The Histidine-Rich Protamine from Ostrich and Tinamou Sperm. A Link between Reptile and Bird Protamines †

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The Histidine-Rich Protamine from Ostrich and Tinamou Sperm. A Link between Reptile and Bird Protamines†

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ABSTRACT: We have characterized for the first time the proteins of two different species of palaeognathous birds, *Struthio camelus australis* (ostrich) and *Nothoprocta perdicaria sanborni* (Chilean tinamou). Similar to what had been previously reported in neognaths, the electrophoretic mobility, amino acid composition, and primary structure of the main protamine (P-II) component of these two species of birds are similar. However, in contrast to neognathous birds, the protamines from paleognaths display a higher electrophoretic mobility and a significantly different amino acid composition and protein sequence. The sperm and the main protamine component P-II from the ostrich reveal structural and compositional characteristics intermediate between neognathous birds and reptiles. The marked differences between the protamines and sperm structure of neognaths and paleognaths provide support to a phylogenetic relationship between neornithine birds in which these two groups represent two separate phylogenetic lines. Furthermore, these results shed some additional light on the controversial origin of birds. They provide further molecular support to the fossil record that suggests that reptiles and birds are closely related.

Protamines are a family of sperm nuclear basic proteins (SNBPs) of small molecular mass (<15 000 Da) which have a simple consensus amino acid composition (Arg ≥ 30 mol %, His + Lys + Arg = 45−80 mol %, and Ser + Thr + Gly = 10−25 mol %) (*1*, *2*). They are found in the sperm of vertebrate and invertebrate organisms (*1*, *3*−*7*) and are among the fastest evolving proteins. Their fast rate of divergence makes them very useful for the analysis of the evolution of closely related phylogenetic groups (*8*).

The protamines of vertebrates have been studied in considerable detail [see (*5*, *7*) for reviews]. The first protein members of the protamine family of SNBPs ever characterized were from fish (see ref *9* for an early review). Mammalian protamines have been characterized (reviewed in *5*, *8*), as have those of neognathous birds (*10*−*14*), and amphibians (*15*−*18*). Recently we characterized the protamines of reptiles which provides the protein sequences necessary to determine the phylogeny of vertebrate protamines (*2*). In this study, we discovered that crocodilian protamines have an unusually high content of histidine (*2*).

The available information about bird protamines comes from representative organisms of several Neognathae groups (*8*, *11*, *12*, and Table 1). Although sequence information is only available for a few species in this group (*10*, *13*, *14*), an electrophoretic, cytochemical, and compositional characterization indicated that their protamines have been well preserved since they exhibit little variation compared to protamines from other groups (*11*, *12*). This was attributed to the uniform method of reproduction of this vertebrate class (*11*, *12*) consisting of internal fertilization and egg laying.

For many years, the origin of birds has been the theme of many controversial paleontology and molecular biology studies (see *19* for an extensive review). In a recent example, molecular RNA evidence has been provided in support of a bird−crocodilian relationship (*20*). Based on the fossil record, it has also been proposed that the Neognathae and Paleognathae represent separate phylogenetic lines of the neornithine (modern birds) (*21*). Our results provide further evidence in favor of these propositions. sperm.

MATERIALS AND METHODS

Living Organisms and Sperm Collection. Semen samples were collected from 25 mature male ostriches (*Struthio camelus australis*) from the Oudtshoorn district, Western Province, Republic of South Africa, by digital massage of the deferent duct papillae (*22*, *23*). Immediately after ejaculation, the semen samples were suspended in 9 volumes of 95% ethanol and stored at 4 °C or at room temperature. The primary structure of protamines has been shown to be preserved in samples of male gonadal tissue or sperm when stored in alcohol for long periods of time (months) at room temperature (*24*). Some of the semen samples were immediately frozen after collection and lyophilized.

Testes and vas deferens from 2 year old male tinamous (*Nothoprocta perdicaria sanborni*) (*25*) were obtained from

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Table 1: Amino Acid Composition (mol %) of Protamines from Different Orders of Neornithine Birds

	Neognathae				Palaeognathae		
					Struthioniformes *Struthio camelus australis*		Tinamiformes *Nothoprocta perdicaria sanborni*
amino acid	Galliformes *Gallus gallus*[a]	Anseriformes *Anas platyrhynchos*[b]	Columbiformes *Columba livia*[b]	Psittaciformes Melopsittacus undulatus[b]	P−II	P−I	P−II[d]
His				2.1[c]	**28.9**	**25.8**	**34.8**
Arg	58.5	63.4	63.4	65.7	50.0	48.5	47.8
Thr	1.6	1.2	1.6			1.5	
Ser	17.2	14.0	18.2	13.5	9.1	12.2	6.5
Glx			tr.[e]	4.4		3.4	2.2
Pro	3.5	2.5					
Gly	8.6	6.5	7.1	4.5	9.3	2.9	6.5
Ala	3.2	1.5	1.6			2.3	2.2
Val	1.7	1.9		2.0			
Leu				2.1			
Tyr	6.2	9.0	8.1	5.8	2.7	3.4	

[a] From (*46*). [b] From (*11*). [c] Corresponding to an amino acid that eluted just before histidine at pH 6.4 [Chiva et al. (*11*)]. [d] Amino acid composition calculated from the protein sequence shown in Figure 2. [e] tr.= trace amounts.

birds at the University of British Columbia San Rafael Research Aviary in Surrey, B.C. The animals were sacrificed by cervical dislocation. The testes and vas deferens were then quickly excised, and the tissue was immediately frozen in liquid nitrogen and kept at −70 °C until further processing.

Protein Extraction and Purification. SNBPs were isolated from the semen or vas deferens samples, as described previously (*2*). Buffers used during the isolation of proteins contained Complete protease inhibitor cocktail tablets (Boehringer). In some instances the SNBPs were directly extracted from the semen samples with 0.4 N HCl without previous treatment to assess the effects of the manipulation time on proteolysis of the protein extracts. The HCl−protein extracts were fractionated by reverse-phase HPLC (*2*).

Gel Electrophoresis. Acetic acid (5%)−urea (2.5 M) polyacrylamide gels were prepared as described elsewhere (*26*).

Mass Spectrometry. Molecular masses were determined by mass spectrometry on a VG quattro mass spectrometer (Fisons).

Amino Acid Analysis and Protein Microsequencing. Amino acid analyses were carried out on an ABI Model 420 A derivatizer analyzer system, and protein sequencing was performed on an ABI Model 470 A gas-phase protein sequenator as described elsewhere (*27*).

Electron Microscopy. A portion of the ejaculate was fixed overnight at 4 °C in 4% glutaraldehyde in Millonig's phosphate buffer. After gentle centrifugation and resuspension, the sperm were washed once in Millonig's phosphate buffer, post-fixed in similarly buffered 1% osmium tetroxide for 1 h at room temperature, and given two buffer rinses. After pelleting in glass microhematocrit tubes, the sperm were dehydrated through a graded ethanol series (25%, 50%, 75%, 96%, 100% × 2−10 min per step), cleared in propylene oxide, and embedded in Polarbed 812 epoxy resin. Thin sections were cut with a Reichert OmU4 ultramicrotome using a diamond knife, stained for 5 min each with a saturated solution of uranyl acetate (*28*) and 0.2% lead citrate (*29*), and examined with a Philips 301 or CM10 transmission electron microscope operated at 80 kV.

Sequence Analysis. Protein sequence alignments and similarities were determined with the CLUSTAL W 1.7 multiple sequence alignment program (*30*) and the BLITZ server at EMBL, which uses the best local similarity algorithm (*31*).

RESULTS AND DISCUSSION

We analyzed the nuclear protein composition of the sperm from 25 samples of ostrich semen. We also looked at the protamine composition of the sperm from the vas deferens of several tinamous. We found that the SNBPs of these birds consist of a major electrophoretic band P-II (Figure 1A, lanes 1, 2) plus a few minor bands of lower electrophoretic mobility all of which run within the region corresponding to protamines in a urea−acetic acid PAGE (Figure 1) (*2*). In the ostrich, a small, variable amount of histones was present probably from variation in sexual maturity. The same electrophoretic pattern was observed regardless of the method of preparation of the semen or the time elapsed during the extraction of the proteins (see Materials and Methods). The constancy of the bands within the protamine region was taken as an indication that protein degradation had not occurred during preservation. Proteolysis of protamines is a common occurrence in birds (*10*, *12*). The massive presence of histones observed in tinamou (see Figure 1A, lane 2) is due to the fact that protamines were not extracted from semen but from the vas deferens tissue. From a qualitative point of view, the SNBP patterns of the ostrich and the tinamou resemble those observed in different groups of reptiles (*2*) (see also Figure 1B).

Reverse-phase HPLC analysis of the protein extracts from both species revealed protein microheterogeneity for the proteins corresponding to P-II (results not shown) which has also been observed in SNBPs from reptiles (*2*). In the ostrich, after several rounds of HPLC fractionation, two major protein components for P-II and P-I were purified to homogeneity (as assessed from mass spectrometry analysis). In the tinamou, the P-II component was isolated in a similar fashion. The masses of the monodisperse fractions were found to be 5956.7 ± 1.5 Da for the main P-II component and 7462.6 ± 0.6 Da for the main P-I component of the ostrich and 6281 ± 1.7 Da for the tinamou P-II fraction. The proteins were sequenced by conventional Edman degradation protein microsequencing. The complete amino acid sequence for P-II of the two species of birds analyzed and the partial sequence of P-I of the ostrich are shown in Figure 2. In both, the high

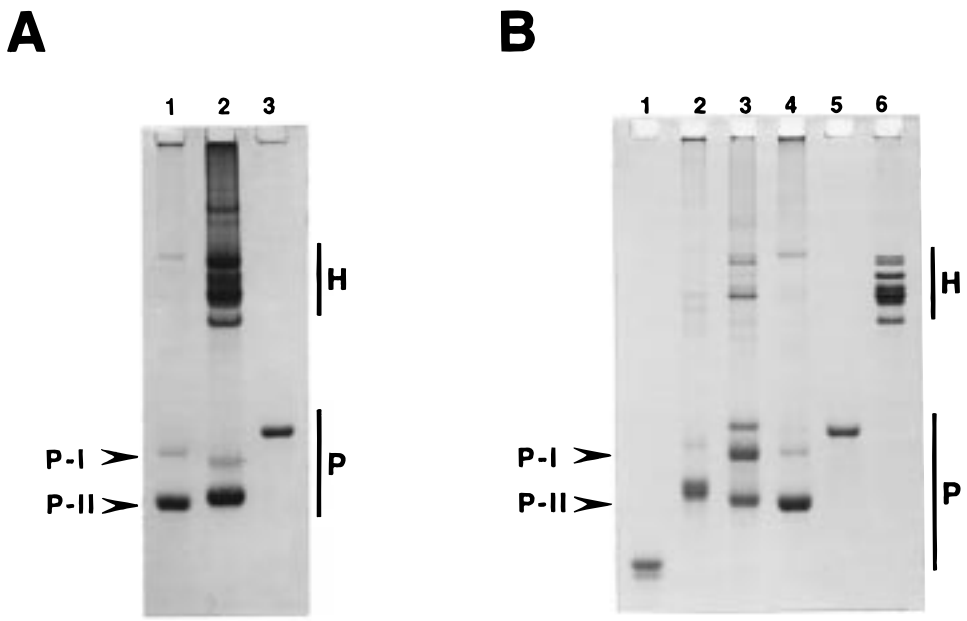

FIGURE 1: (A) Sperm nuclear basic proteins (SNBPs) of different neornithine birds as analyzed by acetic acid−urea (AU) polyacrylamide gel electrophoresis: lane 1, ostrich (*Struthio camelus australis*); lane 2, tinamou (*Nothoprocta perdicaria, sanborni*); lane 3, domestic fowl (*Gallus gallus*). (B) Electrophoretic analysis (AU) of the SNBPs from different vertebrates: (lane 1, salmon (Salmine) (*Oncorhyncus keta*); lane 2, turtle (*Chrysemys picta*); lane 3, alligator (*Alligator mississipiensis*); lane 4, ostrich (*S. camelus australis*); lane 5, domestic fowl (*Gallus gallus*). Lane 6 corresponds to a chicken erythrocyte histone sample used as a marker for histones.

Tn P-II	RRR RHHRRAHGQRHRRHR RHG-----RSHHRRH HRHHHHHSRRRRGRR SRH	46
Os P-II	RRR SHRRRGHGRRHHRHR RHG-----HSHRRHH RR--HHHSRRRRGRR SHY	44
Os P-I	ARYQRSRTRSRSRRR SHRRRGHGRRHHRHR RHG-----HSHRRH• •••••••••••••• •••	56*
Gll	ARYRRSRTRSRSPRS RRRRRRSGRRRSPRR RR-----RYGSARRS RRSVGGRRRRYGSRR RRRRRY	61
Al I	ARYERNRSRSRSRRR RR-WSNHGGRYRRRR TRRSGGGRYGQRRHH R---GGSRRRRRRRR RRRRRR	62
Al II	ARYRHNRSRSRSRHR RRRRGHRGGRYRRRR RR----GRYGHRRHH R---GHSRRRRKRRR SRH---	56
Al III	------------RRR RRRGGHGGGSYRRRR GG-----YYGRRRHH R---GSQRRRRRRRR RRR---	43
Tt I-1	ARYRRNRSRSRSRRR RRRRGGRGGRRGRRR RR------HGQRRRG RR--GRERTRRRRRR RRRSSS	58
Tt II-3	------------RRR RRRRGGRGGRRGRRR RR------HGRRRRG RR--GRERTRRRRRR RRRSS-	45

FIGURE 2: Sequence alignment of the protamines from different birds and reptiles. Tn-PII is the major protamine component (see Figure 1A, lane 2) from tinamou, *N. perdicaria sanborni*. Os P-II and Os P-I are the major and minor components (see Figure 1A, lane 1) from the sperm of ostrich, *S. camelus australis*. Gll is the protamine from rooster (*G. gallus*) (*46*). Al I, Al II, and Al III are protamine components from the sperm of the alligator (*A. mississipiensis*) (*2*). Tt I-1 and Tt II-3 are two representative sequences of the protamines from the sperm of the painted turtle (*C. picta*) (*2*). The dots represent amino acids for which the sequence was not determined. (*) The number of amino acids of Os P-I was established from the molecular mass determined by mass spectrometry and from the amino acid analysis composition (see Table 1).

purity of the proteins produced the complete sequence of P-II from the N-terminus in one single microsequencing run. The molecular mass determined from the sequences was 5958.6 Da for the average molecular mass and 5955.2 Da for the monoisotopic mass of the ostrich P-II protamine and 6281.0 and 6277.4 Da for the respective masses of the P-II protamine of the tinamou. These values are in agreement with those expected from the experimental mass determination. With the ostrich P-I, we were able to sequence only the first 39 amino acids, although the homogeneity was as good as for P-II. Unfortunately this protein was present in small amounts in the SNBP extracts (see Figure 1, lane 4) and not enough material could be purified to proceed further with sequencing. Nevertheless, indirect evidence from the amino acid analysis composition (Table 1) and the molecular mass determined by mass spectrometry (see above) strongly suggest that the sequence of the missing amino acids is identical to that of the last 17 C-terminal amino acids of P-II. The molecular mass of the protein estimated on this assumption was found to be 7464 Da, which is in good agreement with that determined experimentally. This finding is interesting because, as we have recently shown (*2*), the ARYR (X)n $(BS)_3$ N-terminal sequence motif, that is present in the major protamine components of neognathous birds analyzed and in all mammals (*8*), seems to have first appeared in reptiles where it is present as a minor protamine component (*2*). Thus, the significance of the P-I sequence consisting of an N-terminal extension of P-II in ostriches suggests that the major protamine component of birds and mammals may have arisen from insertion of a sequence of DNA corresponding to the N-terminal sequence of P-I into a more primitive gene of P-II which was already present in lower terrestrial vertebrate groups. The reason for the evolutionary selection of the former over the latter remains to be established.

The close relationship of the major SNBP component (P−II) of ostriches and tinamous to the protamines of reptiles (see Figure 2) supports the fossil record and current molecular evidence which suggest that crocodilians and birds are closely related (*20*). However, due to the absence of

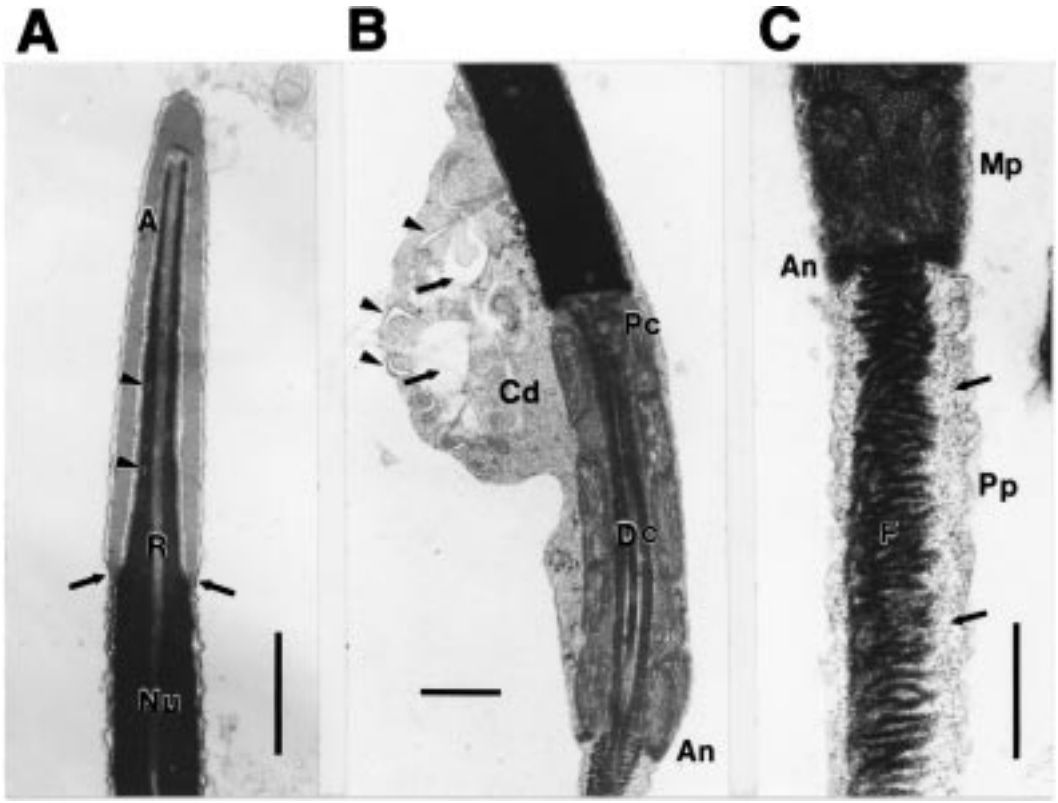

FIGURE 3: Electron micrographs of the head and midpiece of ostrich sperm. (A) A longitudinal section of the tip of the sperm head showing the acrosome (A) covering the tapered tip of the nucleus (Nu), the nuclear invagination containing the acrosomal rod (R), and the posterior ring (arrows). TEM 30750×. Bar = 0.5 μm. (B) A longitudinal section of the base of the head and midpiece. Note the persistent cytoplasmic droplet (Cd) attached at the head/midpiece junction. The droplet contains lipid-like droplets (arrows) and membranous structures (arrowheads). The distal centriole (Dc) extends the entire length of the midpiece, from below the proximal centriole (Pc) to the annulus (An). TEM 19500×. Bar = 0.5 μm. (C) A longitudinal section of the midpiece (Mp)/principal piece (Pp) junction. The proximal region of the principal piece immediately below the annulus (An) displays a wide cytoplasmic zone (arrows) between the fibrous sheath (F) and the plasmalemma. This zone contains fine granular material. The ribs of the fibrous sheath are shown in longitudinal profile due to the plane of section. TEM 35250×. Bar = 0.5 μm.

information on the SNBPs and the sperm organization of bipedal carnivorous dinosaurs, the biochemical information on SNBPs described here cannot rule out the origin of modern birds as well as Archosauria from a basal Archosaurian ancestor (*21*). The biochemical evidence provided by protamines for a link between reptiles and birds, in contrast to a closer bird−mammal relationship (*32*), is also supported by the structural peculiarities of the ostrich sperm shown in Figure 3. Morphological characteristics of sperm have proved to be useful for phylogenetic analysis (*33*, *34*). Ostrich sperm has several morphological features (*35*−*37*) which are also present in rhea (*38*) and tinamou (*39*) sperm, and which distinguishes these from other nonpasserine birds. These include a deep, cylindrical endonuclear canal housing an acrosomal rod; a long distal centriole extending the complete length of the midpiece; and the presence of a ribbed fibrous sheath and a wide cytoplasmic zone between the axoneme and the plasmalemma in the proximal part of the principal piece of the tail (see Figure 3). Based on these characteristics, the sperm of palaeognathous birds show some affinity with certain groups of reptiles such as the Chelonia, Crocodilia, and Sphenodontida (see also *40*, *41*).

The presence of a large amount (30−35%) of histidine in the protamines of paleognaths, which is also present in crocodilians and lizards (*2*) (see also Figure 2), confers a unique status on these proteins and differentiates them from the compositionally homogeneous protamines of the neognathous birds (*11*) (see Table 1). This observation, combined with the fast evolutionary rate of protamines (*8*), provides strong support for the phylogenetic relationships existing among Neornithes (modern birds) as depicted in Figure 4 from Chiappe (*21*) in which the Paleognathae constitute a monophyletic group (*41*−*45*). This conclusion is also supported by the distinctive structural features of the sperm of palaeognathous birds (*40*) which have been described above.

The reason for the appearance of histidine in reptiles and for its massive presence in paleognaths is not clear. It has been hypothesized that zinc, which is very abundant in the spermatozoa of both invertebrates and vertebrates (*47*), may bind to some of the histidine/cysteine residues which occur in mammalian P2 protamines. This could play a role in the way these proteins bind to DNA, possibly through zinc fingerlike motifs (*48*, *49*). In this respect, the presence of histidine in the protamines of reptiles and neognaths may represent a first attempt in this direction and/or it could simply reflect the presence of larger amounts of zinc in these two groups of vertebrates.

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