ACYLATION OF LYSINE AND OF ARGININE-RICH HISTONES WITH CARBAMYL PHOSPHATE AND 1,3 DIPHOSPHOGLYCERATE[1]

Giampietro Ramponi[2] and Santiago Grisolia

Dept. of Biochemistry, University of Kansas Medical Center, Kansas City, Kans.

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Summary: The formation of carbamyl and phosphoglyceryl derivatives of both lysine-rich and arginine-rich histones is demonstrated. Lysine-rich histone reacts more readily than arginine-rich histone. Acyl phosphatase prevents histone acylation. The large production of acyl phosphates and concentration of acyl phosphatase in tissues is pointed out, and it is suggested that acyl phosphatase plays a regulatory role in preventing histone acylation.

Introduction There is presently much interest in histones and in such derivatives as acyl, methyl and phosphoryl histones [1-8]. Phillips [1] detected ∿1 mole of carbohydrate positive material in lysine-rich histone isolated from calf thymus. Carbamyl-P (CP) and other acyl phosphates may react rapidly with lysine residues of proteins, e.g. with glutamic dehydrogenase [9]. Although CP and 1,3 diphosphoglycerate (1,3 PGA) are formed in very large quantities by mammals, the very large concentration of acyl phosphatase in tissues remains unexplained. While an extensive study has not been carried out as yet, experiments with some twenty proteins indicate that their susceptibility to carbamylation at or near neutral pH varies a great deal. As indicated, glutamic dehydrogenase is very readily carbamylated, while CP synthetase, ornithine transcarbamylase and P-glycerate mutase are not. It appeared then of interest to determine if CP and 1,3 PGA would react with histones, proteins known to be easily acylated, and to test whether acyl phosphatase prevents acylation of histones by CP and 1,3 PGA.

This paper answers the first question by demonstrating formation of carbamyl and 3-P-glyceryl histones. Moreover, by demonstrating marked inhibition of histone acylation by acyl phosphatase, it suggests that histone modification by CP and 1,3 PGA may be regulated by acyl phosphatase and that such a role, as

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[2]On leave of absence from the University of Florence, Department of Biochemistry.

well as the formation and/or presence of carbamyl and P-glyceryl histones in tissues, merits investigation.

Materials and Methods Lysine-rich and arginine-rich histones, type III and IV respectively, phosphoglycerate kinase, aldolase, glycerophosphate dehydrogenase and glyceraldehyde phosphate dehydrogenase were SIGMA products. Pyruvate kinase was a Boehringer preparation. CP synthetase, brain acyl phosphatase, enolase and phosphoglycerate mutase were prepared as previously described [10-13]. Sephadex was obtained from Pharmacia. CP was prepared from ^{14}C cyanate [14]. ^{14}C 3-P-glycerate was obtained from the Laboratory of Comparative Biochemistry, San Diego, California.

Proteins were estimated by measuring absorbancy at 230 mμ [15] and by the method of Lowry et. al. [16]. Carbamylated protein, P, and glyceric acid were determined colorimetrically [17-19]. Radioactivity was measured with a Packard Scintillation Counter.

In all experiments, unless specified otherwise, 10 mg protein, 1.0 ml and 2 hours incubation at 38° were used. The reactions were stopped with 10 ml of 25% trichloroacetic acid. After standing overnight at 5°, the samples were centrifuged. The precipitates from the control tubes containing albumin were dissolved in 7 ml of 0.1 N NaOH and the precipitates from the tubes containing histones were dissolved in 5 ml water, 2 ml ethanol and 0.2 ml concentrated HCl (any insoluble material was centrifuged off). Samples were mixed with 70 ml acetone, the precipitates collected by centrifugation and dissolved in 0.01 M NaCl (albumin samples may need a few drops of 0.1 N NaOH). The solutions were fractionated on Sephadex G-25 fine columns (2.5 cm x 44 cm for CP experiments, 1.5 cm x 47 cm for 1,3 PGA experiments) equilibrated and eluted with 0.01 M NaCl. 1.5 ml fractions were collected.

Results and Discussion Carbamylation with CP and with a generating system for CP were tested. Fig. 1 illustrates both by colorimetric and by radioactivity measurements the negligible carbamylation of albumin, while arginine-rich and particularly lysine-rich histone are readily carbamylated. The profiles show

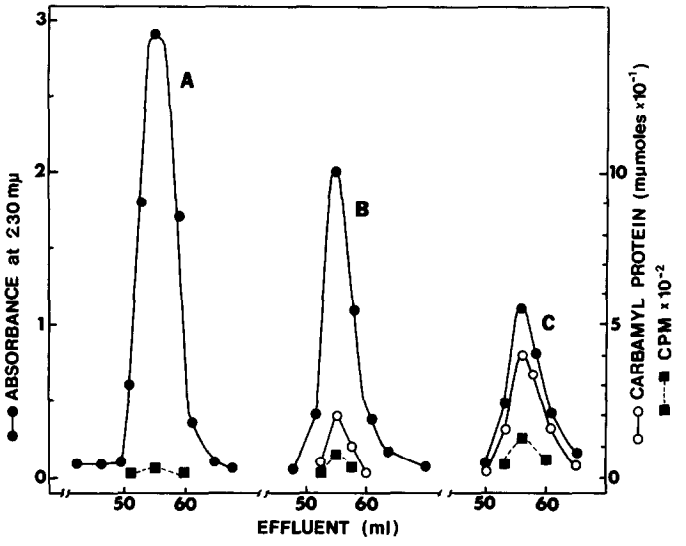

Fig. 1. Profile of incorporation of CP into several proteins. The conditions were as described in the text. 40 mM CP (3200 counts per μmole per min.) and 0.1 M phosphate buffer, pH 7.4, were used. A - albumin, B - arginine-rich histone, C - lysine-rich histone.

TABLE 1. CARBAMYLATION OF LYSINE-RICH AND ARGININE-RICH HISTONES

Experiment	Conditions	I Lysine-rich histone mμmoles/mg	II Lysine-rich histone mμmoles/mg	I Arginine-rich histone mμmoles/mg	II Arginine-rich histone mμmoles/mg
1*	Complete system	170	160	61	54
"	Plus acyl phosphatase	35	34	3	5
2	Complete system	40	37	22	21
"	Without carbamyl-P synthetase	0	0	0	0
"	Plus acyl phosphatase	17	18	8	9

The conditions were as described in the text except that for experiment 1 41 mM CP and 80 mM imidazol buffer, pH 7.4 were used; for experiment 2 the incubations contained: Tris-Cl^- pH 7.4, 50 μmoles; potassium bicarbonate, 50 μmoles (containing 2 μcuries ^{14}C); NH_4Cl, 50 μmoles; acetyl glutamate, 10 μmoles; ATP, 30 μmoles; 3-P-glycerate, 30 μmoles; $MgSO_4$, 40 μmoles; enolase, 60 units[12]; 3-P-glycerate mutase, 50 units[13]; pyruvate kinase, 15 international units; carbamyl-P synthetase, 2.5 units[6,9]; acyl phosphatase, 200 international units[20] in 1.1 ml.

The figures under columns I refer to ^{14}C incorporation. The figures under columns II indicate carbamyl protein measured colorimetrically.

*The specific activity of the CP used in experiment 1 was 3200 counts per min. per μmole.

the coincidence of protein, carbamyl protein and radioactivity incorporation; indeed the amount of incorporation agrees very closely with the amount of carbamyl protein detected.

Fig. 2 shows increased carbamylation of lysine-rich histone with increasing CP. It should be noted that this is the only set of experiments where we found some differences (~15%) between the radioactive and colorimetric measurements. We have no explanation for this. Table 1 illustrates carbamylation with added CP and with CP synthesized de novo with CP synthetase. As shown, acyl phosphatase markedly inhibits carbamylation.

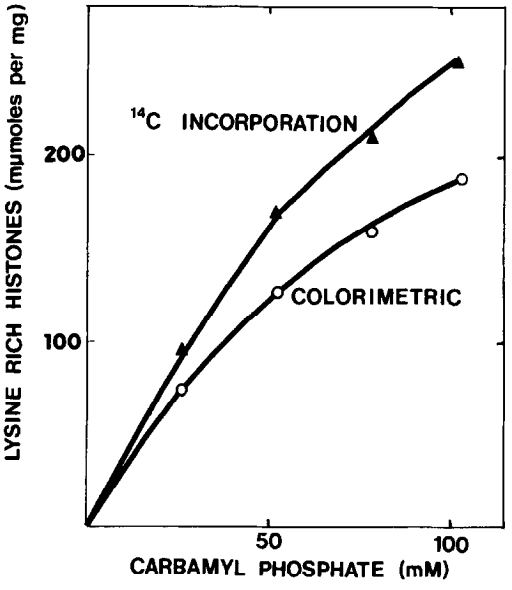

Fig. 2. The influence of CP concentration on the formation of carbamyl histone. The conditions were as described in the text except that the CP (3200 counts per μmole per min.) was added as indicated.

Fig. 3 illustrates incorporation of 1,3 PGA with arginine-rich histone and particularly with lysine-rich histone. To check whether phosphate as well as the carbon chain of 1,3 PGA remained attached to the protein other experiments were carried out; the fractions containing the bulk of protein were concentrated by lyophilization after separation in columns. The concentrated fractions were then analyzed. As shown in Table 2, the agreement between phosphate and ^{14}C

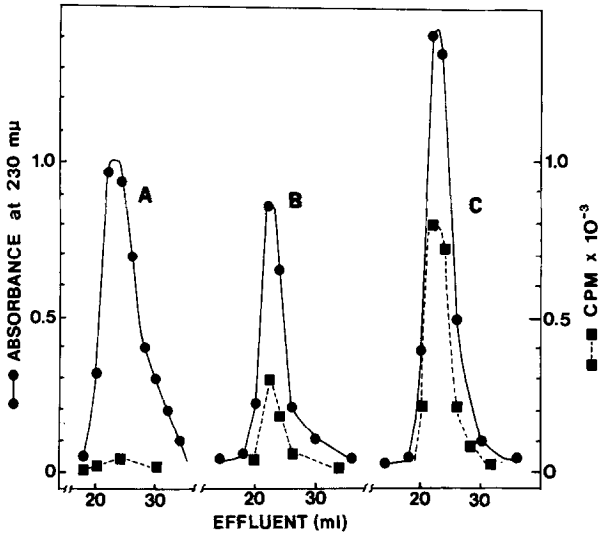

Fig. 3. Profile of incorporation of 1,3 PGA into several proteins. The conditions were as indicated in the text except as follows: sodium phosphate buffer, pH 7.4, 160 μmoles, NAD 5 μmoles, fructose diphosphate 5 μmoles (containing 1.5 μcuries ^{14}C), aldolase 100 μg, glyceraldehyde phosphate dehydrogenase 100 μg, glycerophosphate dehydrogenase 100 μg. The reaction was started by addition of aldolase. At 10, 20 and 30 min. 5 μmoles of fructose diphosphate in 10 μl were added. Total incubation 40 min. at 25°. A - albumin, B - arginine-rich histone, C - lysine-rich histone.

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incorporation is excellent. Moreover, the amount of modified histone is larger with lysine-rich than with arginine-rich histone. Also, in agreement with CP experiments, acyl phosphatase almost completely prevents histone acylation. Table 2 also illustrates acylation of histones even when using the very thermodynamically unfavorable P-glycerate kinase system for 1,3 PGA synthesis.

It should be noted that under the conditions used there is very little acylation of albumin but extensive acylation of histones with CP or 1,3 PGA. Whether CP is added or made de novo, there is rapid carbamylation. As shown in Fig. 4 CP and 1,3 PGA compete for histone acylation; this agrees with the fact that acetyl-P, CP as well as 1,3 PGA are substrates in several reactions, e.g. acyl phosphatase carbamate-acetate kinases, carbamyl-P-synthetase and several transcarbamylases [10].

TABLE 2. INFLUENCE OF COMPONENTS IN FORMATION OF P-GLYCERYL HISTONES

Experiment	Conditions	mμmoles P incorporated per mg histone	mμmoles ^{14}C incorporated per mg histone
1	Complete*	26	26
"	No triosephosphate dehydrogenase	3	3
"	No glycerophosphate dehydrogenase	5	5
"	Plus 200 units brain acyl phosphatase	--	3
2	Lysine-rich histone	4.7	5
"	Arginine-rich histone	2	2
"	Albumin	0	0

For experiment 1, the conditions were as indicated in the text and in legend of Fig. 3 using lysine-rich histone. There were 14 and 15 mμmoles of P and ^{14}C incorporated respectively when arginine-rich histone was used. There was less than 4 mμmoles of ^{14}C incorporated when histones were replaced by albumin. For experiment 2, a different generating system for 1,3 PGA was used as follows: Triethanolamine buffer, pH 7.4, 50 μmoles, EDTA, 10 μmoles, 7.5 μmoles $MgSO_4$, 2.5 μmoles glutathione, 30 μmoles ATP, 80 μmoles KCl, 50 μmoles PEP, 0.1 mg of phosphoglycerate kinase, all in 1 ml. The reaction was started by addition 50 μl containing 350 nanomole (1.1 μcurie) of ^{14}C 3-P-glycerate. At 10 min. 0.1 ml containing 30 μmoles of 3-PGA was added. At 25 min. 10 μl containing 10 μg pyruvate kinase was added. 50 min. total incubation at 25°. There was no or negligible incorporation when pyruvate kinase or P-glycero kinase were omitted.

*Glycerate detected in these samples; unfortunately the color changes in the presence of protein and the method[18] becomes semi-quantitative.

To determine whether carbamylation from CP proceeds via cyanate, carbamylation of histones with KCNO was tested and found to proceed more readily than with CP. Therefore a time and concentration study with CP should show an increase in carbamylation due to spontaneous decomposition of CP to cyanate. This was not the case. Moreover, acyl phosphatase interferes markedly with carbamylation, and since the product of CP phosphatase action is not cyanate but carbamate the carbamylation studies reported here must proceed via CP. However, carbamylation with cyanate may be of practical interest in that carbamyl histones could be formed as artifacts when using high concentration of urea to solubilize nuclear protein.

[21]

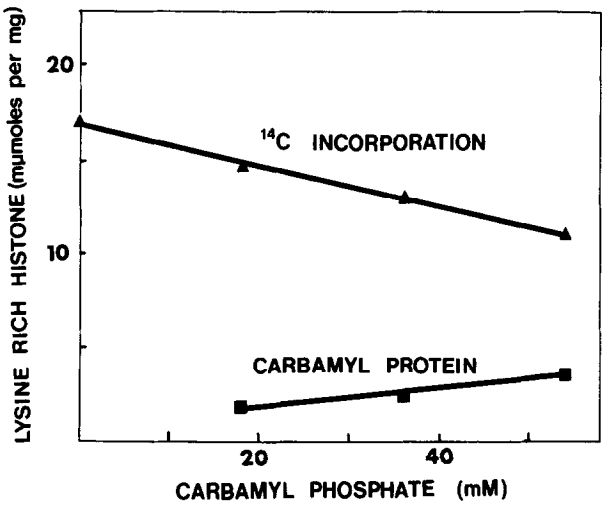

Fig. 4. Competition for acyl histone formation with 1,3 PGA and CP. The conditions were as described in the legend of Fig. 3, except that CP was added as indicated.

Whether there are enzymes catalyzing formation of the acyl histone here described (ornithine transcarbamylase is inactive) and whether they are substrates, as is the case for other histone derivatives [22,23] remains to be investigated. Acyl histones may be formed readily in biological systems simply by chemical interaction; some acyl phosphates are made in very large quantities, e.g. for a 70 kg man ∿1 mole CP per day in ∿1 kg of liver!, and ∿2 to 4 moles of 1,3 PGA (via glycolysis) mostly in muscle and liver. Therefore additional studies including formation, existence, role and particularly regulation by acyl phosphatase, seem of interest.

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