MULTIPLICITY OF HISTONE DEACETYLASE FROM CALF THYMUS

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Received 20 November 1972

1. Introduction

Histone is modified enzymatically with acetyl, methyl and phosphoryl groups, and enzymes catalyzing these reactions may play an important role in the regulation of gene expression [1]. Recently, the multiplicity and organ specificity of histone acetylase were reported [2]. These features of the enzyme may be compatible with the proposed role of histone acetylation in the complex process of gene regulation.

Histone deacetylase was demonstrated first in an extract of calf thymus [3, 4] and subsequently in extracts of various animal and plant tissues [5, 6]. The enzyme from calf thymus acts on the specific ε-*N*-acetyllysyl residues in arginine-rich histone fractions, f2a1 and f3 [7]. However, the enzyme has not yet been purified and the question as to whether the deacetylation of histone is achieved by multiple enzymes, as is the case with histone acetylation, remains to be solved.

We report here the partial purification of histone deacetylase from calf thymus. During the purification, we obtained the results which suggested the multiplicity of the enzyme.

2. Methods

[^{14}C]Acetate labeled histone was prepared as described previously [4]. Histone fractions, f2a1 and f3 were separated by gel filtration on a Bio-Gel P-60 column [8, 9]. Soluble chromatin was prepared according to the method of Zubay and Doty [10]. Assay of histone deacetylase activity was carried out as described previously [4]. Protein concentration was determined by the method of Lowry et al [11]. Hydroxylapatite was prepared by the method of Tiselius [12].

Histone deacetylase was extracted from calf thymus (8.4 g) by homogenization in 0.05 M phosphate buffer, pH 7.0 (20 ml) in a Potter-Elvehjem homogenizer. The homogenate was centrifuged at 10,000 *g* for 15 min and the supernatant was used as the crude enzyme preparation. The crude enzyme was frozen, thawed and centrifuged at 70,000 *g* for 30 min. To the supernatant solution (20 ml), hydroxylapatite (packed volume 40 ml, equilibrated with 0.01 M phosphate buffer, pH 7.0) was added. After stirring for 15 min, the hydroxylapatite was collected by low speed centrifugation. The enzyme was then eluted from the hydroxylapatite with 40 ml of 0.25 M phosphate buffer, pH 7.0, containing 5% (v/v) glycerol, 1 mM $MgCl_2$ and 1 mM 2-mercaptoethanol, with stirring for 60 min. The extraction was repeated with 20 ml of the same buffer for 15 min and the eluates were combined and concentrated with Diaflo, UM-10 membrane filter to 10 ml. The solution was then applied on a Sepharose 4B column and eluted as described in the legend for fig. 1. The Sepharose 4B eluate was then subjected to DEAE-cellulose column chromatography as described in the legend for fig. 2.

3. Results and discussion

The elution pattern of histone deacetylase from a Sepharose 4B column is presented in fig. 1. Two not-well resolved peaks appeared. Calibration of the column by filtration of urease, catalase, γ-globulin and D-amino acid oxidase gave estimated molecular

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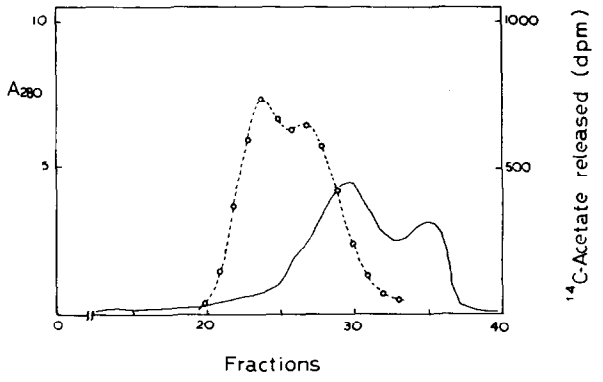

Fig. 1. Gel filtration on Sepharose 4B. A column (1.7 cm × 75 cm) of Sepharose 4B was equilibrated with 0.05 M phosphate buffer, pH 7.0 containing 5% glycerol, 1 mM 2-mercaptoethanol and 1 mM $MgCl_2$ (Buffer A). The hydroxylapatite eluate (10 ml, 129 mg protein) was applied on the column and eluted with the same buffer. Fractions of 5.4 ml were collected at a flow rate of 36 min per fraction. The absorbance at 280 nm was recorded (——) and histone deacetylase activity (o–o–o) was assayed by incubating 0.5 ml aliquots of the fractions with labeled whole histone (0.96 mg, 1430 dpm) at 37° for 15 min in a final volume of 2.8 ml in 0.05 M phosphate buffer, pH 7.0.

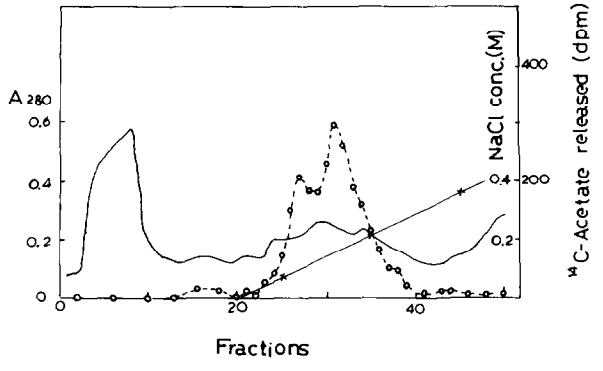

Fig. 2. DEAE-cellulose column chromatography. The Sepharose 4B eluate (fraction no. 21–29 of fig. 1) was applied on a DEAE-cellulose column (1.2 × 12 cm), which was previously equilibrated with buffer A (see the legend for fig. 1). The column was washed with 65 ml of the same buffer before a linear NaCl gradient started. The gradient was produced from 100 ml each of the buffer A and the buffer A containing 0.4 M NaCl (×–×–×). Fractions of 5.4 ml were collected at a flow rate of 12 min per fraction. The enzyme activity (o–o–o) was assayed by incubating 0.5 ml aliquots of the fractions with labeled whole histone (0.96 mg, 1430 dpm) at 37° for 30 min in a final volume of 2.8 ml in 0.05 M phosphate buffer, pH 7.0. The absorbance at 280 nm was recorded (——).

Table 1
Deacylation rate of f3 and f2a1 histone fractions.

Enzyme fraction	Total protein	Deacetylation rate (dpm [^{14}C] acetate released/hr/mg protein)		
	(mg)	f3	f2a1	f3/f2a1
Crude extract	400	310	300	1.0
Supernatant at 70,000 *g*	350	410	530	0.8
Hydroxylapatite eluate	130	3,900	1,200	3.3
Sepharose 4B eluate *				
Fraction no. 24	3.8	6,000	3,000	2.0
Fraction no. 27	12.0	740	910	0.8
DEAE-cellulose eluate *				
Fraction no. 27	1.3	9,500	3,700	2.6
Fraction no. 31	1.6	10,800	6,100	1.8

An aliquot of each enzyme fraction was incubated with f2a1 (830 dpm, specific activity 2,330 dpm/mg) or f3 (790 dpm, specific activity 2,850 dpm/mg) at 37° for 15 min and released [^{14}C] acetate was extracted and counted as described previously [4].

* Each fraction was assayed for deacetylase activity with whole histone as a substrate (see figs. 1 and 2). Only the indicated fractions were assayed for f3- and f2a1-deacetylation. When assayed with whole histone as a substrate, recovery of the total enzyme activity from the Sepharose 4B column was 34% and that from the DEAE-cellulose column was 32%.

Table 2
Activity toward soluble chromatin.

Enzyme fraction	[^{14}C] Acetate released			
	Soluble chromatin		Free histone	
	(dpm)	Degree of deacetylation (%)	(dpm)	Degree of deacetylation (%)
Sepharose 4B eluate				
Fraction no. 24	0	0	1,279	98
Fraction no. 27	18	1	1,168	90

Soluble chromatin (2,060 dpm, 0.63 mg protein and 0.51 mg DNA) or whole histone (1,300 dpm, 0.24 mg protein) was incubated with each enzyme fraction (109 μg protein for frac. no. 24 or 180 μg protein for frac. no. 27) at 37° for 50 min.

weights of about 550,000 (first peak) and 300,000 (second peak).

When the Sepharose 4B eluate was chromatographed on DEAE-cellulose column, two distinct peaks of the enzyme activity appeared (fig. 2). The enzyme was purified about 30-fold.

During the course of purification, various fractions were tested for the deacetylation of f3 and f2a1 histones. As shown in table 1, there is a significant change of the ratio of the deacetylation rate of the two histone fractions, suggesting that the enzyme is heterogeneous with respect to substrate specificity. It is of interest that a marked activation of f3-deacetylating activity was observed at the stage of the hydroxylapatite treatment. This result suggests that an inhibitor specific for the deacetylation of f3 histone was removed by the treatment. The two fractions separated by the Sepharose 4B column were distinguished by the different substrate specificity, however, they did not simply correspond to the two enzyme peaks separated by DEAE-cellulose chromatography.

In eukaryotic cells, histone is present as a complex with DNA, RNA and nonhistone proteins and the complex is called chromatin [13]. It is rather surprising to find that neither of the two enzyme fractions separated by the gel filtration on a Sepharose 4B column could deacetylate chromatin-bound histone (table 2). It has been shown that a crude extract of nuclei of calf thymus could deacetylate both free and chromatin-bound histones [14]. The deacetylation of chromatin-bound histone may involve a complex process.

During the preparation of this paper, Vidali et al. [15] reported that a histone deacetylase was purified over 500-fold from calf thymus nuclei and that its molecular weight is 150,000 – 160,000, which is significantly smaller than those of the enzymes described here. The reason for the discrepancy remains to be elucidated.

Acknowledgements

We wish to thank Drs. V.G. Allfrey and G. Vidali for generously supplying a preprint of their paper and Dr. N. Tamiya for his support. This work was supported in part by grants from the Ministry of Education of Japan.

References

[1] V.G. Allfrey, in: Histones and Nucleohistones, ed. D.M.P. Phillips (Plenum Publishing Co., London, 1971) p. 241.
[2] D. Gallwitz, FEBS Letters 13 (1971) 306.
[3] A. Inoue and D. Fujimoto, Biochem. Biophys. Res. Commun. 36 (1969) 146.
[4] A. Inoue and D. Fujimoto, Biochim. Biophys. Acta 220 (1970) 307.
[5] P.R. Libby, Biochim. Biophys. Acta 213 (1970) 234.
[6] D. Fujimoto, J. Biochem. (Tokyo) 72 (1972) 1269.
[7] A. Inoue and D. Fujomoto, J. Biochem. (Tokyo) 72 (1972) 427.
[8] T.Y. Shih and G.D. Fasman, Biochemistry 10 (1971) 1675.
[9] M.T. Sung, G.H. Dixon and O. Smithies, J. Biol. Chem. 246 (1971) 1358.
[10] G. Zubay and P. Doty, J. Mol. Biol. 1 (1959) 1.
[11] O.H. Lowry, N.J. Rosebrough, A.L. Farr and R.J. Randall, J. Biol. Chem. 193 (1951) 265.
[12] A. Tiselius, S. Hjerten and O. Levin, Arch. Biochem. Biophys. 65 (1956) 132.
[13] J. Bonner, M.E. Dahmus, D. Fambrough, R.C. Huang, K. Marushige and D.Y.H. Tuan, Science 159 (1968) 47.
[14] A. Inoue, H. Kaneta, K. Huriuchi, H. Kikuchi and D. Fujimoto, Seikagaku 43 (1971) 439.
[15] G. Vidali, L.C. Boffa and V.G. Allfrey, J. Biol. Chem., in press.