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Isolation and characterization of two protamines St1 and St2 from stallion spermatozoa, and amino-acid sequence of the major protamine St1

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Two protamines, St1 and St2, were isolated from stallion sperm nuclei, where they represent about 75 and 25%, respectively, of the total basic protein complement. The primary structure of protamine St1 (49 residues; $M_r \approx 6600$) has been determined. The structure of this protamine is compared to the amino-acid sequence of other mammalian protamines already known.

Introduction

In vertebrates, during the late steps of spermiogenesis, histones or intermediate proteins are replaced by smaller, highly basic proteins called protamines. These proteins, of which the molecular size ranges from 30–65 residues, are very rich in arginine (about 40–70%) [1]. Lysine is generally absent, except in rat protamine [2], in three of the four protamines found in dog-fish [3], and human sperm [4]. In contrast with teleost and avian protamines, which lack cysteine [5,6], dog-fish and mammalian protamines contain a high level of cysteine (about 10–15%), which induces further condensation and stabilization of the sperm chromatin through the formation of disulfide cross-links during the transit of spermatozoa in epididymis.

Protamines exhibit an important structural diversity from species to species [7], and even within the same species [3,4,8].

Generally, mammalian spermatozoa contain a single protamine and no other basic protein [7,9–12]. However, two protamines have been found in mouse [13] and stallion sperm (this paper), whereas in human sperm a variety of basic proteins, including protamines, histones, specific histones and intermediate proteins, are present [4].

This paper deals with the characterization of two stallion protamines St1 and St2 and reports the amino-acid sequence of the major protamine St1, previously described by Monfoort et al. [14] as the unique stallion protamine.

Materials and Methods

All operations to isolate the stallion protamines were carried out at 1–3°C.

Preparation of sperm nuclei

Two to four ejaculates obtained from stallions, routinely used for artificial insemination, were immediately diluted with 300 ml of 10 mM Tris-

Abbreviations: PMSF, phenylmethylsulfonyl fluoride; Gdn, guanidinium.

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HCl (pH 8.0)/3.5 mM PMSF (buffer A) at 2°C, and were sonicated for 1 min in an MSE 150 W ultrasonic disintegrator at maximum power. The suspension was centrifuged at $1500 \times g$ for 15 min. The pellet was resuspended in 25 ml buffer A, sonicated in an ice-bath with eight 20-s bursts of ultrasound every 30 s at maximum power, then diluted to 150 ml with buffer A, and centrifuged as above. The pellet was sonicated once again as above in 40 ml buffer A. The sperm nuclei were then purified by centrifugation at $1000 \times g$ during 30 min through 1.5 M sucrose in buffer A. The nuclei were resuspended in 50 ml of 1% Triton X-100 in buffer A, and left for 2 h at 2°C. After centrifugation as above, they were treated at 37°C for 1 h by 25 ml of 10 mM sodium desoxycholate in buffer A and washed twice with 100 ml of buffer A.

Purification of stallion protamines

Nuclei were treated with 0.28 M 2-mercaptoethanol in 0.5 M Tris-HCl (pH 8.5)/1.2 M NaCl/4 M urea at 37°C for 2 h under nitrogen. Iodoacetamide was added up to 0.5 M and the incubation was continued for 2 h in the same conditions. One volume of cold 0.5 M HCl was then added and the basic proteins were extracted for 1 h at 2°C with magnetic stirring. The insoluble material was removed by centrifugation at $17000 \times g$ for 30 min. The supernatant was dialysed and lyophilised.

About 16 mg of protamine fraction were obtained from $7 \cdot 10^9$ spermatozoa.

The protamine fraction was then submitted to ion-exchange chromatography on a column of carboxymethyl-cellulose CM-52 (Whatman 1.6×18 cm), using a linear gradient of guanidinium chloride (0.7–1.6 M) in 50 mM lithium acetate (pH 5.0) [7].

Analytical gel electrophoresis

Protein samples were analysed by electrophoresis on polyacrylamide slab gels in 0.9 M acetic acid/2.5 M urea, according to the method of Panyim and Chalkley [15]. Gels containing sodium dodecyl sulfate were not usable, since protamines are insoluble in presence of this detergent.

Amino-acid analysis

Amino-acid analyses of stallion protamines

were performed on a Beckman 119 CL amino-acid analyser, after hydrolysis in vacuo at 110°C for 24 and 72 h in 6 M HCl (1 ml/mg protein) with one drop of 1% phenol to avoid excessive degradation of tyrosine.

Sequence analysis

For sequence determination, the *S*-carboxamidomethylated protamine St1 was submitted to automated Edman degradation and to cleavage with thermolysin (EC 3.4.24.4).

Automated Edman degradation of the protamine St1 (about 300 nmol) was firstly performed in liquid phase on a Beckman 890 C sequencer, using a 0.33 M quadrol program in the presence of polybrene [16]. Moreover, protamine St1 (10 nmol) was sequenced in two different runs on an Applied Biosystems 470 A gas-phase protein sequencer using a 02 n vac program. Phenylthiohydantoin derivatives of amino acids were identified by reverse-phase high-pressure liquid chromatography on a column of C_{18} μ Bondapak (Waters Associates) as described previously [17].

Protamine St1 (2 mg; about 280 nmol) was hydrolysed with thermolysin in 1 ml of 0.1 M *N*-methylmorpholine acetate (pH 8.0) at 40°C for 4 h, using an enzyme-to-substrate ratio of 1:70 by weight. The thermolysin peptides were subsequently separated by reverse-phase high-pressure liquid chromatography on a column of C_{18} μ Bondapak as indicated in [18]. Manual Edman degradation of the thermolysin C-terminal peptide was performed as described previously [19].

Carboxypeptidase (EC 3.4.17.2) analysis of the protamine was performed as described previously [7].

Results and Discussion

On polyacrylamide gel electrophoresis, the acid-soluble fraction of stallion sperm nuclei appeared to consist of two protamines, St1 and St2 (Fig. 1B, lane 1). The relative mobility of St2 was 0.85 of that of St1. As calculated from densitometric scanning of the electrophoretic analysis, protamines St1 and St2 represent 75 and 25%, respectively, of the total nuclear basic proteins of stallion-sperm chromatin.

Stallion protamines were separated by ion-ex-

change chromatography on a carboxymethyl-cellulose column. Protamine St1 was eluted as a symmetrical and sharp peak by 0.96–0.99 M guanidinium chloride, while the protamine St2 was eluted by 1.25–1.28 M guanidinium chloride (Fig. 1A). Analytical gel electrophoresis indicated that protamines St1 and St2 were purified to homogeneity (Fig. 1B, lanes 2 and 3). Setting apart human sperm where, besides four major protamines, specific basic proteins and histones are present [4], the stallion is, after the mouse [11], the second mammalian species in which two protamines have been found in the sperm.

Fig. 1. Purification of the two stallion protamines St1 and St2. (A) Ion-exchange chromatography of basic nuclear sperm proteins on a CM-cellulose column, eluted with a linear gradient of guanidinium chloride. The peak eluted at the beginning is due to non-protein material (not precipitable with 20% trichloroacetic acid). (B) Electrophoretic analysis of stallion sperm protamines on acid urea polyacrylamide slab gel. Gels were scanned at a wavelength of 620 nm. (1) Whole basic nuclear sperm proteins; (2) protamine St1 from chromatographic fraction 2; (3) protamine St2 from chromatographic fraction 3.

The amino-acid composition of stallion protamines is presented in Table I. Stallion St1 (49 residues; $M_r \approx 6600$) is little different from other mammalian protamines and is mostly characterized by the absence of phenylalanine and histidine. Our results are in agreement with the amino-acid composition established previously by Monfoort et al. [14], except that St1 was found to contain seven cysteine residues instead of six.

The minor stallion protamine, St2, is quite distinct from the major stallion protamine and, more generally, from the other mammalian protamines, by a larger size (about 80 residues), a high content in tyrosine, a lower amount of cysteine and the presence of proline. Preliminary structural investigations have indicated that St2 consists of at least two closely related variants, the separation of which has remained unsuccessful up to now.

Automated Edman degradation of protamine St1 was first performed in a Beckman 890 C

TABLE I

AMINO-ACID COMPOSITION OF STALLION PROTAGMINES AND OF THE C-TERMINAL THERMOLYSIN PEPTIDE OF PROTAMINE St1, USED FOR THE SEQUENCE DETERMINATION

The amounts of each amino acid were calculated from duplicate analyses performed on 24 and 72 h hydrolysates. The values for threonine and serine were obtained by linear extrapolation to zero-hydrolysis time. Results are expressed as mol%. Numbers in parentheses are the numbers of residues derived from the final sequence.

Amino acid	Major protamine St1 (mol%)	Minor protamine St2 (mol%)	C-terminal thermolysin peptide (molar ratios)
S-Carboxymethyl cysteine	15.8 (7)	7.6	1.1 (1)
Threonine	2.1 (1)	1.4	
Serine	8.0 (4)	7.1	
Glutamic acid	6.2 (3)	1.0	
Proline	0.0	3.5	
Glycine	0.0	2.2	
Alanine	1.4 (1)	2.3	
Valine	6.0 (3)	3.1	0.9 (1)
Leucine	2.1 (1)	3.3	0.9 (1)
Tyrosine	3.9 (2)	9.2	
Histidine	0.0	1.5	
Arginine	54.5 (27)	57.8	5.1 (5)
Total residues	49

Fig. 2. Amino-acid sequence of stallion protamine St1. Methods used to confirm the carboxy-terminal sequence of the protamine are indicated as follows: — manual Edman degradation of the carboxy-terminal thermolysin peptide, and — carboxypeptidase hydrolysis. Tandemly repeated sequences are in boxes.

liquid-phase sequencer. In two different runs, the sequence became uninterpretable beyond residue 28. These results are rather surprising if we consider that the sequence of stallion protamine St1 is very close to that of ram protamine, which was determined completely by liquid-phase sequencing [7]. The complete amino-acid sequence of St1 was then established after two runs in a gas-phase sequencer. In both experiments positive identifications of phenylthiohydantoin derivatives of amino acids were achieved through the C-terminal residue (Fig. 2).

The carboxy-terminal sequence of protamine St1 was confirmed from structural data provided

by the carboxy-terminal thermolysin peptide (Table I and Fig. 2), and by digestion of the protein with carboxypeptidase B. The comparison of stallion protamine St1 with other mammalian protamines (Fig. 3) calls for the following remarks:

The N-terminal sequence

$${}^1\text{Ala-Arg-Tyr-Arg-Cys-Cys}^6$$

is common to bull [10,20], ram [7], boar [12], mouse [13], rat [2], and human protamines P1 [4,21,22].

A serine or a threonine residue is always present at positions 8, 10 and 12 in all mammalian protamines, except human protamines HP2 and HP3 [4,22,23].

A highly basic domain (residues 12–28), containing two identical octapeptides tandemly repeated (sequences 13–20 and 21–28), is common to stallion, ram and bull protamines. An almost identical sequence is found in boar and mouse protamines. This domain is likely to be the primary binding site of these protamines to DNA.

The carboxy-terminal sequence (residues 29 to C-terminus) of mammalian protamines is much more variable. However, considering that the change Leu $\rightarrow$ Val $\rightarrow$ Ile at position 43 is conservative, the sequence 35–45 is common to stallion, ram, bull and boar protamines (Fig. 3).

Stallion protamine St1 contains several potential sites of phosphorylation on serine residues 8, 10, 12 and 29, and one on threonine residue at position 41. It must be emphasized that serine at

Fig. 3. Sequence homologies between mammalian protamines from stallion, ram [7], bull [10,20], boar [12], mouse [13] and human P1 [4,21,22]. Numbering refers to alignment position for maximum homology and not to sequence position. The boxes correspond to strong homologies between the different protamines. Hydroxyamino acids at positions 8, 10, 12 are circled.

position 29 is located in a sequence specifically recognized by the cyclic AMP-dependent protein-kinase, such as B-X-Ser, where B is lysine or arginine, and X any amino acid except proline [24,25]. Protamine phosphorylation and dephosphorylation are known to be important processes occurring in spermatogenesis [26,27] for correct binding to DNA and chromatin condensation.

The transition of late spermatids to mature spermatozoa is associated with the dephosphorylation of phosphorylated protamines. Mammalian protamines are generally dephosphorylated before the spermatozoa leave the testis. Subsequently, during the transit of spermatozoa in the epididymis, oxidation of the sulfhydryl groups leads to intermolecular disulfide linkages which strengthen the condensation of sperm chromatin.

Mechanisms which in mammalian spermatogenesis take place during the double protein transition – histones → intermediate proteins → protamines – are not yet fully understood. More information on these proteins and their chemical modifications is necessary to elucidate these mechanisms.

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