 M HCl and lyophilized. In some cases the lyophilized material was decarboxylated by heating at 110°C for 24 h [5].

2.3. *Purification of decarboxylated sperm Gla-protein*

Decarboxylated sperm Gla-protein (d-SGP) was purified from the lyophilized material by high performance liquid chromatography (HPLC) using a Beckman 112 gradient system. The first purification step was on a mono-Q anion exchange column (Pharmacia, 50 × 5 mm). The lyophilized proteins (20 mg) were dissolved in a 0.02 M Tris-HCl buffer (pH 8.5) containing 0.1 M KCl (buffer A), adsorbed on the ion exchange gel in the same buffer, and then eluted with a linear gradient from 0.1 to 1 M KCl in 20 mM Tris-HCl, pH 8.5, at room temperature. The flow rate was adjusted to 60 ml/h, the absorbance was monitored at 229 nm, and fractions of 0.5 ml were collected. The presence of d-SGP in the various fractions was determined by incubating 0.1 ml aliquots in carboxylating reaction mixtures (0.25 ml) containing microsomes from normal bovine liver (see below). The amount of $^{14}CO_2$ incorporated into the exogenous substrate was taken as a measure for the amount of d-SGP present in the fractions. The peak fractions were pooled, concentrated and purified further on a TSK-3000 SW size exclusion column (Beckman, 300 × 7.5 mm) in buffer A.

2.4. *Various assays*

The vitamin K-dependent incorporation of $^{14}CO_2$ was measured by incubating reaction mixtures (0.25 ml) containing 4 mg microsomal proteins, 0.15 M KCl, 1 M $(NH_4)_2SO_4$, 20 mM Tris-HCl, pH 7.5, 10 mM dithiothreitol, 0.4 mM vitamin K hydroquinone, 0.4% (w/v) CHAPS, 12% (v/v) ethylene glycol, 0.01 mCi $NaH^{14}CO_3$ and exogenous substrate as indicated. After 1 h at 25°C 1 ml of 5% (w/v) trichloroacetic acid was added to the mixtures, which were then boiled for 1 min and counted in Atomlight (NEN).

Protein-bound Gla-residues were determined after alkaline hydrolysis of the proteins and analysis of the hydrolysate on a Nucleosil 5-SB column (Chrompack 250 × 4.6 mm) as described by Kuwada and Katayama [6]. Polyacrylamide gel electrophoresis was performed in slab gels as described by Laemmli [7] and protein concentrations were measured according to Lowry et al. [8]. Amino acid analysis was performed on an LKB 4400 Analyzer fitted with a 4.6 × 200 mm stainless steel column packed with Ultropac 8 cation exchange resin. The column was eluted with lithium-containing buffers according to the manufacturer's prescriptions.

3. RESULTS

Fresh human sperm was separated into spermatozoa and seminal plasma and the sperm cells were extracted with 50 mM EDTA in 0.5 M KCl. After alkaline hydrolysis, Gla could be directly identified in the crude sperm cell extract (fig.1A). Although varying amounts of Gla (10–30%) were also found in the seminal plasma, we have focussed our attention on the Gla-containing protein

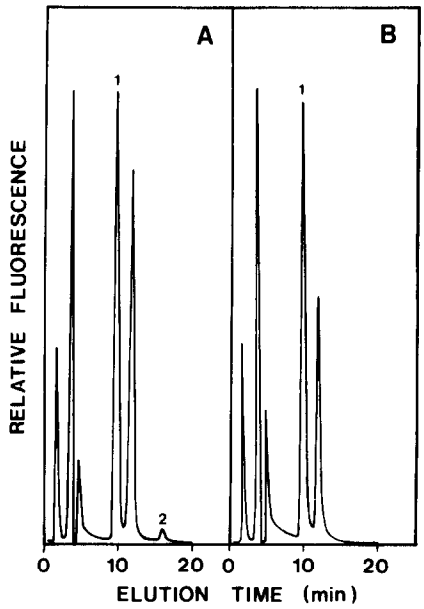

Fig.1. γ-Carboxyglutamic acid determination in sperm cell proteins. The presence of γ-carboxyglutamic acid (Gla) was established in alkaline hydrolysates of a crude cell extract before (panel A) and after (panel B) thermal decarboxylation of the proteins. The Gla-determination was performed by HPLC analysis as described in section 2. Peaks 1 are Glu and peak 2 is Gla.

derived from the spermatozoa. When the crude sperm cell extract mentioned above was submitted to thermal decarboxylation, the Gla-residues were readily converted into Glu (fig.1B). A second observation was that, in contrast to the starting material, the d-SGP could be used as an exogenous substrate for the in vitro carboxylating enzyme system from normal bovine liver. Human liver carboxylase could not be used because of its high level of endogenous substrate [8]. After the reaction mixtures had been incubated for 1 h at 25°C they were supplemented with trichloroacetic acid. The carboxylated d-SGP was recovered in the supernatant and gel filtration chromatography (HPLC) in SDS showed the label to be exclusively present in a protein peak with a molecular mass of about 34 kDa (fig.2A).

Since the Gla-determination is a technique too laborious for the screening of series of column fractions, we decided to decarboxylate the sperm Gla-protein in an early stage of its purification and to use the carboxylase assay for the detection of d-SGP in the various fractions. The purification of d-SGP was accomplished by HPLC using ion exchange and size exclusion columns (fig.2B and C). This procedure resulted in a preparation which migrated as a single peak during gel filtration chromatography in the absence and presence of SDS (1%, [w/v], fig.2D). The recovery of d-SGP from 20 ml semen is shown in table 1. SDS-polyacrylamide gel electrophoresis of the purified material was performed in the absence and presence of 20 mM dithiothreitol (fig.3). Under non-reducing conditions a single band was obtained, with an apparent molecular mass of 28 kDa. Under reducing conditions a low molecular mass peptide chain was dissociated from the heavy chain. From the fact that this dissociation apparently does not cause a substantial decrease of the molecular mass of the heavy chain, we conclude that the light chain may be smaller than indicated by its position in the gel. From these experiments we concluded that d-SGP is a two-chain molecule with an estimated molecular mass of approx. 30 kDa.

The amino acid composition of purified d-SGP was determined after acid hydrolysis and is given in table 2. Based on the assumption that only 1 methionine residue is present per molecule d-SGP a molecular mass of 28 kDa was calculated, from

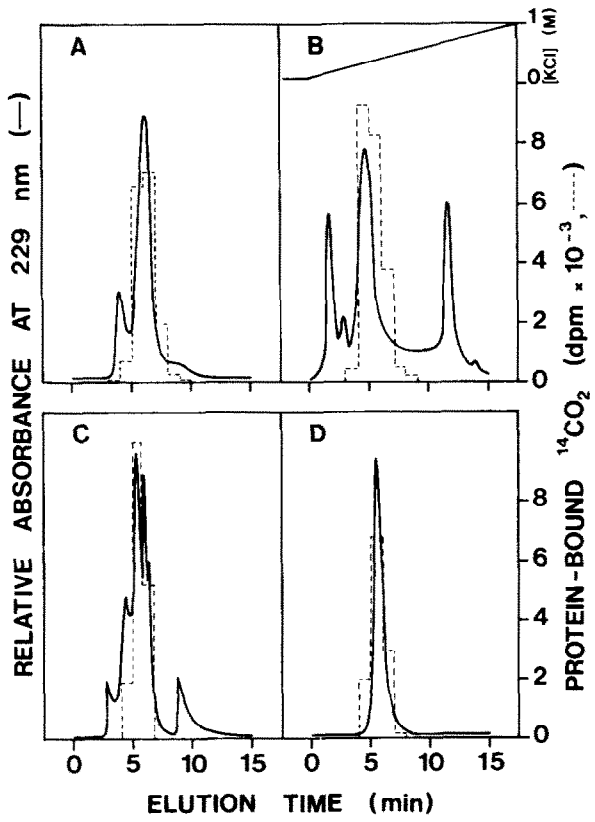

Fig.2. HPLC analysis of the decarboxylated γ-carboxyglutamic acid-containing protein from sperm. (A) Crude decarboxylated sperm Gla-protein (d-SGP, 0.5 mg) was added to normal cow liver carboxylase and incubated in the presence of vitamin K hydroquinone and $NaH^{14}CO_3$ under standard conditions. After 1 h, 1 ml of trichloroacetic acid (5%, w/v) was added and after centrifugation the supernatant was neutralized with 1 M NaOH. SDS was added to a final concentration of 1% (w/v). A sample of 0.05 ml of this preparation was applied to a TSK-3000 SW column in 0.1 M NaCl, 20 mM Tris-HCl, pH 7.5, and 0.1% (w/v) SDS. The flow rate was 1 ml/min. Fractions of 1 ml were collected and counted. The data in this diagram are corrected for the various dilutions and represent the total $^{14}CO_2$ incorporation in 0.5 mg d-SGP. (B) Crude d-SGP (20 mg) was applied to a mono-Q anion exchange column as described in section 2. Fractions of 1 ml were collected and aliquots thereof (0.05 ml) were tested for the presence of d-SGP in the bovine liver carboxylase assay. (C) The proteins eluting between 4 and 6 min were concentrated and applied to a TSK-3000 column in buffer A. The peaks were collected and tested for the presence of d-SGP as described above. (D) The protein peak eluting between 5 and 6 min was collected, concentrated and rechromatographed in buffer A on the same column.

Table 1

Purification of the decarboxylated γ-carboxyglutamic acid-containing protein from sperm

Step	Protein (mg)	Recovery (%) of		Purification (-fold)
		Gla	Substrate	
1. Ejaculate (20 ml)	3600	100	–	1
2. Spermatozoa	640	72	–	4
3. EDTA-extract	46	45	–	35
4. QAE-eluate	20	40	–	72
5. Decarboxylated 4	20	–	100	72
6. Mono Q	3	–	135	648
7. TSK-3000 SW	0.6	–	61	1464
8. TSK-3000 SW rechromatography	0.4	–	56	2016

Since the thermal decarboxylation of the sperm Gla-protein routinely occurred after step 4, only the recovery of protein-bound Gla-residues was measured during the purification steps 1–4. The purification factor (72-fold) is based on the assumption that only one Gla-containing protein is present in the starting material. In step 5 the preparation was decarboxylated and could serve as a substrate for hepatic carboxylase. The latter assay was used to measure the recovery of decarboxylated sperm Gla-protein

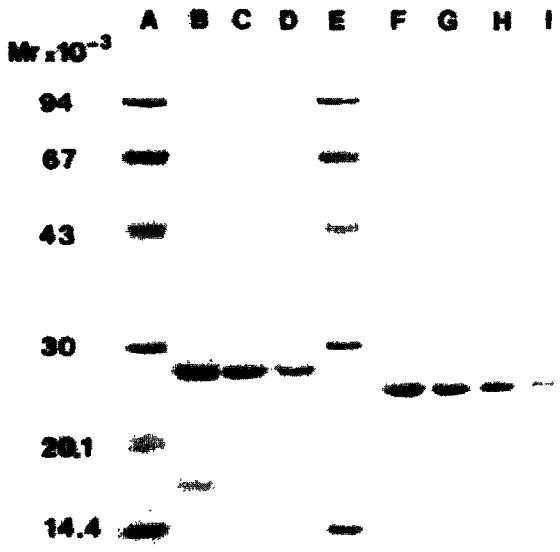

Table 2

Amino acid composition of the decarboxylated γ-carboxyglutamic acid-containing protein from sperm

Amino acid residue	Concentration in hydrolysate (μM)	Number of residues per protein molecule
Asx	444	22.2
Thr	261	13.1
Ser	359	18.0
Glx	777	38.9
Pro	156	7.8
Gly	283	14.2
Ala	137	6.9
Val	196	9.8
Cys	432	21.6
Met	20	1.0
Ile	183	9.8
Leu	216	10.8
Tyr	105	5.3
Phe	64	3.2
Lys	306	15.3
His	162	8.1
Arg	121	6.1
Trp	n.d.	n.d.

The amino acid composition of the purified protein was determined after acid hydrolysis. The number of residues per protein molecule was calculated on the assumption that one residue of Met is present per molecule. The data are not corrected for eventual hydrolysis losses. Trp was not determined because it is destroyed during acid hydrolysis

the given amino acid composition, which is in good agreement with our estimate from the HPLC gel filtration and polyacrylamide gel electrophoresis experiments. After the purification procedure for d-SGP had been established we also tried to purify non-decarboxylated sperm Gla protein and to determine its Gla-content. Although the presence of Gla could be clearly established in the purified

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Fig.3. SDS-polyacrylamide gel analysis of the decarboxylated γ-carboxyglutamic acid-containing protein from sperm. (lanes A and E) Marker proteins, reduced; (lanes B, C and D) decarboxylated sperm Gla-protein, reduced (20, 15 and 10 μg, respectively); (lanes F, G, H, and I) decarboxylated sperm Gla-protein, non-reduced (25, 20, 15 and 10 μg, respectively).

protein, for unknown reasons its Gla-content varied from batch to batch, so that it was impossible for us to make a good estimate of the amount of Gla-residues per molecule native protein.

4. DISCUSSION

The presence of carboxylase has been demonstrated in the testes of rat [2,10], steer [1], goat, ram and stallion (unpublished) thus suggesting that testicular carboxylation systems are indeed very common to mammalian species, including man. Though there was no experimental proof, it seemed plausible that the endogenous substrate of testicular carboxylase might be a constituent of spermatozoa. Indeed Gla was detected in the sperm cell wash and after thermal decarboxylation the preparation contained a protein that could serve as a substrate for bovine hepatic and testicular carboxylase. The isolation procedure resulted in the purification of a hitherto unknown protein, the nascent form of which was designated as sperm Gla-protein. Initial characterization of its decarboxylated form (d-SGP) was accomplished by determining its M_r and its amino acid composition (table 2). Striking is the very high content of Glx. It is likely, therefore, that the native protein contains a high amount of Glu and Gla residues and that it thus bears a considerable negative charge. Possibly this negative charge destabilizes the Gla-residues to some extent so that they are partially lost during various steps of purification of the sperm Gla-protein. This would explain the batch to batch variation in the Gla-content of purified sperm Gla-protein. Further research concerning the function of the sperm Gla-protein is in progress in our laboratory.

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