Purification and characterization of two basic spermatid-specific proteins isolated from the dog-fish *Scylliorhinus caniculus*

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In dog-fish spermatid nuclei two intermediate proteins S_1 and S_2 replace histones before the setting down of protamines. These spermatid-specific proteins were isolated by carboxymethyl-cellulose chromatography and purified by high pressure liquid chromatography. S_1 and S_2 are characterized by a high content of basic residues and by the lack of cysteine and phenylalanine. The determination of their amino acid composition and of their N- and C-terminal sequences prove that each protein corresponds to a specific molecule which can be considered neither as a histone hydrolytic product nor as a protamines precursor.

Spermiogenesis *Nuclear basic spermatid-specific proteins*

1. INTRODUCTION

Spermatogenesis in animal species is characterized by dramatic morphological and biological changes in nuclear organization (for a recent review, see [1]). As spermatogenesis proceeds, various modifications occur in the nature of proteins associated with DNA and the result is the progressive condensation of chromatin. In spermatozoa, somatic histones are generally replaced by other typical low-M_r, arginine-rich proteins called protamines such as in fishes and molluscs or protamine-like proteins such as in mammals. In some of these species, for instance in the rat, ram, stump-tail macaque, man [2–11] and dog-fish [12,13], protamines do not replace directly the somatic histones. Specific and generally heterogeneous basic proteins are transiently associated with DNA in elongating spermatid nuclei before the setting down of protamines. From autoradiographic observations, it has been deduced that a similar situation could occur in mouse spermiogenesis [14].

Such a complex protein transition occurs during dog-fish spermiogenesis. The microelectrophoretic analysis of basic protein changes shows that this process can be divided into 3 steps [13]. At the beginning of nuclear elongation (stage 3 of spermiogenesis), two spermatid-specific proteins also called intermediate proteins S_1 and S_2 appear. The gradual increase in the amount of the two proteins, whilst the nuclear elongation of spermatids proceeds, is accompanied by a progressive but incomplete loss of histones (stage 4a). In the final stages of nuclear shaping (stages 4b, 4c, 5) the 4 protamines (Z1, Z2, Z3 and S4) found in mature spermatozoa appear, whereas the S_1 and S_2 contents of nuclei gradually decrease. In mature intratesticular spermatozoa (stage 5) S_2 then S_1 have been completely eliminated and only the 4 protamines remain in the nuclei.

The work reported here deals with the isolation and characterization of the two intermediate proteins S_1 and S_2 from dog-fish. Their amino acid composition and their amino- and carboxy-terminal sequences reveal that each corresponds to a unique protein species and can be considered neither a histone hydrolytic product nor a precursor of the protamines.

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2. MATERIALS AND METHODS

Dog-fish testes were collected at the Station de Biologie Marine de Concarneau (France). After excision, gonads were frozen in liquid nitrogen and stored at −80°C until use. All operations up to chromatographic purification were performed at 4°C.

2.1. *Preparation of basic proteins from spermatid nuclei*

Testicular zone B (young spermatids 1–4a) was finely minced and homogenized in 50 mM Tris–HCl (pH 7.4) containing 3.3 mM $CaCl_2$, 0.25 M sucrose and 0.1 mM di-isopropylfluorophosphate (DFP). The homogenate was filtered through 6 layers of surgical gauze and centrifuged at 2500 × *g* for 20 min. The nuclear pellet was then rehomogenized in the same buffer containing 2.2 M sucrose (19 ml/g of testis) and was centrifuged at 47000 × *g* for 1 h.

Purified nuclei were resuspended in 50 mM Tris–HCl containing 0.14 M NaCl, 0.1 mM DFP and were centrifuged at 2500 × *g* for 20 min. The nuclei were washed 3 more times with the same buffer and finally with 90% ethanol.

Basic nuclear proteins were extracted from nuclei by gentle stirring in 0.25 N HCl (2 ml/g of testis) overnight and precipitated either by 6 volumes of acetone at −30°C for 48 h or by 20% trichloroacetic acid at 0°C for 2 h. After centrifugation, proteins were washed successively with acetone acidified with concentrated HCl and finally with acetone. The proteins dried in vacuo were redissolved in distilled water and lyophilized.

2.2. *Isolation of intermediate proteins*

The acid-soluble proteins were reduced in 8 M urea, 0.5 M NaCl containing 20 mM dithiothreitol (DTT) at 37°C for 1 h under nitrogen, and then alkylated at 37°C for 1 h by adding 25 mM iodoacetamide. The alkylated proteins were loaded onto a carboxymethyl cellulose (Whatman, CM 52) column (2.6 × 30 cm) equilibrated with 0.05 M sodium acetate, pH 6 buffer containing 0.5 M NaCl. After washing the column with the equilibration buffer, the two intermediate proteins S_1 and S_2 were eluted together from the column by 2 M NaCl in the same buffer.

2.3. *Purification of intermediate proteins S_1 and S_2 by high pressure liquid chromatography*

The purification of proteins S_1 and S_2 was performed by reverse phase high pressure liquid chromatography using a Waters Associates ALC/GPC 244 apparatus equipped with a U6K sample injector and a Cecil 212 variable wavelength ultraviolet monitor. The protein sample was loaded onto a C_{18} micro-Bondapak column (9 μm, 0.39 × 30 cm) equilibrated with 10% acetonitrile in 0.05% trifluoroacetic acid. Proteins were eluted with a linear gradient of acetonitrile (10–25%) in 0.05% trifluoroacetic acid for 30 min.

2.4. *Analytical gel electrophoresis*

Protein preparations were controlled by polyacrylamide gel electrophoresis as in [15]. Samples dissolved in 8 M urea, 0.9 M acetic acid, 0.5 M β-mercaptoethanol were run in slab gels (14 × 20 × 0.075 cm) containing 17% acrylamide and 6.25 M urea.

2.5. *Amino acid analysis*

Protein samples were hydrolysed in vacuo at 110°C in 6 M HCl for 24 h and 72 h. One drop of 1% phenol was added to prevent excessive degradation of tyrosine. Amino acid analyses were performed on a Beckman 119 CL amino acid analyser.

2.6. *Amino-terminal sequence determination*

The amino-terminal sequences of proteins S_1 and S_2 were determined by manual Edman degradation performed as in [16]. Phenylthiohydantoin derivatives of amino acids were identified by high pressure liquid chromatography on a column of C_{18} micro-Bondapak (Waters) as in [17].

2.7. *Carboxy-terminal analysis*

Protein S_1 (20 nmol) dissolved in 0.45 ml of 0.1 M *N*-methylmorpholine acetate (pH 8.0) was digested at 37°C with DFP-treated carboxypeptidase B (Sigma) using an enzyme-to-substrate ratio of 1:50 (w/w). After 3 h digestion, a 0.2-ml aliquot was taken off, acidified with glacial acetic acid and freeze-dried. $PhMeSO_2F$-treated carboxypeptidase A (Millipore) was then added using an enzyme-to-substrate ratio of 1:25 (w/w). After 2 h

digestion, a 0.2-ml aliquot was taken off and treated as above. The released amino acids were analysed on the amino acid analyser.

The carboxy-terminal analysis of protein S_2 (20 nmol) was carried out as indicated for protein S_1 but the aliquots were taken off after 1 h digestion with carboxypeptidase B and after 1 h digestion with carboxypeptidase A.

3. RESULTS AND DISCUSSION

The acid-soluble proteins were extracted from spermatid nuclei of zone B of the testis, enriched in stages 1–4a, to prevent contamination by protamines. These basic proteins are a complex mixture of histones, non-histone proteins and intermediate proteins S_1 and S_2 (fig.1, lane a). The two intermediate proteins were separated from other proteins by chromatography on carboxymethyl-cellulose as shown in fig.2. The histones and non-histone proteins were eluted in fractions 1a and 1b by 0.5 M NaCl whereas S_1 and S_2 were eluted together in fraction 2 by 2 M NaCl (fig.1, lane b). On overloaded gels, S_1 and S_2 appear slightly contaminated with components of lower mobilities.

Proteins S_1 and S_2 were separated by reverse phase high pressure liquid chromatography (fig.3). Pure S_1 and S_2 were obtained in fractions 2 and 3, respectively as assessed by polyacrylamide gel electrophoresis (fig.1, lanes c and d). A protein presently designated by 'X' was eluted in the first

Table 1

Amino acid compositions of proteins S_1 and S_2 from the dog-fish *Scylliorhinus caniculus* expressed as mol/100 mol[a]

Amino acids	S_1	S_2
Asp	8.0	9.0
Thr[b]	4.7	3.2
Ser[b]	6.5	7.6
Glu	3.8	6.8
Pro	6.0	6.8
Gly	3.2	2.4
Ala	0.0	7.4
Half cys	0.0	0.0
Val	5.8	4.2
Met	2.1	0.7
Ile[c]	3.2	1.9
Leu[c]	6.1	10.0
Tyr	3.4	4.5
Phe	0.0	0.0
His	3.7	2.5
Lys	9.3	19.4
Arg	34.0	13.9
Arg/Lys	3.65	0.71

[a] Average values of at least 4 analyses
[b] Values for threonine and serine were obtained by linear extrapolation to zero hydrolysis time
[c] 72-h hydrolysis values

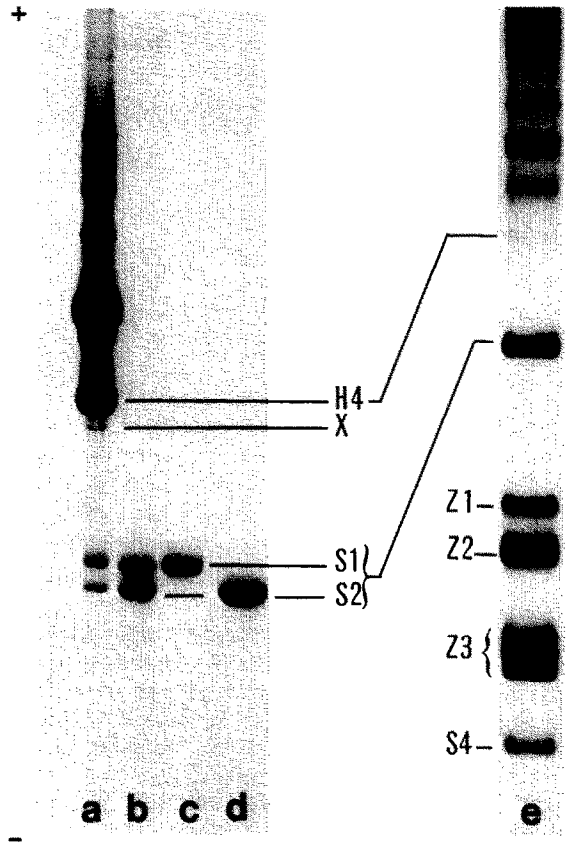

Fig.1. Electrophoretic control of spermatid-specific proteins at different steps of purification. Electrophoreses were performed as described in section 2 and gels were stained with Coomassie brilliant blue R 250 for 1 h [20]. Lane a: Acid-soluble proteins extracted from testicular zone B nuclei; Lane b: Fraction 2 eluted from the carboxymethyl-cellulose column (fig.2); Lanes c and d: Fractions 2 and 3, respectively, obtained by high pressure liquid chromatography as described in fig.3; Lane e: Acid-soluble proteins extracted from testicular zone C nuclei (stages 4b–5). Z_1, Z_2, Z_3 (heterogeneous in spermatid nuclei) and S_4: protamines.

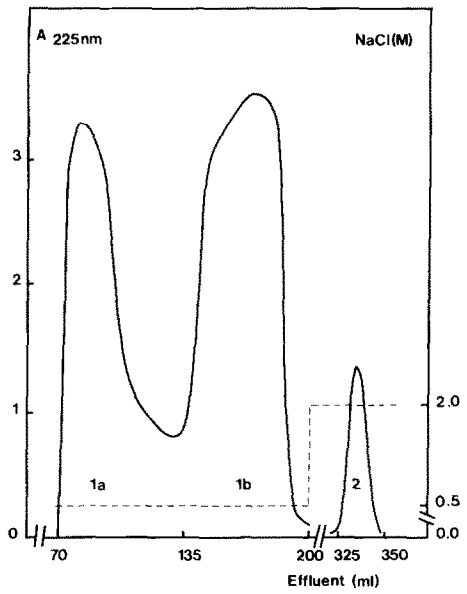

Fig.2. Isolation of intermediate proteins by carboxymethyl-cellulose chromatography. 60 mg of acid-soluble proteins (fig.1, lane a) were reduced and alkylated as described in section 2 and the protein sample was loaded onto a CM 52 column (2.6 × 30 cm) equilibrated with 0.05 M sodium acetate buffer (pH 6) containing 0.5 M NaCl. The column was washed with 200 ml of equilibration buffer; then S_1 and S_2 were eluted by 2 M NaCl in acetate buffer. The flow rate was 20 ml/h and fractions of 2.5 ml were collected.

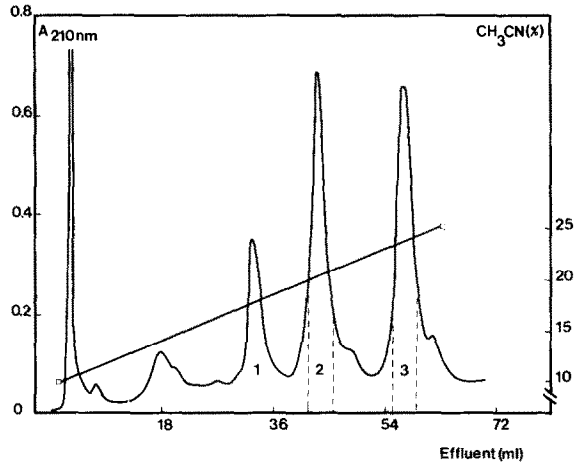

Fig.3. Separation of proteins S_1 and S_2 by high pressure liquid chromatography. 1 mg of the fraction containing proteins S_1 and S_2 (fraction 2 in fig.2) was dissolved in 60 μl of 0.01 N HCl, 8 M urea and kept overnight at 4°C. The protein solution was diluted with 20 μl of 0.01 N HCl, centrifuged and loaded on a micro-Bondapak column (9 μm, 0.39 × 30 cm) equilibrated with 10% acetonitrile in 0.05% trifluoroacetic acid. The proteins were eluted with a linear gradient from 10–25% in acetonitrile in 0.05% trifluoroacetic acid for 30 min. The flow rate was 2 ml/min and fractions of 1.2 ml were collected.

peak at 17% acetonitrile concentration. This protein 'X' migrates a little faster than histone H4 on acid-urea gel electrophoresis (fig.1, lane a). The characterization of this protein is now in progress.

The amino acid compositions of intermediate proteins S_1 and S_2 are presented in table 1. Both proteins are devoid of phenylalanine and cysteine, but are mainly characterized by a high content of basic residues which represent 47% and 35.8% of the total number of residues in S_1 and S_2, respectively. They differ markedly from each other by their arginine to lysine ratio: 3.66 in S_1 in which arginine accounts for 34% of the total number of residues; 0.72 in S_2 which contains 19.4% of lysine. Moreover S_1 differs from S_2 by the lack of alanine and by a less hydrophobic character which explains its smaller retention time in reverse phase high pressure liquid chromatography. The amino-terminal sequences of S_1 and S_2 were found to be (H) Thr-Lys-Ser-Arg-Tyr-Arg- and (H) Val-Lys-Ser-Arg-Tyr-Ser-, respectively. Arginine was found at the carboxy-terminus of S_1 and the carboxy-terminal sequence of S_2 was established as -Arg-Leu (OH). Taking into account all these results, each protein can be considered as a specific molecule.

The determination of the relative M_r of S_1 and S_2 by SDS polyacrylamide gel electrophoresis was rendered impossible because the two proteins are insoluble in sodium dodecyl sulphate as are protamines. However, their electrophoretic mobilities in acid-urea gels (fig.1, lane e) as well as the data of the amino acid analyses indicate that their M_r must be intermediate between that of histone H4 (M_r ~11300) and that of protamine Z1 (M_r ~7200). Their amino acid compositions and especially their very high content in basic residues as well as their amino terminal sequences exclude that proteins S_1 and S_2 could be histone proteolytic products [18]. Moreover, the lack of alanine in S_1 and of cysteine in both proteins leads to the conclusion that neither of them can be regarded as

high M_r precursor of protamines ([19] and M. Gusse et al., unpublished), since all 4 protamines contain alanine and 3 of them (Z1, Z2 and S4) are cysteine-rich. Consequently, proteins S_1 and S_2 are basic nuclear proteins which are characteristic of spermatid nuclei at mid-spermiogenesis.

On the other hand, dog-fish intermediate proteins S_1 and S_2 share no common feature with spermatid-specific basic proteins previously characterized in rat testis [2–6] and human testis [11].

So, these spermatid-specific proteins also appear to be species- or perhaps class-specific proteins. The respective primary structure of proteins S_1 and S_2 are presently under investigation and their determination would enable us to get an insight into their biological role which is presumed to be the replacement of histones and the onset of chromatin condensation which is completed by protamines.

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REFERENCES

[1] Chevaillier, Ph. (1983) 4th Internat. Congress of Spermatology, Seillac (France), Nijhoff, The Hague, in press.
[2] Kistler, W.S., Noyes, C., Hsu, R. and Heinrikson, R.L. (1975) J. Biol. Chem. 250, 1847–1853.
[3] Grimes, S.R., Meistrich, M.L., Platz, R.D. and Hnilica, L.S. (1977) Exp. Cell Res. 110, 31–39.
[4] Platz, R.D., Grimes, S.R., Meistrich, M.L. and Hnilica, L.S. (1975) J. Biol. Chem. 250, 5791–5800.
[5] Meistrich, M.L., Brock, W.A., Grimes, S.R., Platz, R.D. and Hnilica, L.S. (1978) Fed. Proc. 37, 2522–2525.
[6] Meistrich, M.L., Bucci, L.R., Brock, W.A., Trostle, P.K., Platz, R.D., Grimes, S.R. and Burleigh, B.D. (1980) Fed. Proc. 39, 1884.
[7] Meistrich, M.L., Trostle, P.K. and Brock, W.A. (1981) in: Bioregulators of Reproduction (Jagiello, G., Vogel, H.J. eds) pp.151–166, Academic Press, New York.
[8] Loir, M. and Lanneau, M. (1978a) Exp. Cell Res. 115, 231–243.
[9] Loir, M. and Lanneau, M. (1978b) Biochem. Biophys. Res. Commun. 80, 975–982.
[10] Lanneau, M. and Loir, M. (1982) J. Reprod. Fert. 65, 163–170.
[11] Kistler, W.S., Geroch, M.E. and Williams-Ashman, H.G. (1975) Invest. Urol. 12, 346–350.
[12] Gusse, M. and Chevaillier, Ph. (1978) Cytobiol. 16, 421–443.
[13] Gusse, M. and Chevaillier, Ph. (1981) Exp. Cell Res. 136, 391–397.
[14] Mayer, J.F. and Zirkin, B.R. (1979) J. Cell Biol. 81, 403–410.
[15] Panyim, S. and Chalkley, R. (1969) Arch. Biochem. Biophys. 130, 337–346.
[16] Edman, P. and Henschen, A. (1975) in: Protein Sequence Determination (Needleman, S.B. ed) 2nd ed., pp.232–279, Springer, Berlin, New York.
[17] Hermann, J., Titani, K., Ericsson, L.H., Wade, R.D., Neurath, H. and Walsh, K.A. (1978) Biochemistry 17, 5672–5679.
[18] Von Holt, C., Strickland, W.N., Brandt, W.F. and Strickland, M.S. (1979) FEBS Lett. 100, 201–218.
[19] Sautière, P., Briand, G., Gusse, M. and Chevaillier, Ph. (1981) Eur. J. Biochem. 119, 251–255.
[20] Steck, G., Leuthard, P. and Burk, R.R. (1980) Anal. Biochem. 107, 21–24.