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Anion-mediated lysine–arginine interaction

Evidence in *Chaetopterus variopedatus* sperm protamine

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Chaetopterus ariopedatus sperm protamine is a stable oligomer. Specific amino acid side chain modifications show that the oligomeric structure depends on anion-mediated lysine–arginine interactions. The occurrence of this type of interaction is confirmed by the finding that poly-L-arginine readily forms aggregates with poly-L-lysine or with the native but not with the protamine with carbamylated ε-amino groups.

Ionic interaction; Protein structure; Protamine; *Chaetopterus variopedatus*

1. INTRODUCTION

Lysine–arginine interactions between protein side chains has not been recognized so far as a bond participating in the stability of tertiary and quaternary structures of proteins. This type of interaction, mediated by a chloride ion, has been reported so far only between the terminal amino group of Val-1 α_1 chain and the guanidino group of Arg-141 α_2 chain in deoxyhemoglobin [1]. NMR evidence has been presented on chloride ions binding to hemoglobin [2]. The unusual findings that the protamine of the marine worm, *Chaetopterus variopedatus*, has an oligomeric structure and that it is precipitated from its solution by addition of SDS [3], prompted a study to identify the groups contributing to those properties. The only difference in amino acid composition between the oligomeric protamine of *Ch. variopedatus* and fish sperm protamines, which have no tendency to form oligomers, is the presence of lysine residues in addition to the arginines. For this reason the possible implication of lysines in the stabilization of the oligomeric structure of the protamine was investigated. Studies on model compounds, such as poly-L-lysine and poly-L-arginine, were performed to completely exclude the contribution of other residues.

2. MATERIALS AND METHODS

2.1. *Materials*

Chemical reagents, poly-L-lysine and poly-L-arginine of approximately 11,000 M_r and molecular weight marker proteins were obtained from Sigma (USA). Gel filtration media were purchased from Pharmacia (Sweden); Cl^- Dowex 1 × 8, 200–400 mesh, was from Bio-Rad (USA). *Ch. variopedatus* protamine was prepared, and purity was determined, according to De Petrocellis et al. [3].

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2.2. *Amino acid side chain modifications*

Carbamylation of lysine ε-amino groups of protamine was performed by overnight reaction of 1 mg protein in 0.1 ml of 6 M urea, at 30°C, with 0.4 M cyanate. The mixture was then diluted with water to 1 ml and extensively dialyzed against water to remove excess reagent. Residual free amino groups were titrated with 2,4,6 trinitrobenzen-sulfonic acid [4].

Deguanidination of arginines was performed in aqueous solution by addition of 1 vol. of hydrazine to 0.1 ml of 10 mg/ml of protamine, followed by heating at 75°C for 1 h exactly as described [5]. Ornithines, formed by deguanidination of arginines, were determined by amino acid analysis.

2.3. *Amino acid analyses*

Samples were hydrolyzed in vacuo at 110°C for 20 and 60 h in 6 N HCl. After removal of HCl over NaOH in vacuo the hydrolyzates were filtered and analyzed on a Beckman Mod 119 CL amino acid analyzer.

2.4. *Gel filtration chromatography*

Sephadex G-100 columns, 0.4 × 35 cm and 1 × 30 cm, both equilibrated with 100 mM NaCl, 10 mM Tris, pH 7.2, were used and eluted at a flow rate of 0.5 ml/h and 2 ml/h, collecting respectively, fractions of 0.125 ml and 0.2 ml. Samples loaded in the gel filtration experiments contained 200 μg of protamine in 0.02 ml. The protein concentration in each fraction was determined by Coomassie blue staining [6]. Molecules in the eluted fractions were analyzed on 18% SDS-PAGE as reported [7], loading about 20 μg of protein in each slot.

2.5. *Deionization of the protamine*

Cl^- was exchanged with OH^- on a Dowex 1 × 8 resin, as suggested by the manifacturer, and packed in a 1 × 10 cm chromatography column, in distilled water. 0.5 ml of a 10 mg/ml protamine solution, extensively dialysed against water, was loaded and eluted from the column over a period of 3 h.

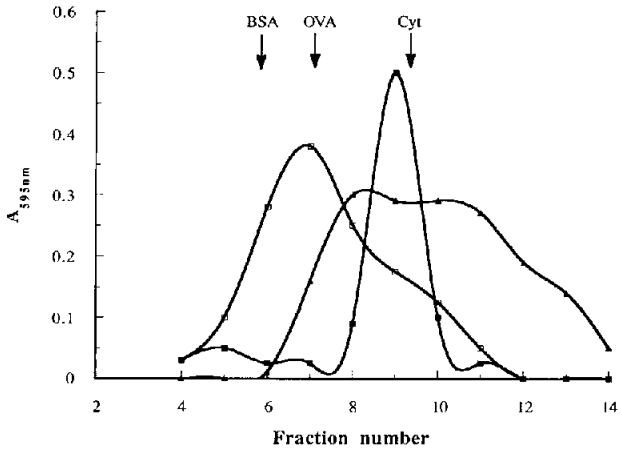

Fig. 1. Elution profiles from a 0.4 × 35 cm Sephadex G-100 column, equilibrated with 10 mM Tris, 100 mM NaCl, pH 7.2, of native (□), carbamylated (■) and deguanidinated (▲) derivatives of *Ch. variopedatus* sperm protamine. Arrows show the position of peak elution of the indicated marker proteins: BSA, bovine serum albumin (65 kDa); OVA, chicken ovalbumin (45 kDa); Cyt, cytochrome *c* from horse heart (13 kDa). For other details see section 2.

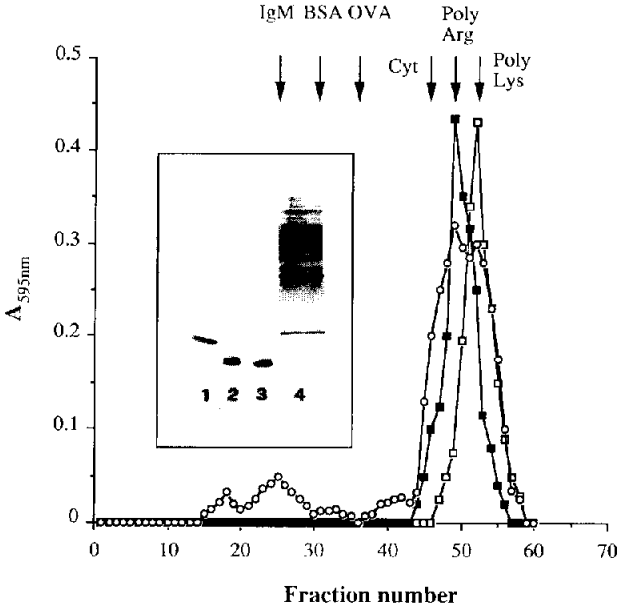

Fig. 2. Elution profiles from a 1 × 30 cm Sephadex G-100 column of poly-L-arginine (■), poly-L-lysine (□) and their 1:1 mixture (○). Conditions as in Fig. 1. IgM, Cohn fraction II bovine γ-globulin (140 kDa). Insert: SDS-PAGE patterns, lanes 1–4 of marker cytochrome *c*, poly-L-lysine, poly-L-arginine, and pooled fractions 20–30, respectively.

3. RESULTS AND DISCUSSION

The oligomeric state of *Ch. variopedatus* protamine depends on the presence of both free lysine amino groups and arginine guanidino groups, as apparent by comparing gel filtration patterns of the native and modified molecules (Fig. 1). In fact, while the native protamine is eluted in a volume corresponding to 45 kDa, the molecule with 95% carbamylated lysine ε-amino groups shows an elution profile corresponding to 16 kDa, the true molecular weight as derived from amino acid sequence studies (manuscript in preparation). The relevance of the guanidino groups is shown by the dramatic effects on the gel filtration of the protamine in which only 50% of the arginines are substituted with ornithines, as obtained by the deguanidination reaction (Table I). Both lysine and arginine side chains are almost unreactive in the native protamine. The high percent value of modified lysines was possible by performing the carbamylation reaction in 6 M urea. This denaturing condition could not be used for the deguanidination reaction, and change of reagent concentration, temperature of repeating cycles of the reaction invariably caused fragmentation of the protamine into smaller peptides. The low reactivity of the amino acid side chains of the protamine has a counterpart in the inability to remove the naturally occurring chloride ion. Attempts to exchange Cl^- with OH^- by slow filtration over a period of 3 h of a protamine solution, previously extensively dialysed against water, on a Dowex column charged with OH^-, gave no appreciable result. The low reactivity of the amino acid side chains and the inability to remove Cl^- from the protamine solutions is expected if these components are engaged in the formation of bonds.

To obtain independent and more direct evidence of the occurrence of lysine–arginine interactions, poly-L-arginine and poly-L-lysine were used as model compounds because each of them has only one type of amino acid residue. These molecules, when individually gel filtered, are eluted from Sephadex G-100 columns in

Table I

Amino acid composition (mol%) of native and of deguanidinated *Chaetopterus variopedatus* protamine

Sample	Amino acid*								
	T	S	P	G	A	V	K	R	Orn
Native	2.1	20.8	2.2	2.7	10.9	5.3	14.4	41.7	
Modified	2.1	21.2	2.1	3.3	11.7	5.9	14.2	19.1	20.1

*Only the amino acids present in the molecule are reported

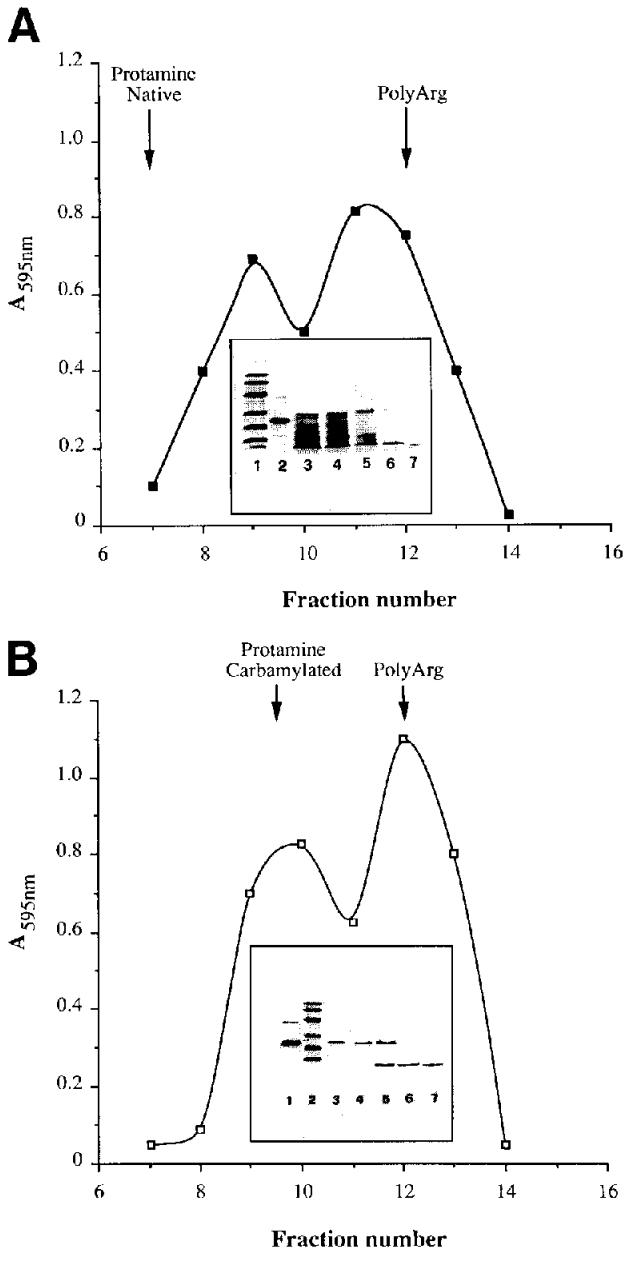

Fig. 3. Elution profiles from a 1 × 30 cm Sephadex G-100 column of 1:1 mixtures of poly-L-arginine and native (A) or carbamylated (B) protamine. Arrows show the peak elution of the indicated molecules and markers when individually filtered on the same column. Insert of A: lanes 1–7, SDS-PAGE analysis of molecular weight markers, carbamylated protamine, column fractions 9, 10, 11, 12, and poly-L-arginine, respectively. Insert of B: lanes 1–7, SDS-PAGE analysis of carbamylated protamine, molecular weight markers, column fractions 8, 9, 10, 11, and poly-L-arginine, respectively. Other conditions as in Fig. 1.

a volume corresponding to their sizes (Fig. 2). The elution profile of their 1:1 mixture shows the formation of multiple discrete forms of higher molecular sizes. SDS-PAGE analysis shows the presence in those bands of both the individual polymers and of their aggregated forms (insert of Fig. 2). It is evident that aggregates are formed only when the two polymers are mixed together, allowing lysine and arginine side chains to interact. A similar conclusion can be derived from the gel filtration analyses of mixtures of poly-L-arginine with native protamine (Fig. 3A) and with carbamylated protamine (Fig. 3B). The carbamylated protamine is eluted in the same volume both when filtered alone (position of the arrow) or in a mixture with poly-L-arginine, indicating no interaction between the two molecules (Fig. 3B). In contrast, native protamine shows different elution volumes when filtered alone or in a mixture with poly-L-arginine (Fig. 3A). Consistently, SDS-PAGE analyses of eluted fractions (inserts of Fig. 3) show aggregates between poly-L-arginine and the native but not the carbamylated protamine. Interestingly, the aggregates (inserts of Figs. 2 and 3) are not dissociated in the presence of SDS. This surprising result is in line with the previous finding that native protamine is soluble in water but is precipitated upon addition of SDS [3].

The interaction between lysine and arginine side chains, through the intermediate action of an anion, as demonstrated here for *Ch. variopedatus* sperm protamine and for model compounds, might contribute to the properties of cationic proteins. Particularly, one would expect that protamines that contain, in addition to arginines, also lysines, should show the occurrence of aggregated oligomeric forms. The effects of side chain modifications in histones, in which lysine and arginine residues are abundant and occur in different ratios, might similarly derive from perturbation of this type of interaction.

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