PHOSPHORYLATION OF HISTONE-LIKE COMPONENTS DURING SPERMIOGENESIS IN THE SEA URCHIN

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1. Introduction

Recently Ruiz-Carrillo [1] in our laboratory found that the very lysine-rich histone ϕ1 obtained from unripe male gametes of the sea urchin *Arbacia lixula* had the same amino acid composition as the equivalent component obtained from ripe sperm. However, the electrophoretic mobility of the protein was significantly reduced in the unripe cells. These results suggested to us that this protein was modified during gametogenesis in such a way that its net charge changed. Preliminary experiments showed that this behavior was due to the fact that this protein was heavily phosphorylated in unripe gametes. In this paper we present evidence which shows that phosphorylation also occurs in other sea urchin species as well as in other histone fractions during male gametogenesis.

2. Materials and methods

Unripe *Paracentrotus lividus* male gonads were homogenized in 0.25 M sucrose, 3 mM $CaCl_2$, and the nuclei recovered by centrifugation. After washing with 0.15 M NaCl, histone fractions ϕ1 and ϕ2b were prepared by method II according to Johns [2] as described elsewhere [1, 3]. Electrophoresis was carried out as described by Panyim and Chalkley [4] with slight modifications. Amino acid analyses were obtained in an Unichrom Beckman instrument. The amino acid analyses of the fractions were identical in ripe and unripe gametes. Their amino acid compositions are given in table 1.

Dephosphorylation experiments were carried out in 1 M Tris HCl buffer, pH = 8.0, at 37°–45°. The histone concentration was approx. 1 mg/ml. The length of the treatment and the concentration of enzyme varied in each case. The enzyme used was *E. coli* alkaline phosphatase (Worthington).

Table 1
Amino acid composition of histone-like components from *Paracentrotus lividus* spermatozoa.

Amino acid	ϕ1	ϕ2b
Lys	24.3	13.1
His	0.5	1.3
Arg	11.8	15.7
Asp	1.9	4.3
Glu	2.8	7.1
Thr	2.3	6.2
Ser	6.5	9.7
Pro	7.5	4.9
Gly	5.4	8.7
Ala	26.4	8.8
Met	1.5	0.2
Val	3.5	7.7
Ile	1.4	3.3
Leu	2.5	3.9
Tyr	1.1	2.6
Phe	0.5	1.6

No corrections have been made for hydrolytic losses. The amino acid compositions are expressed as moles/100 moles of all amino acids found.

The histone fractions ϕ1 and ϕ2b used in these experiments contained in all cases two or three bands, as shown in fig. 1 (a) and fig. 2 (a). The fast moving band is identical in electrophoretic mobility with the

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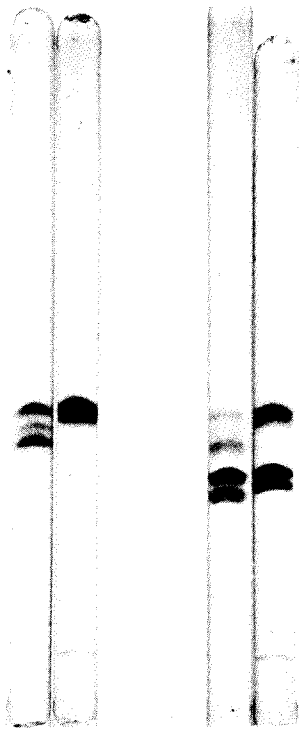

Fig. 1. Polyacrylamide gel electrophoresis of histones: a) ϕ2b component from unripe gametes; b) the same protein after treatment with alkaline phosphatase (8 μg/ml, 2 hr, 37°). The three bands have merged into a single one which has the same mobility of ϕ2b from ripe sperm; c) a mixture of ϕ1 and ϕ2b from unripe gametes; d) the same mixture after treatment with phosphatase (8 μg/ml, 2 hr, 37°). Under these conditions only the ϕ2b component is completely dephosphorylated. The slow ϕ1 component increases its mobility slightly, probably indicating partial dephosphorylation. The faint slow moving bands in frames *b* and *d* are due to the phosphatase present.

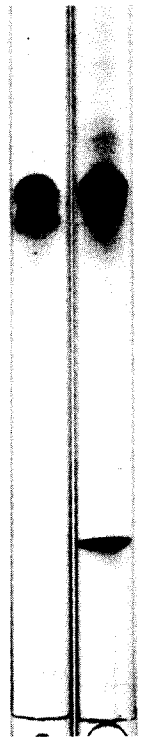

Fig. 2. Polyacrylamide gel electrophoresis of histone fraction ϕ1 before (a) and after (b) treatment with alkaline phosphatase (45 μg/ml, 1 hr, 45°). Under these more extreme conditions the ϕ1 histone is fully dephosphorylated.

equivalent component from ripe sperm. The fact that several bands are found may indicate that either the cells or the histone fractions in a single cell are heterogeneous. Probably both factors contribute to the heterogeneity found. However it should be noted that Ruiz-Carrillo [1] working with fractionated *A. lixula* cells was able to obtain a highly purified ϕ1 fraction which contained only the component with low electrophoretic mobility. This result showed that there are some unripe cells in the gonad which only contain this low mobility phosphorylated component. It should also be noted that the presence of three bands in ϕ2b may indicate that changes in phosphate content in this protein may take place in a discontinuous manner as gametogenesis proceeds. The three bands might correspond to proteins with 0, 1 or 2 phosphorylated residues.

Figs. 1 and 2 show the electrophoretic behavior of these proteins before and after treatment with alkaline phosphatase. After this treatment, the mobility of unripe cell histones increases and becomes identical with that of the sperm components. When a mixture of histones ϕ1 and ϕ2b is treated with a small amount of enzyme, only the ϕ2b component is dephosphorylated, as shown in fig. 1 (d). A more drastic treatment with higher amounts of enzyme is required in order to fully dephosphorylate the very lysine-rich histone ϕ1, as shown in fig. 2. This result indicates that the action of the phosphatase on fraction ϕ1 is not very efficient, probably due to the highly charged nature of this protein, which may interfere with the

Table 2
Estimation of the minimum phosphorylation of histones from electrophoretic mobilities

Histone fraction	Basic less acidic (%)	Decrease in electrophoretic mobility (%)	Equivalent number of positive charges (%)	Number of phosphorylated residues (%)
ϕ1	33.5	8.8	3.0	1.5
ϕ2b	22.5	10.5	2.4	1.2

All the amino acid data are expressed as moles/100 moles of total amino acids in the protein. The decrease in electrophoretic mobility is the average of several runs. In the results shown in the first column it is assumed that one third of the acidic groups are uncharged (in the amide form).

hydrolytic action of the enzyme.

In order to confirm the presence of phosphoserine, amino acid analyses were carried out after mild hydrolysis of the proteins (2 N HCl, 10 hr at 110°). Under these conditions, phosphoserine is only partially destroyed [5] and could be detected in all of the samples studied. The yield of phosphoserine detected in a pure ϕ1 component from *A. lixula* was 19% of all the serine content. Due to the low yield of this type of analysis, the amount of phosphorylation is probably higher.

The extent of phosphorylation can also be estimated from the changes in electrophoretic mobility, since each phosphorylated serine will neutralize two positive charges of the protein. The calculation is shown in table 2. The results obtained indicate that every 100 amino acid residues of the protein contain 1.5 serine plus threonine phosphorylated residues in ϕ1 and 1.2 in ϕ2b. These are only minimum values, since the calculation assumes that the shape of the protein does not change upon phosphorylation, whereas in practice it should be expected to contract due to the lower charge density. The measured difference in mobilities will therefore be less than the difference expected if the protein did not change in shape. Therefore it is likely that a higher proportion of all the serine and threonine residues are phosphorylated in the ϕ1 and ϕ2b histones from unripe male gametes.

It is interesting to note that Ingles and Dixon [6] found a similar phosphorylation of protamine during trout gametogenesis. They suggested that phosphorylation took place in order to partially neutralize the charges of the newly synthesized protamine to avoid interference with cell metabolism. This is also probably true in this case, since the very basic histones ϕ1 and ϕ2b are also synthesized during early gametogenesis. The fact that phosphorylation of sperm proteins occurs both in trout and sea urchins suggests that this process may take place also in other species as a general mechanism to prevent interference of newly synthesized basic proteins with cellular processes.

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