Volume 296, number 1, 82–86 FEBS 10559 January 1992

Enzymes involved in the dynamic equilibrium of core histone acetylation of *Physarum polycephalum*

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Received 28 October 1991

DEAE-Sepharose chromatography of extracts from plasmodia of the myxomycete *Physarum polycephalum* revealed the presence of multiple histone acetyltransferases and histone deacetylases. A cytoplasmic histone acetyltransferase B, specific for histone H4, and two nuclear acetyltransferases A1 and A2 were identified; A1 acetylates all core histones with a preference for H3 and H2A, whereas A2 is specific for H3 and also slightly for H2B. Two histone deacetylases, HD1 and HD2, could be discriminated. They differ with respect to substrate specificity and pH dependence. For the first time the substrate specificity of histone deacetylases was determined using HPLC-purified individual core histone species. The order of acetylated substrate preference is H2A≫H3≅H4>H2B for HD1 and H3>H2A>H4 for HD2, respectively; HD2 is inactive with H2B as substrate. Moreover histone deacetylases are very sensitive to butyrate, since 2 mM butyrate leads to more than 50% inhibition of enzyme activity.

Histone acetylation; Histone acetyltransferase; Histone deacetylase; Butyrate; Chromatin; *Physarum*

1. INTRODUCTION

Core histones are subject to reversible posttranslational acetylation at distinct lysine residues within the N-terminal protein domains. The biological function of this modification is far from clear, experimental evidence has been presented that histone acetylation might be involved in different nuclear processes [1,2].

The dynamic equilibrium of histone acetylation is maintained by two enzyme activities, histone acetyltransferase and histone deacetylase. Multiple histone acetyltransferases have been investigated and characterized in a wide variety of organisms from yeast to mammals [3–10]. These investigations led to a basic classification into nuclear A-type enzymes, acetylating histones in nucleosomes and all core histones in vitro, and cytoplasmic B-type enzymes, not active with nucleosomes, which acetylate histone H4 in vitro.

Histone deacetylases are also present as multiple enzyme activities in a variety of fungi, plants and animal cells [11–15]. In pea three enzyme fractions with deacetylase activity have been characterized [15]. Histone deacetylases of mammalian origin were found to be strongly inhibited by butyrate in millimolar concentrations. In contrast, a weak effect of butyrate on deacetylases has been described for plants [15,16] and *Physarum* [17].

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Despite the numerous data on biological effects of histone acetylation, little is known about the role of the involved enzymes. We have recently separated and characterized multiple histone acetyltransferases and deacetylases in *Zea mays* and have demonstrated that the cooperative action of these enzymes is specifically involved in the complex differentiation program during the germination of maize embryos [18,19]. We suggest that analysis of histone acetyltransferases and deacetylases could provide the key for a better understanding of the biological significance of this modification. Since a huge body of data is available on histone acetylation in *Physarum* [2,20–27] it seemed warranted to separate and characterize enzymes involved in histone acetylation in this organism. We demonstrate the presence of multiple forms of histone acetyltransferase and histone deacetylase, which differ with respect to substrate specificity and kinetic properties. These properties and the experimental conditions for enzyme separation should provide a sound basis for specific experiments during proliferation and differentiation of *Physarum polycephalum*.

2. MATERIALS AND METHODS

2.1. *Materials*

Materials were purchased from the following suppliers: [^{14}C]acetyl-CoA from ICN Biomedicals (Costa Mesa, CA, USA), novobiocin from Boehringer (Mannheim, Germany), DEAE-Sepharose CL-6B from Pharmacia (Uppsala, Sweden), Nucleosil 300-5 C_4 from Macherey-Nagel (Düren, Germany).

2.2. *Culture conditions*

Microplasmodia of the myxomycete *Physarum polycephalum* (strain

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M_3b, a Wis1 isolate) were cultivated in submersed shake culture in semi-defined nutrient medium [28] under sterile conditions at 24°C with reciprocal shaking.

2.3. *Preparation of plasmodial extracts*

Microplasmodia were collected by centrifugation at 300 × g for 1 min, washed twice with distilled water and suspended in 100 ml of buffer A (0.25 mM EDTA, 10 mM 2-mercaptoethanol, 75 mM Tris-HCl, pH 7.9). Cells were disrupted in a Braun blendor for 30 s twice at maximum speed and further homogenized by 10 strokes at 200 rpm in a Potter-Elvehjem homogenizer. Ammonium chloride was added to the homogenate up to 0.7 M (from a 4 M stock solution in buffer A). The homogenate was stirred for 30 min on ice and centrifuged at 100 000 × g for 1 h. Proteins were precipitated from the supernatant with 90% ammonium sulfate. After 1 h on ice proteins were recovered by centrifugation at 27 000 × g for 30 min. The sediment was resuspended in 40 ml of buffer B (10 mM NH_4Cl, 0.25 mM EDTA, 10 mM 2-mercaptoethanol, 10% (v/v) glycerol, 15 mM Tris-HCl, pH 7.9) and dialyzed against the same buffer. The dialysate was centrifuged at 27 000 × g for 10 min and the clear supernatant was then loaded onto a DEAE-Sepharose CL-6B column (0.7×15 cm) previously equilibrated with buffer B. After washing the column with 10 vols. of buffer B, the retained proteins were eluted with 90 ml of a linear NH_4Cl gradient (10–350 mM) in buffer B at a flow rate of 6 ml/h. Fractions of 2 ml were recovered and assayed for histone acetyltransferase and histone deacetylase activities.

2.4. *Enzyme assays*

Histone acetyltransferase activity of chromatographic fractions was assayed using chicken erythrocyte whole histones as substrate according to [3]. Histone deacetylase activity was determined according to [15] using [^{3}H]acetate-prelabelled chicken erythrocyte histones [18] as substrate.

2.5. *Specificity of histone acetyltransferases*

This was done essentially as described recently [18], but in the presence of an excess of proteins (apoferritin 350 μg/ml; insulin 350 μg/ml) to protect histones from proteolytic degradation. Histones were analyzed by SDS-polyacrylamide gel electrophoresis [29] and the incorporation of radiolabel into histones was detected by fluorography performed essentially as described [30] using preflashed Amersham Hyperfilm-MP.

Quantitative evaluation of fluorograms was done with a Hirschmann densitometer system. Integrated labelling densities of individual histone bands of the fluorogram were standardized by dividing by the integrated densities of the corresponding Coomassie blue-stained bands, determined from the original gels. The resulting standardized specific labelling density (SLD) was therefore a measure of the specific activity of the individual histones. To obtain a profile of individual histone acetyltransferase activities, the SLD values for each histone species were plotted against the eluted fractions of the chromatographic separation [4].

2.6. *Separation and purification of chicken erythrocyte histones by HPLC*

Chicken erythrocyte whole histones were fractionated as described [37] with the following modifications. The separations were performed on a Nucleosil 300-5 C_4 column (0.8 mm internal diameter × 250 mm). The freeze-dried histones were dissolved in 0.2% (v/v) TFA containing 50 mM 2-mercaptoethanol and 500–800 μg protein samples were injected onto the column. Histones were chromatographed within 55 min at room temperature at a constant flow of 1.5 ml/min using a multistep acetonitrile gradient starting at 57% (v/v) A/43% B (solvent A, water containing 0.1% TFA; solvent B, 70% acetonitrile and 0.1% TFA). The concentration of solvent B was increased linearly in the following order: from 43% B to 55% B (during 15 min), from 55% to 62% (2 min), from 62% to 65% (25 min), from 65% to 75% (5 min) and then maintained at 75% (8 min). The fractions collected were freeze-dried in the presence of 10 mM 2-mercaptoethanol. Our H2A fraction only contained H2A.1, which represents the majority of H2A; H2A.2 represents less than 10% of total H2A in chicken erythrocytes. The H3 fraction contained H3.2, since chicken erythrocytes do neither contain H3.1 nor H3.3.

2.7. *Specificity of histone deacetylases*

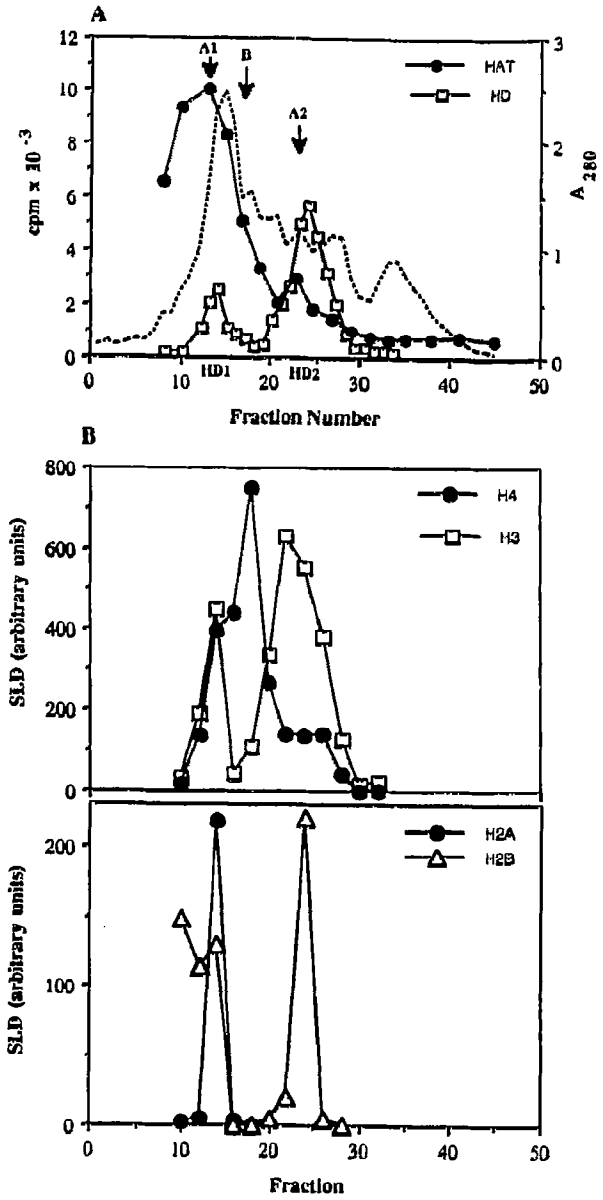
To determine the substrate specificity of histone deacetylases chromatographic peak fractions were analyzed with HPLC-purified, radioactively prelabelled, individual histone species as substrates in the deacetylase assay as described [15]. The assays were done in triplicates using between 7-10 μg of individual histone, in order to yield 25 000 cpm per assay with a specific labelling of each histone species of 2.5 to 3.5 × 10^6 cpm/mg protein.

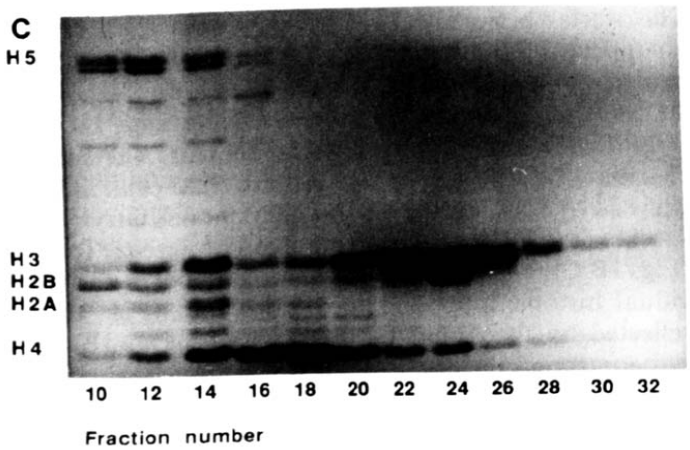
3. RESULTS AND DISCUSSION

Elution of ammonium sulfate precipitated microplasmodial extracts from a DEAE-Sepharose column with a linear NH_4Cl gradient separates histone acetyltransferase activity into two apparent peaks (Fig. 1A). One broad peak elutes in fractions 8 to 18, the second elutes in fractions 20 to 28. Histone deacetylase activity is separated into two distinct peaks; HD1 elutes in fractions 12 to 17, HD2 in fractions 20 to 30.

Since the rather broad chromatographic histone acetyltransferase peaks could consist of different histone acetyltransferases, chromatographic fractions were tested for substrate specificity towards individual histone species by electrophoretic analysis of histones with subsequent fluorography and quantitative densitometric evaluation. The resulting SLD values (Fig. 1B) represent the ratio between the amount of radioactivity in an individual histone band of the fluorogram (Fig. 1C) and the amount of protein present in the corresponding band of the Coomassie blue-stained gel. This method eliminates slight differences in the amount of protein loaded to each lane.

The fluorogram (Fig. 1C) together with detailed densitometric evaluation revealed two distinct histone acetyltransferases with different substrate specificities within the first chromatographic peak. An enzyme activity A1 is present in fractions 12 to 16, which acetylates all core histones with a preference for H3 and H2A. A second enzyme activity, histone acetyltransferase B is present in fractions 14 to 24 which is specific for H4. The overlap between A1 and the major B activity causes an apparent preference of A1 for histone H4. However, analysis of every single fraction of A1 and B clearly shows that A1 does not have a preference for H4, but only for H3 and H2A (result not shown). The second chromatographic histone acetyltransferase peak in fractions 20 to 30 is homogeneous and contains enzyme A2 specific for H3 and H2B with a main preference for H3 (Fig. 1B,C). Note that the intensity of label in the individual histone species in the fluorogram (Fig. 1C) is reflected by the different SLD-scales in Fig. 1B. The rather strong labelling of H2B in fraction 24 (Fig. 1C) is due to slight proteolytic degradation of H3; therefore the SLD value of H2B in fraction 24 (Fig. 1B) is clearly





overestimated. Nevertheless we cannot completely rule out the presence of a further enzyme specific for H2B.

Unfortunately, extremely active proteases are present in the extracts; although we could inhibit these proteases by inclusion of excess amounts of heterologous proteins (insulin, apoferritin) in the assay mixture, slight proteolytic degradation in certain chromatographic fractions could not be completely eliminated.

It also has to be mentioned that in the first chromatographic fractions, which contain very little protein, one observes non-enzymatic, chemical acetylation of histones. This chemical acetylation preferentially occurs on histones H5 and H2B and can be inhibited with increasing salt concentrations [18,31].

Densitometric evaluations of fluorograms of extracts from isolated nuclei and cytoplasmic compartment after DEAE-Sepharose chromatography revealed that A1 and A2 are nuclear enzymes, whereas B is cytoplasmic (data not shown).

Comparison of our results with previous data from lower eukaryots and plants reveals a general pattern of histone acetyltransferases. The cytoplasmic B-enzyme is specific for histone H4, regardless of the experimental system [4,18]. At least two nuclear A-type enzymes are present in these eukaryotic systems, among which one enzyme is always highly specific for histone H3; in yeast [4], maize [18] and *Physarum* the acetylation of H3 and H4 is considerably stronger than the acetylation of H2A and H2B. In yeast and maize only one of the H2 species is accepted as substrate by one of the histone acetyltransferase forms. However, only *Physarum* contains nuclear enzymes acetylating H2A as well as H2B. It is also worth mentioning that the cytoplasmic B-enzyme of *Physarum* is bound to the DEAE-Sepharose less strong than in yeast and maize; for this reason it eluted from the column between the nuclear enzymes A1 and A2. In yeast as well as in maize the B-enzyme was most closely bound to the chromatographic matrix, therefore

Fig. 1. Enzyme activity and substrate specificity of histone acetyltransferases and enzyme activity of histone deacetylases after separation by DEAE-Sepharose chromatography. Ammonium sulfate precipitated extracts of microplasmodia of *Physarum* were applied to a DEAE-Sepharose CL-6B column and eluted with 90 ml of a linear NH_4Cl-gradient (10–350 mM). Chromatographic fractions were analyzed for histone acetyltransferase and histone deacetylase activity (A). Protein was measured by A_{280} recording (dashed line). After incubation of chromatographic fractions with [^{14}C]acetyl-CoA and chicken erythrocyte histones, the novobiocin precipitate was analyzed by SDS-polyacrylamide gel electrophoresis with subsequent fluorography (C). The specific labelling density (B) was calculated as the ratio of the intensity of label in an individual histone band in the fluorogram and the amount of protein present in the corresponding Coomassie blue-stained band. A1, A2 and B (arrows) indicate the position of histone acetyltransferases as determined in B and C, HD1 and HD2 in the bottom indicate the position in the chromatographic profile of histone deacetylases.

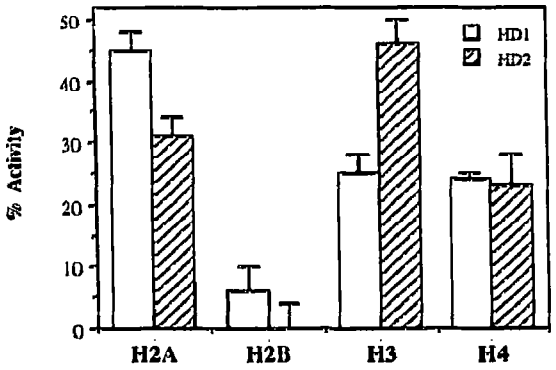


Fig. 2. Specificity of histone deacetylases HD1 and HD2 towards individual core histone species. Chicken erythrocyte histones, prelabelled in vivo with [^{3}H]acetate, were separated and purified into individual histone species by HPLC and used as substrate in the histone deacetylase enzyme assay. Peak fractions of HD1 or HD2 were used as enzyme source. Values are expressed as % of the total histone activity of HD1 and HD2, respectively, after subtraction of background due to nonenzymatic deacetylation. 100% = 5.96×10^5 cpm/mg histone (HD1), 1.61×10^5 cpm/mg histone (HD2). Assays were done in triplicate (standard deviation indicated).

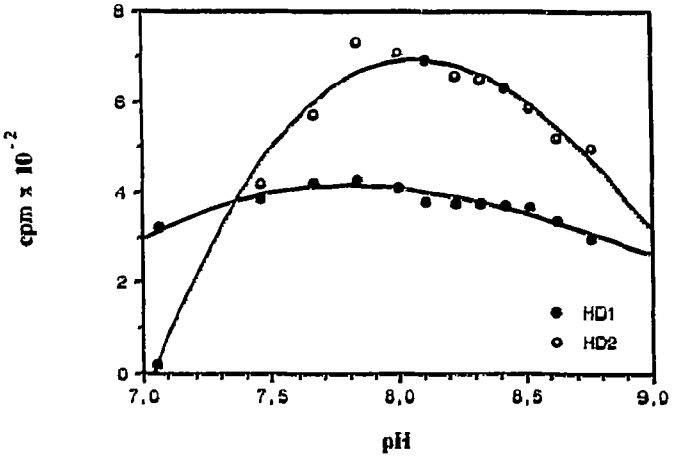


Fig. 3. pH dependence of HD1 and HD2. Chromatographic peak fractions were analyzed in the histone deacetylase enzyme assay at various pH values.

eluting as the last histone acetyltransferase activity [4,18].

In order to test the substrate specificity of histone deacetylases HD1 and HD2, we analyzed chromatographic peak fractions from both enzymes with HPLC-purified individual core histone species. Fig. 2 shows that HD1 and HD2 differ significantly; only HD1 can deacetylate H2B, whereas HD2 does not at all accept H2B as substrate. The order of substrate preference is H2A≫H3≅H4>H2B for HD1, but H3>H2A>H4 for HD2 (Fig. 2). An unexpected result was that, although HD2 was the more active enzyme when measured with total core histones, HD1 was the predominant enzyme, when analyzed with purified individual histone species (result not shown). This could be an indication that HD2 probably requires the formation of a structure, such as histone aggregates or nucleosome-like particles, that assemble in a mixture of total histones; the formation of prenucleosomal particles in vitro was previously reported [32].

Furthermore the two deacetylases differ in their pH dependence (Fig. 3). Whereas HD1 does not exhibit a pronounced pH dependence between pH 7 and 9, HD2 has a clear optimum at pH 8.

Since a weak inhibition by butyrate was previously reported for *Physarum* deacetylase activity [17], we also tested the effect of this compound on the activity of HD1 and HD2. Contrary to the previous results we find a striking sensitivity of both enzymes towards butyrate; 1 mM leads to almost 50% inhibition, 5 mM depress enzyme activity for more than 70%. Waterborg and Matthews [17] found a 25% inhibition with 10 mM; in contrast to their conclusion *Physarum* deacetylases are among the most sensitive enzymes towards butyrate inhibition. The discrepancy between the two sets of data are most likely due to the experimental conditions. Whereas Waterborg and Matthews [17] used total isolated nuclei as enzyme source in their assay, we used enzymatic peak fractions of the DEAE-chromatographic eluate. The strong inhibitory effect of butyrate on *Physarum* histone deacetylases explains the pronounced hyperacetylation of H4 and the dramatic cellular effects of 1 mM butyrate reported earlier [21,33]. In general, the inhibition of histone deacetylase activity of *Physarum* by butyrate is similar to mammalian cells, where butyrate has been shown to act as a non-competitive inhibitor [14].

It is interesting that both deacetylases have a high specificity for histone H2A, although acetyltransferases are preferentially active with H3 and H4. At present we do not have a sound explanation for this finding, but it could be interpreted in terms of the proposed role of

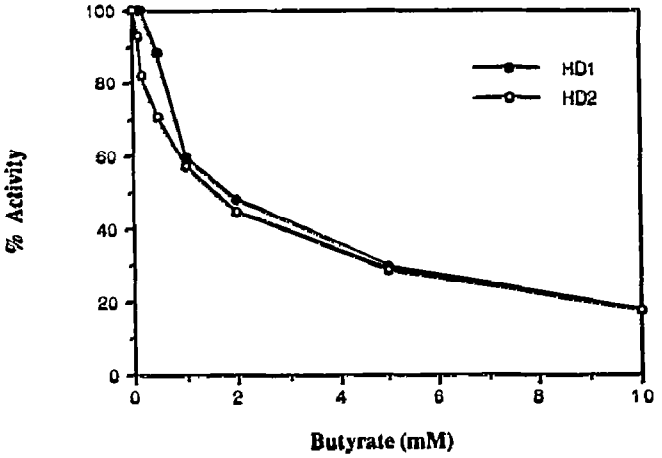


Fig. 4. Inhibition of histone deacetylases by butyrate. Chromatographic peak fractions of HD1 or HD2 were incubated in the presence of increasing concentrations of butyrate. Residual activity is expressed as % of control activity.

H2A and H2B during transcription; it has been suggested that removal of an H2A/H2B dimer from nucleosomes at distinct chromatin sites is a prerequisite for the transcriptional machinery to pass through the nucleosome [34,35]. This suggestion got additional experimental support by the selective in vivo acetylation of H2A and H2B during transcription in the *Physarum* cell cycle [2,26] with the subsequent assembly of newly synthesized H2A and H2B into chromatin [36]; from these results it was concluded that acetylation of H2A and H2B is essentially involved in the structural changes in nucleosomes during transcription [2]. The high specificity of HD1 and also HD2 for H2A represents a circumstantial evidence for this hypothesis. Since only a small subset of chromatin is involved in transcription at a given time, the percentage of H2A and H2B which has to be specifically acetylated could be rather low, as reflected by the relatively low affinity of histone acetyltransferases A1 and A2 for H2A and H2B. This is in line with results of Nelson [38], who found a more rapid turnover of acetate groups in H2A and H2B in comparison to H3 and H4; deacetylation of H2A and H2B occurred faster, whereas deacetylation of H3 and H4 was in the order of hours. Therefore the regulation of such a transient, transcription-related acetylation could occur on the level of deacetylation and the presence of a deacetylase with high affinity for H2A seems suggestive.

Acknowledgements: The authors wish to thank Mrs. M. Edlinger and Ms. A. Devich for expert technical assistance. The continuous interest and valuable discussions with Dr. G. Stöffler and Dr. E. Georgieva are gratefully acknowledged. G. López-Rodas is recipient of a research fellowship from the Programa sectorial de Formación de Profesorado y Personal investigador del Ministerio de Educación y Ciencia (Spain). This project was supported by Grant P7989 to P.L. from the Fonds zur Förderung der wissenschaftlichen Forschung and the Dr. Legerlotz Stiftung.

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