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HISTONE DEACETYLASE FROM CALF THYMUS

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SUMMARY

1. An enzyme activity which catalyzed the deacetylation of histone was found in calf thymus extract.

2. [^{14}C]Acetyl-labeled histone, prepared by incubating calf thymus nuclei with sodium [^{14}C]acetate, served as substrate and treatment of the histone with pronase destroyed most of the susceptibility to the enzymatic deacetylation. [^{14}C]-Acetyl-labeled histone prepared by chemical acetylation with [^{14}C]acetic anhydride served as substrate to a lesser extent.

3. The enzyme had a pH optimum at 7.0.

4. The enzyme reaction was inhibited by sulfhydryl reagents.

5. Chromatographic profile on DEAE-cellulose indicated the multiplicity of the enzyme. Large molecular weight of the enzyme was indicated by the gel filtration on Sephadex G-200.

6. Most of the enzyme activity was found in the soluble fraction rather than in the nuclear fraction.

7. The mouse liver extract had little deacetylase activity and had an inhibitory effect on the deacetylation reaction by calf thymus extract.

INTRODUCTION

It is known that histone can be acetylated, phosphorylated and methylated. Such enzymatic modifications may participate in the regulation of the gene expression by altering the structure of chromatin or the binding of histones to DNA (for a recent review, see ref. 1).

Acetyl groups in histones are attached to the terminal α-amino groups[2] or to the ε-amino groups of the specific lysine residues[3,4].

Turnover of these acetyl groups in histones is much more rapid than the turnover of histones themselves[5].

We will report here the presence and the properties of an enzyme activity which catalyzes deacetylation of histones in calf thymus. A preliminary report of the work has appeared[6].

Abbreviation: PCMB, *p*-chloromercuribenzoate.

METHODS

Preparation of enzyme

Calf thymus (2.1 g) was finely minced with scissors and homogenized in 0.14 M NaCl (5.0 ml) in a Potter–Elvehjem homogenizer at 0° for 3 min. The homogenate was centrifuged at 10 000 × *g* for 15 min. The supernatant was frozen at −20°, thawed at room temperature and centrifuged at 12 000 × *g* for 30 min. The supernatant was used as the enzyme preparation.

Preparation of substrate

Labeled histone was prepared by incubating calf thymus nuclei with sodium [1-^{14}C]acetate (20 μC/approx. 3.0 g nuclei, specific activity 40 mC/mmole, purchased from Daiichi Chemical Co., Tokyo) according to the method of GERSHEY *et al.*[3] except that 0.25 M sucrose–1 mM spermidine phosphate adjusted to pH 7.1 was used instead of 0.25 M sucrose–1 mM spermidine solution. The neutral medium resulted in a high incorporation of radioactive acetate into histone. Whole histone was extracted with 0.25 M HCl at 0° for 18 h from the thoroughly washed nuclei, precipitated by adding 10 vol. of acetone to the extract and collected by centrifugation. After being washed with acetone, the whole histone was dissolved in 2 mM HCl and lyophilized.

The presence of [^{14}C]acetyl groups in the histone was confirmed by the correlation of the radioactivity peaks to the histone fractions in the CM-cellulose chromatography, according to the method of JOHNS *et al.*[7]. Histone fractions corresponding to f1, f2, f3 accounted for 26, 32 and 22% of the total radioactivity applied on the column and 25, 36 and 15% of the total proteins measured by absorption at 280 mμ, respectively.

VIDALI *et al.*[8] reported that all of the radioactivity in the labeled histone prepared under these conditions was recoverable as [^{14}C]acetate after acid hydrolysis and that no hydroxylamine-labile acetyl groups were present, *i.e.* no O-acetyl was present. We confirmed these observations. When histone was treated with 2 M HCl at 110° for 46 h, 98% of the radioactivity became volatile. When histone was treated with 10.3% hydroxylamine (pH 6.6) at room temperature for 5 h (ref. 9) and recovered by adding 9 vol. of acetone, the recovered histone retained 97% of the radioactivity of the original histone.

Acetylation of histone with [^{14}C]acetic anhydride

Whole histone sulfate from calf thymus (60 mg) was treated with [1-^{14}C]acetic anhydride (20 μC, 1.1 μmoles) in 2.5 ml of 2 M sodium acetate (pH 8.1) at 0° for 4 h. After the treatment, histone was recovered by precipitation with ethanol (5.0 ml) and washed thoroughly with it.

About 20% of the [^{14}C]acetyl groups in the preparation was hydroxylamine-labile.

Assay of enzyme activity

The enzyme activity was determined by measuring the release of radioactive acetic acid from labeled histone. The assay procedure, employed routinely, is as follows: [^{14}C]Acetyl-labeled histone was incubated with the enzyme preparation at

37° in a final volume of 0.6 ml in 0.025 M Tris–HCl buffer (pH 7.3) or in 0.025 M sodium phosphate buffer (pH 7.0). After the incubation, the reaction mixture was acidified by the addition of 0.1 ml of 1 M HCl containing 5 μmoles of carrier acetic acid. Acetic acid was extracted by shaking with 3 ml of ethylacetate. 2 ml of the organic layer was taken, mixed with the scintillator solution and the radioactivity was measured by a Kobe Kogyo liquid scintillation counter, Model GSL-111. Values were corrected for the efficiency of the extraction with ethylacetate and for the efficiency of the counting (approx. 80%).

Determination of protein concentration

Protein concentration was determined by the method of LOWRY *et al.*[10] with bovine serum albumin as standard.

RESULTS

Extraction efficiency of [^{14}C]acetate from reaction mixture

Since no simple method for the determination of labeled acetic acid in the reaction mixture is known, extraction of labeled acetic acid with various solvents was tested. Ethyl acetate and ethyl ether were more effective for the extraction of acetic acid than cyclohexane, benzene or methylene chloride.

Since ethyl acetate has a higher boiling point and could be handled more easily than ethyl ether, ethyl acetate was used as the solvent. The extraction efficiency varied with the volume of the reaction mixture. The standard curve of extraction efficiency was figured against the volume of the acidified solution (Fig. 1).

Time-course of the reaction

Fig. 2 shows the time-course of the deacetylation reaction when labeled

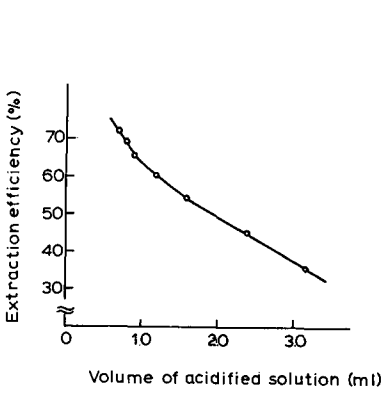

Fig. 1. Extraction efficiency of [^{14}C]acetate from reaction mixture. 3 ml of ethyl acetate were shaken vigorously with 0.125 M HCl–0.016 M acetic acid solution containing sodium [^{14}C]acetate (7240 disint./min) and left standing for 30 min. 2 ml of the organic layer was taken, mixed with scintillator and the radioactivity was measured.

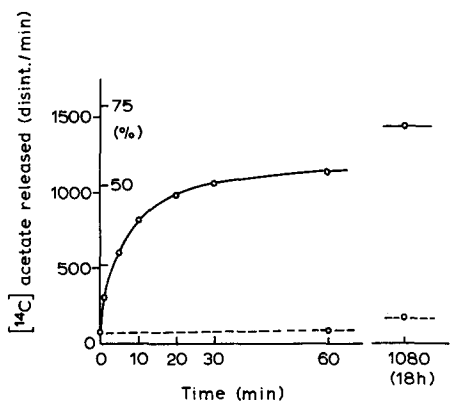

Fig. 2. Time-course of the deacetylation reaction. The labeled histone (2.5 mg, 2100 disint./min) was incubated with (○—○) or without (○— — —○) the calf thymus extract (4.0 mg protein) at 37° in a final volume of 0.6 ml in 0.025 M Tris–HCl buffer (pH 7.3).

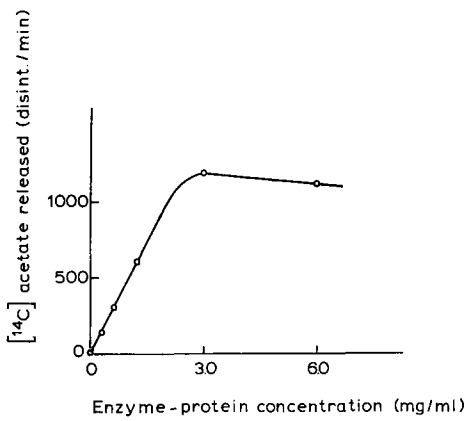

Fig. 3. Effect of enzyme-protein concentration. Labeled histone (0.22 mg, 2650 disint./min) was incubated at a various enzyme concentration at 37° for 30 min in 0.6 ml of 0.025 M Tris–HCl buffer (pH 7.2).

histone was incubated with the calf thymus extract. After a 60-min incubation, about 55% of the [^{14}C]acetyl groups was released. Without the enzyme, no significant deacetylation occurred.

Effect of enzyme-protein concentration

There is a linear relationship between the release of [^{14}C]acetate and the concentration of the enzyme extract up to 3 mg/ml (Fig. 3).

Confirmation of the liberation of acetate

In order to confirm the liberation of [^{14}C]acetate and to check the possible contamination of [^{14}C]acetate-labeled-amino acids or [^{14}C]acetate-labeled peptides in the ethyl acetate extract, acetic acid was separated by azeotropic distillation from the ethyl acetate extract. Radioactivity in the distillate (*i.e.* [^{14}C]acetic acid) accounted for about 90% of the ethyl acetate-extractable radioactivity (radioactivity: 1044 disint./min in the extract and 935 disint./min in the distillate).

Substrate specificity

As shown in Table I, treatment of the [^{14}C]acetate-labeled histone with pronase destroyed most of the susceptibility to enzymatic deacetylation. The results indicate that the deacetylase attacks the acetyl groups in a large molecule and is different from other known aminoacylases. The results, therefore, ruled out the possibility that the acetate liberation from histone resulted from the cooperation of proteases with aminoacylase (EC 3.5.1.14) or acyllysine deacylase (EC 3.5.1.17).

After the incubation of labeled histone (3.3 mg, specific activity 9030 disint./min per mg) with the calf thymus extract (10 mg protein) for 20 min at 37°, histone was recovered from the incubation mixture by precipitation with ethanol, extraction with 0.125 M H_2SO_4 and reprecipitation with ethanol. The recovered histone fraction showed decreased specific activity (1320 disint./min per mg).

On polyacrylamide gel electrophoresis[11], the recovered histone gave five bands

TABLE I

SUBSTRATE SPECIFICITY; COMPARISON OF THE DEACETYLATION OF THE HISTONE PREPARATIONS

[^{14}C]Acetyl-labeled histone (2.5 mg) was incubated with the calf thymus extract (4.0 mg protein) at 37°.

Substrate	*Activity in substrate (disint./min)*	*Incubation time (min)*	*[^{14}C]Acetate released*	
			Activity (disint./min)	*Degree of deacetylation (%)*
Labeled histone	2100	0	97	5
		10	912	44
		30	1017	49
Pronase-digested labeled histone*	2100	0	36	2
		10	133	6
		30	157	7
Chemically acetylated histone	10000	0	349	3
		10	524	5
		30	888	9

* Labeled histone (2.5 mg, 2100 disint./min) was digested with pronase (0.014 mg, purchased from Kaken Chemical Co., Tokyo) in 0.3 ml of Tris–HCl buffer (pH 7.3) at 37° for 60 min. After the digestion, the solution was heated at 100° for 15 min and served for the deacetylation reaction.

corresponding to major histone fractions and two bands which were not present in the original histone. It would appear that the deacetylation of histone was not accompanied by degradation of histone.

Labeled histone prepared by incubating nuclei with [^{14}C]acetate served as a better substrate than the chemically acetylated histone (Table I). After 10 min incubation, 44% of [^{14}C]acetyl groups in the biologically acetylated histone was released, while only 5% of [^{14}C]acetyl groups was released in the case of the chemically acetylated histone.

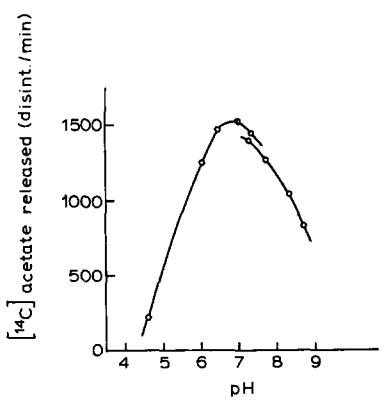

Fig. 4. pH optimum. Labeled histone (0.22 mg, 2650 disint./min) was incubated with the enzyme preparation (2.9 mg protein) at 37° for 20 min in 0.025 M phosphate buffer (pH 4.6–7.35) or in 0.025 M Tris–HCl buffer (pH 7.3–8.77) in a final volume of 0.6 ml.

TABLE II

EFFECTS OF INHIBITORS ON DEACETYLASE ACTIVITY

The enzyme preparation, preincubated with inhibitor at room temperature for 10 min, was assayed with labeled histone at 37° for 6 min in 0.7 ml of 0.03 M phosphate buffer (pH 7.0).

Inhibitor	*Concentration (mM)*	*Inhibition (%)*
Sodium acetate	12.5	76
	1.25	26
NH_4Cl	12.5	3
Spermidine phosphate	1.25	4
EDTA	1.4	14
Mercaptoethanol	1.3	17
3',5'-AMP	1.4	20
Monoiodoacetic acid	1.4	90
	0.14	53
PCMB	1.3	79
	0.13	13
$HgCl_2$	1.4	98
	0.14	61

pH optimum

The effect of pH on deacetylation reaction was examined. The pH optimum was found at 7.0 as shown in Fig. 4.

Effect of inhibitors on deacetylase activity

Effects of various inhibitors on the deacetylase activity were examined (Table II). Acetate, the product of the reaction, inhibited the deacetylation reaction at a high concentration. NH_4^+ and spermidine, which is a polycationic molecule having amino groups, had no effect on the reaction. EDTA and cyclic 3',5'-AMP inactivated the enzyme activity slightly. The deacetylase was inhibited effectively by sulfhydryl reagents such as iodoacetate, *p*-chloromercuribenzoate (PCMB) and Hg^{2+}. The results suggest the involvement of a sulfhydryl group at the active site of the enzyme. Mercaptoethanol, however, had no accelerating effect on the deacetylase.

Effect of NaCl concentration on deacetylase activity

A high ionic strength inhibited the deacetylation reaction as shown in Fig. 5. The enzyme activity decreased gradually as NaCl concentration increased. At a high concentration of 1.5 M NaCl, 86% inhibition of the enzyme activity was observed.

DEAE-cellulose chromatography

Fig. 6 shows the results of the chromatography on DEAE-cellulose with a linear gradient of NaCl. The enzyme activity was eluted at the NaCl concentration of 0.05–0.3 M. Four not well-resolved peaks of the deacetylase activity were obtained. This elution pattern, suggesting the multiplicity of the enzyme, was reproducible.

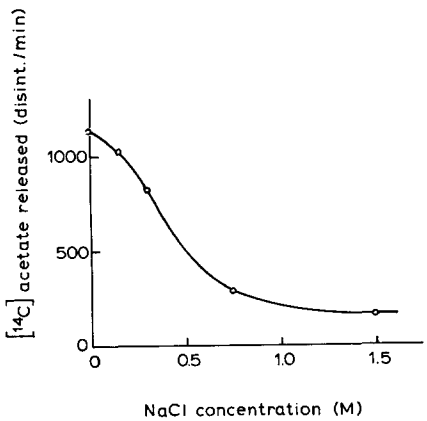

Fig. 5. Effect of NaCl concentration on deacetylase activity. 5 ml of the enzyme preparation were dialyzed against 100 ml of 0.05 M phosphate buffer (pH 7.0) at 0°. Labeled histone (0.22 mg, 2950 disint./min) was incubated at 37° for 20 min with 0.1 ml of the dialyzed enzyme solution (1.6 mg protein) at various concentrations of NaCl in a final volume of 0.6 ml in 0.03 M phosphate buffer (pH 6.6–6.7).

Sephadex G-200 gel filtration

Fig. 7 shows the results of the gel filtration on Sephadex G-200. The peak of the enzyme activity was one fraction behind the exclusion peak of the column. The results suggest that the deacetylase has a considerably high molecular weight.

Localization of deacetylase activity in calf thymus

Table III shows the distribution of the deacetylase activity. Most of the enzyme

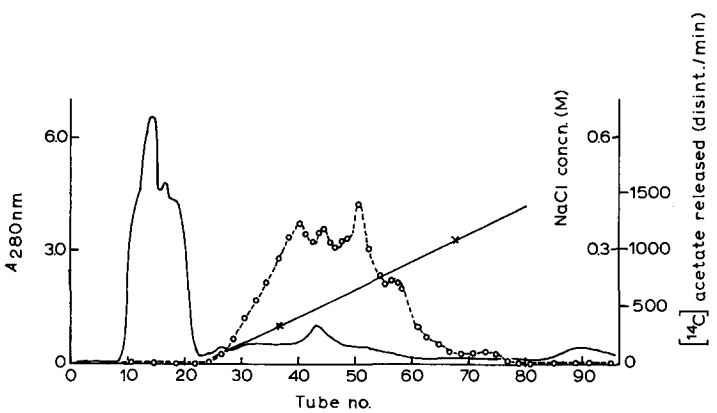

Fig. 6. DEAE-cellulose chromatography. The enzyme solution (685 mg protein) was applied on a DEAE-cellulose column (3.0 cm × 26 cm), which was equilibrated with 0.05 M phosphate buffer (pH 6.8). The column was washed with 75 ml of the same buffer before a linear NaCl gradient was started. The gradient was produced from 750 ml each of 0.05 M phosphate buffer containing 0.05 M NaCl and the buffer containing 0.66 M NaCl (×—×). Fractions of 15 ml were collected at a flow rate of 56 ml/h. The absorbance (———) at 280 mμ was recorded. The enzyme activity (○ -- ○) was assayed as follows: 0.2-ml aliquots of fractions and labeled histone (0.33 mg 4420 disint./min) were incubated at 37° for 60 min in a final volume of 0.7 ml in 0.02 M phosphate buffer (pH 7.0).

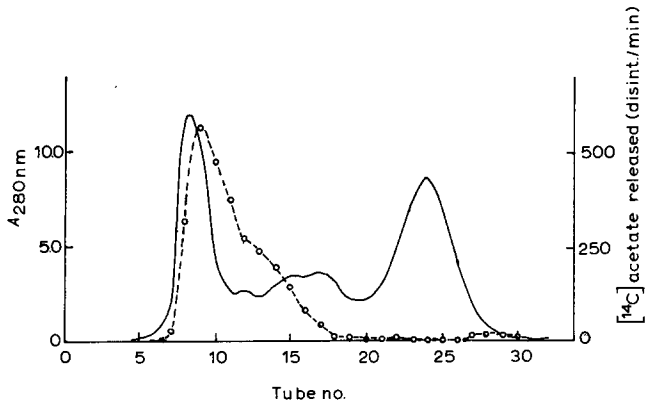

Fig. 7. Sephadex G-200 gel filtration. A column (1.5 cm × 39 cm) of Sephadex G-200 was equilibrated with 0.1 M NaCl–0.05 M phosphate buffer (pH 7.0). The enzyme preparation (145 mg protein) in 3.3 ml of the same buffer was charged. Fractions of 2.7 ml were collected at a flow rate of 6 ml/h. The absorbance (———) at 280 mμ was recorded. The enzyme activity (○— — —○) was assayed as follows: 0.3-ml aliquots of fractions were incubated with labeled histone (5.6 mg, 1250 disint./min) at 37° for 120 min in a final volume of 0.6 ml in 0.025 M phosphate buffer (pH 7.0).

activity was found in the 100 000 × *g* supernatant obtained by centrifuging the tissue homogenate.

Inhibitory effect of mouse liver extract on deacetylation reaction

To demonstrate the presence of the deacetylase activity in other species, mouse liver extract was tested by incubation with labeled (calf thymus) histone (Table IV). The deacetylase activity found in the mouse liver extract was only 9% of that of calf thymus. Futhermore, the deacetylase activity of calf thymus was inhibited by the mouse liver extract, and dialysis did not change the inhibitory effect of the mouse liver extract. These results indicate that an inhibitory factor is

TABLE III

LOCALIZATION OF DEACETYLASE ACTIVITY IN CALF THYMUS

The enzyme extract was prepared from 2.1 g of calf thymus in 5.0 ml of 0.14 M NaCl as described in METHODS. 100 000 × *g* supernatant was obtained by centrifuging 6 ml of the enzyme extract at 100 000 × *g* for 60 min at 0°. The residual pellet was suspended in 6.0 ml of 0.14 M NaCl by a homogenizer. The suspension was designated as 100 000 × *g* pellet. Nuclei were isolated from 2.1 g of calf thymus and homogenized in 5.0 ml of 0.14 M NaCl. 0.2 ml each of the enzyme solutions was assayed by incubating with labeled histone (0.22 mg, 2650 disint./min at 37° for 30 min.

Fractions	*[^{14}C]Acetate released (disint./min)*
Enzyme extract	1162
Nuclei	81
100 000 × *g* supernatant	1322
100 000 × *g* pellet	188

TABLE IV

INHIBITORY EFFECT OF MOUSE LIVER EXTRACT ON DEACETYLATION REACTION BY CALF THYMUS EXTRACT

Mouse liver extract was prepared by the same method as described with calf thymus. 0.2 ml of the mouse liver extract was dialyzed against 100 ml of 0.05 M phosphate buffer (pH 7.0) at 0° for 15 h and used as "dialyzed mouse liver extract". Labeled histone (0.22 mg, 2950 disint./min) was incubated at 37° for 5 min in 0.7 ml of 0.03 M phosphate buffer (pH 7.0) with 0.1 ml of the calf thymus extract and/or 0.1 ml of the mouse liver extract.

Enzyme preparation	*[^{14}C]Acetate released (disint./min)*	*Enzyme activity (%)*
Calf thymus extract	800	100
Calf thymus extract + mouse liver extract	203	25
Calf thymus extract + dialyzed mouse liver extract	197	25
Mouse liver extract	74	9

present in the mouse liver extract and that the factor seems to be a large-molecular substance.

DISCUSSION

Histone deacetylase extracted from calf thymus was examined. The enzyme catalyzes the deacetylation reaction of histone to give acetic acid and deacetylated histone.

Labeled histone prepared by incubating calf thymus nuclei with radioactive acetic acid could serve as a better substrate than the chemically acetylated histone. [^{14}C]Acetyl groups in histones labeled biologically are known to attach to ε-amino groups of the specific lysine residues[4]. The chemically acetylated histone has not been characterized, but acetyl groups are probably introduced nonspecifically into α-amino groups of N-terminal amino acids, ε-amino groups of a number of lysine residues and hydroxy groups of hydroxy amino acids. The deacetylase demonstrated here may be considered to have a high degree of specificity to distinguish specific acetyl groups in the histone molecule.

The chromatographic pattern on DEAE-cellulose suggested the multiplicity of the enzyme. It is possible that each enzyme fraction corresponds to an individual histone fraction.

Distribution studies revealed a higher deacetylase activity in the soluble fraction of tissue homogenate than in the nuclear fraction. Interestingly, similar observations have been reported that the enzymes which are thought to function in the nucleus, such as DNA polymerase[12], histone phosphatase[13] and histone acetylase[14], are present predominantly in cytoplasm rather than in nuclei. The different enzyme levels in cytoplasm and in nuclei may reflect the operation of a control mechanism for the enzyme activity in nuclei.

There is considerable evidence for a correlation between the acetylation of histones and the increase in RNA synthesis[15]. If histone acetylation and deacetylation are involved in gene activation and repression, a mechanism which controls selective acetylation and deacetylation of the histones associated with the specific genes

should be present. Recently, it was reported that other chromosomal components, chromosomal RNA and/or nonhistone proteins may be involved in the regulation of gene expression, and they may function as a base-sequence detector for the specific genes[16–18]. Studies on the effect of the RNA and nonhistone proteins on the susceptibility of histones to the deacetylase are now in progress in our laboratory.

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