Biochimica et Biophysica Acta, 429 (1976) 742—749

BBA 67772

MULTIPLE FORMS OF HISTONE ACETYLTRANSFERASES IN THE CYTOSOL OF CALF ENDOMETRIUM

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(Received September 11th, 1975)

Summary

The histone acetyltransferase (EC 2.3.1.-) activity of calf endometrium cytosol has been separated into three separate activities by stepwise chromatography on DEAE-cellulose. In addition to differential elution from the DEAE-cellulose, the three activities are differentiated by their pH optima, preferences for histone subfractions as substrates, and stability to heat denaturation. Peak I has an optimum of pH 8.7 and preferentially acetylates histones F2b and F3; Peak II has an optimum of pH 8.5, and preferentially acetylates histone F2a1 followed by histone F2b; Peak III has an optimum of pH 9.5, and had similar specificity to Peak II. Peak III is appreciably more stable at 60°C than is Peak II. None of the peaks transferred acetate to other proteins tested or to tRNA. These studies suggest the presence of multiple histone acetyltransferases in tissue cytosols.

Introduction

The turnover of acetyl groups in histones in rapidly dividing cells and non-dividing cells under hormonal stimulation has been reported to be due to the enzymatic acetylation of histones by histone acetyltransferases (EC 2.3.1.-) [1, 2]. These enzymes have been shown to be present in nuclei of various tissues from several species [3,4]. In addition, cytoplasmic activities of these enzymes have also been reported in rat uterus [4] or kidney [5] and calf thymus [6]. Recently, Gallwitz demonstrated the presence of two activities in nuclei from either rat liver or kidney [7,8]. In this report we show evidence for the existence of multiple forms of histone acetyltransferases in the cytosol of calf endometrium which differ in specificity towards the acetylation of histones in vitro.

Materials and Methods

Calf thymus histone, batch HLY/ICA was purchased from Worthington Biochemicals. [1-^{14}C]-Acetyl-Coenzyme A (56 mCi/mol) was purchased from New England Nuclear Corp. Bovine hemoglobin, bovine serum albumin, and casein were purchased from Nutritional Biochemicals, $(NH_4)_2SO_4$ (enzyme grade) from Mann Research Labs, Whatman DE-22 from H. Reeve Angel and Co., and *Escherichia coli* tRNA from General Biochemicals. Sepraphore III strips were purchased from Gelman Instrument Co. Calf uteri were obtained frozen from a local abbatoir. Calf thymus histone fractions were prepared by the method of Johns [9,10], and were a generous gift from Dr. L. Pothier at this Institute. Acrylamide gel electrophoresis carried out by the method of Panyim and Chalkley [11] showed that each histone fraction was at least 95% pure.

Enzyme assays were carried out in the presence of 10 μmol Tris · HCl buffer, pH 8.5, 20 μmol KCl, 150 μg calf thymus histone, 103 pmol of [1-^{14}C] acetyl-CoA and enzyme in a final volume of 100 μl. A boiled enzyme control was always included to monitor non-enzymatic transfer of acetate from acetyl-CoA to histones. Incubations were carried out in duplicate at 25° C for 10 min and the reaction stopped by chilling and the addition of 50 μl of cold 3% trichloroacetic acid. After mixing, 50 μl were spotted on a dry Sepraphore III strip. The histones were fixed and stained as previously described [4] and the spot was cut out, and counted in 5 ml of scintillation fluid. Enzyme activity is reported as dpm incorporated into 1 μg of histone in 10-min incubation period. Protein concentrations were determined by either the biuret [12] or Lowry [13] procedures, using bovine serum albumin as standard.

The sensitivity of the radioactive acetate incorporated into histones towards hydroxylamine was determined by a modification of the method of Nohara et al. [14]. After stopping the reaction, 150 μl of either neutral 4 M hydroxylamine hydrochloride or water was added to duplicate incubation tubes. The mixtures were allowed to stand at room temperature for 1 h at 25° C before spotting and then counted for radioactivity. Under these conditions, there was no significant loss of radioactivity.

Preparation of Enzyme. Endometrium from thawed calf uterus was homogenized in 6 vols. of 0.32 M sucrose at high speed in a Waring Blender in three bursts of 30 s each (unless otherwise stated all procedures were carried out at 0—4° C). The homogenate was filtered through a nylon cloth and then centrifuged at 18 000 rev./min (Sorvall SS-34 rotor) for 30 min. The pellet was discarded and solid $(NH_4)_2SO_4$ was added to the supernatant. The protein which precipitated between 30 and 60% saturation was taken up in 0.01 M Tris, pH 8.0, containing 1 mM mercaptoethanol and 0.1 mM EDTA (Medium A) and dialyzed against several changes of this solution. The dialyzed material was applied to a small column of DEAE-cellulose which had been equilibrated with Medium A. 20 mg of protein were applied per ml of bed column. The column was washed with Medium A and then in a stepwise manner successively eluted with 0.1, 0.2 and 0.3 M KCl dissolved in 0.05 M Tris · HCl, pH 8.0. The column was then washed with 0.4 M KCl in 0.05 M potassium phosphate buffer, pH 7.0, and finally with 0.4 M KCl in 0.1 M potassium phosphate, pH 7.0. All solutions also contained 1 mM mercaptoethanol and 0.1 mM EDTA. The elution

with each solution was continued until the absorbance of the effluent at 280 nm progressively decreased and then elution was started with the next solution.

Results

Using the above procedure three separate peaks of enzyme activity were routinely found. Fig. 1 shows the pattern of elution of one typical run on the DEAE-cellulose column. A small additional amount of enzyme was eluted in the 0.05 M phosphate buffer system but the amounts were quite low and variable. Table I summarizes the purification of these enzyme fractions. Peak I was recovered at a lower specific activity than the $(NH_4)_2SO_4$ fraction, whereas Peaks II and III were further purified over this fraction.

Fig. 2 shows the pH dependence of the three enzyme peaks. Peak I has a broad band with maximum activity at pH 8.7. Peak II shows a sharp optimum at pH 8.5. The optimum pH for Peak III is apparently at pH 9.5, but this is confused by the change in buffer systems, since the enzyme is less active in glycine buffer than in Tris at pH 8.5. Also included in Fig. 2 is the effect of pH on the non-enzymatic reaction. Although non-enzymatic acetylation of his-

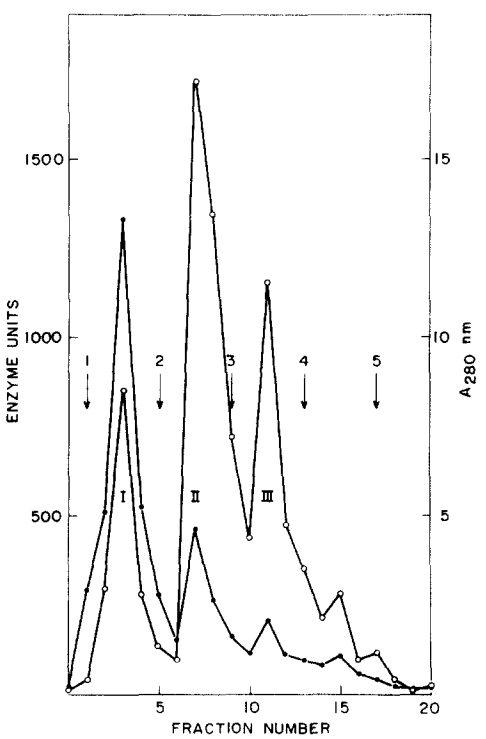

Fig. 1. Separation of histone acetyltransferases of calf endometrium cytosol by stepwise elution from DEAE-cellulose. The closed circles represent absorbance at 280 nm and the open circles represent enzyme activity. The arrows indicate changes made in the elution buffer. The numbers above the arrows indicate the following elution buffers: 1—3, 0.05 M Tris · HCl, pH 8.0, containing 0.1, 0.2 and 0.3 M KCl, respectively; 4, 0.05 M potassium phosphate buffer, pH 7.0, containing 0.4 M KCl; 5, 0.10 M potassium phosphate buffer, pH 7.0, containing 0.4 M KCl.

TABLE I

PURIFICATION OF HISTONE ACETYLTRANSFERASES FROM CALF ENDOMETRIUM

Fraction	Volume (ml)	Protein (mg/ml)	Enzyme units * (per ml)	Specific activity (units/mg protein)	Recovery (%)
Cytosol	275	6.36	380.0	59.7	100.0
30–60% $(NH_4)_2SO_4$ cut	21.0	30.8	5030.0	163.3	101.0
DEAE-cellulose					
Peak I	33.5	7.7	625.0	81.2	19.8
Peak II	35.0	3.2	1320.0	412.5	43.7
Peak III	51.8	1.2	497.5	414.6	24.4

* 1 unit is the total dpm incorporated per μg histone in the incubation mixture at 25° C for 10 min.

tones has been reported [15] there is little or no non-enzymatic reaction under our assay conditions (i.e. in the presence of 0.2 M KCl).

Table II shows the activities of the three enzymes towards various substrates. Peak I shows a broad specificity to all histone fractions, although the activities are somewhat low when compared to the other two peaks. Neither casein, hemoglobin nor ribonuclease are acetylated by this fraction. Both Peak II and Peak III are most active with the F2al histone. However, the F2b histone also serves as a good substrate. F1, F2a2 and F3 histones are acetylated at much lower rates than the F2al and F2b histones. Neither of these enzymes incorporated acetate into any other non-histone macromolecules.

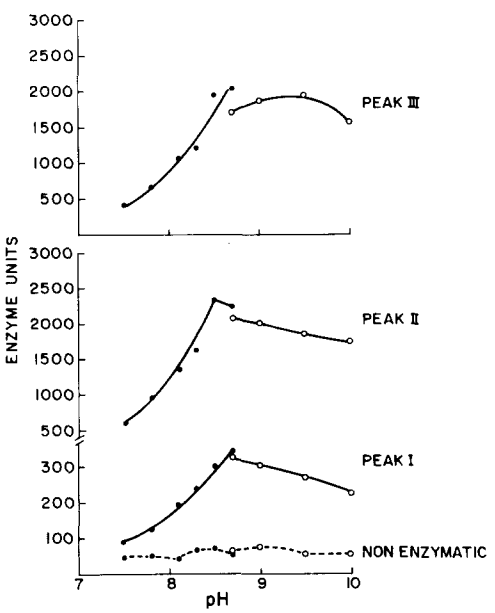

Fig. 2. pH activity of calf endometrium cytosol histone acetyltransferases. The enzymes after partial purification by DEAE-cellulose chromatography were assayed at the indicated pH values by measuring the incorporation of [^{14}C]acetate into calf thymus histones. ●———●, 0.05 M Tris · HCl buffer; ○———○, 0.05 M glycine/NaOH buffer; - - - - - -, non-enzymatic acetylation.

TABLE II

SPECIFICITY OF HISTONE ACETYLTRANSFERASES OF ENDOMETRIUM CYTOSOL IN THE TRANSFER OF [^{14}C] ACETATE FROM [^{14}C] ACETYL-CoA TO VARIOUS ACCEPTORS

Enzyme assays were performed as described in the text in a final volume of 0.1 ml. For each enzyme peak, the pH of the buffer was the optimum (Fig. 2), and the amount of histone was previously determined to be optimum.

Acceptor	Radioactivity incorporated into acceptor (cpm)		
	Peak I	Peak II	Peak III
Heated enzyme with total histone, 150 μg	15.9	17.1	18.5
Total histone, 150 μg	220.0	1384.0	1257.0
F1 histone, 150 μg	264.0		
F1 histone, 50 μg		110.0	66.0
F2a1 histone, 50 μg	147.0		
F2a1 histone, 25 μg		1430.0	1146.0
F2a2 histone, 50 μg	168.0	332.0	238.0
F2b histone, 50 μg	206.0	893.0	682.0
F3 histone, 50 μg	189.0	209.0	190.0
Albumin, 150 μg	0.0	0.0	2.8
Albumin, 300 μg	0.0	0.0	0.0
Casein (bovine), 150 μg	0.0	0.0	11.1
Casein (bovine), 300 μg	18.0	0.0	0.0
Hemoglobin (bovine), 150 μg	5.4	0.0	0.0
Hemoglobin (bovine), 300 μg	11.8	0.0	0.0
RNAase (bovine pancrease), 150 μg	13.2	0.0	0.0
RNAase (bovine pancreas), 300 μg	15.2	0.0	0.0
tRNA (*E. coli*), 20 μg	0.0	0.0	0.0
tRNA (*E. coli*), 200 μg	0.0	0.0	0.0

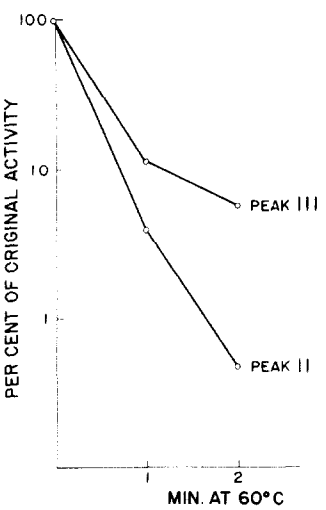

Fig. 3. Effect of heat on activity of calf endometrium cytosol histone acetyltransferases. Peaks II and III were placed in a water bath at 60°C for the indicated times before removal, chilling and assay.

Because Peaks II and III showed very similar activities toward histone subfractions, further work was carried out to distinguish these two enzymes. Fig. 3 shows the result of one heat denaturation study in which the two enzymes were exposed to 60° C before assay. Peak II is obviously much more sensitive to the denaturing conditions than is Peak III.

Discussion

The results presented in this report demonstrate that the cytosol from calf endometrium contains at least three distinct histone acetyltransferase activities which may be separated by stepwise elution on DEAE-cellulose. If elution were carried out by a gradient method, rather than the stepwise procedure, three peaks were also obtained. However, the middle peak was poorly resolved, whereas the rest of the two activities emerged in broad peaks and were present in relatively low concentrations. The stepwise elution allowed us to obtain the activities in sharp peaks as well as in a concentrated form. Exclusion chromatography of the 30—60% $(NH_4)_2SO_4$ cut on Sephadex G-100 showed 30% of the enzyme activity eluted with the void volume proteins, and a further 70% emerged in a broad peak after the void volume. Thus, stepwise elution was chosen as the method of choice for separating the histone acetyltransferases.

Although histone acetyltransferase activity has been reported in nuclei of rat uterine tissue [4], calf thymus [6] or rat brain [3], it is unlikely that the three cytosol activities seen in calf endometrium are due to contamination by the nuclear activity during the extraction procedure, since our unpublished results indicate that the nuclear enzyme activity is very tightly bound to chromatin and cannot be solubilized by the extraction procedure used for cytosol activities. Our findings are in contrast with the report by Horiuchi and Fujimoto [6]. They found only one activity in calf thymus cytosol which acetylated only the F2al histone. However, this may be due to tissue difference in the localization of histone acetyltransferases. Pestana and Pitot [16] studied a soluble enzyme from rat liver which acetylated lysine-rich and arginine-rich histones. Their finding may reflect a mixture of enzymes. Nohara et al. [14] have reported the presence of a soluble histone acetyltransferase activity in the pigeon liver. However, their report did not ascertain the number of histone acetyltransferases activities present or the characteristics of the enzyme.

The enzyme activities reported by us are distinguished by their differences in elution patterns on DEAE-cellulose, their pH optima, their different reactivity with histone subfractions, and their sensitivities to heat denaturation. Table II shows that Peak I has a substrate specificity markedly different from that of Peaks II and III. Under optimal conditions of pH and histone concentrations, Peak I acetylates all histone subfractions at similar rates. Peaks II and III, on the other hand, preferentially acetylated histone F2al and histone F2b. The other histone fractions are relatively poorly acetylated compared to these two fractions. Although Peaks II and III have similar substrate specificities, the data of Figs. 2 and 3 suggest that they are different enzymes. Lue et al. [22] have reported the extraction of histone acetyltransferases from rat liver nuclei. However, they were able to show that form I (AT I) was very similar to form II (AT II) after bound histone had been removed. Although it is possible that Peaks II

and III are also only differentiated by the presence of bound histone in Peak II, we consider this unlikely, since these enzymes are found in the cytosol of the endometrium homogenate under very mild conditions of homogenization when little or no histones would be expected to be extracted from the nucleus.

All of the enzymes acetylate histone F1 poorly which is in agreement with Vidali et al. [17] who reported that histone F1 from calf thymus did not incorporate appreciable amounts of acetate. However, the existence of enzymes which do incorporate acetate into histone F1 supports the findings of Libby [5,18,19] and Shepherd et al. [20] that in uterus and Chinese hamster ovary cells, histone F1 is acetylated. It has been reported that F2a1 and F3 histones undergo extensive acetyl group turnover [21], but the cytosol enzymes present in calf endometrium acetylate histone F3 very poorly compared to histone F2a1, and histone F2b which has also been reported to be poorly acetylated in vivo [17]. These observations suggest that studies made on intact tissues must be supplemented by studies on purified enzymes.

The enzyme studied here seem to be specific for histones. Other proteins, such as albumin, casein, hemoglobin, and ribonuclease do not serve as substrates for any of the three peaks. A tRNA-acetylating enzyme has recently been reported [16], and we have therefore also tested tRNA as a substrate with the three peaks from uterine cytosol, but none of the enzymes could utilize tRNA, as substrate. Acetylated histones formed by the three enzymes were exposed to 2 M hydroxylamine; since no radioactivity was lost by this treatment, the enzymes are all *N*-acetyltransferases. The function of these histone acetyltransferases in the cytosol of calf endometrium needs further study and may very well lead to the understanding of the regulation of cellular proliferation in hormone-responsive tissues.

Acknowledgement

Portions of this work were supported by Grant P-612 of the American Cancer Society.

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