# Histone-Specific Acetyltransferases from Calf Thymus. Isolation, Properties, and Substrate Specificity of Three Different Enzymes†

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ABSTRACT: Three enzymes which transfer acetate from acetylcoenzyme A specifically to histones were isolated from calf thymus. The isolation procedure involved tissue homogenization and sonication in the presence of 20% glycerol and 1 M ammonium sulfate. Two enzyme activities A and B were resolved by DEAE-cellulose chromatography. Histone acetyltransferase A was further purified by chromatography on hydroxylapatite, phosphocellulose, and DEAE-Sephadex. Chromatography of enzyme activity B on Sephadex G-200 in the presence of 0.3 M KCl resulted in the separation of two active fractions B and C. The molecular weights as determined by gel chromatography were 120 000, 98 000, and >200 000 for enzymes $A_1$, $B_1$, and $C_1$, respectively. Histone acetyltransferases A and B are distinguishable by the degree of inhibition of activity by using *p*-(chloromercuri)benzoate, *N*-ethylmaleimide, and iodoacetamide. Modification of histones by the isolated enzymes resulted in the formation of $N^{\epsilon}$-acetyllysine as the only acetylation product. Calf thymus histone acetyltransferases A, B, and C were characterized by following the kinetics of in vitro acetylation of purified calf thymus histones H2a, H2b, H3, and H4. Whereas enzymes A and C transfer acetate to all five histones, enzyme B nearly exclusively acetylates histones H4 and H2a. The substrate specificity of histone acetyltransferases A and B was further investigated by comparing tryptic fingerprints of in vivo and in vitro acetylated histones H2a, H2b, H3, and H4. It was found that in vitro modification of histones H2a and H4 with acetyltransferase B resulted in fingerprints comparable to the in vivo pattern whereas enzyme A gave several additional labeled peptides. The tryptic fingerprints of histones H2b and H3 after in vitro acetylation with enzyme A were similar but not identical with the in vivo pattern. In contrast, numerous additional labeled peptides resulted from in vitro acetylation of histones H2b and H3 with enzyme B.

The DNA of eucaryotic cells is periodically folded by complexing with the four histones H2a, H2b, H3, and H4 which results in the formation of defined particles, the nucleosomes (Kornberg, 1977). The limited number of histones as well as their extraordinary evolutionary and metabolical stability has often led to the view that the primary function of these proteins would be to simply pack and condense the DNA within the nucleus. However, changes in chromatin structure are very likely to occur during transcriptional processes, and it has been suggested that modification of histones such as acetylation, methylation, and phosphorylation is of profound significance for the regulation of gene expression in mammalian cells (Allfrey, 1971; Hnilica, 1972). The acetylation of histones appears to be of special importance: multiple but specific lysine residues within histones H2a, H2b, H3, and H4 can be reversibly acetylated, whereas in histone H1—which is apparently not involved in the basic nucleosome structure—no such modification has been found. All the $N^{\epsilon}$-acetylated lysine residues are located in the N-terminal part of the four histones which is thought to bind to DNA in the nucleosome core. In view of the significant change in the overall charge of the histones due to acetylation, it is very likely that these specific substitutions affect the histone binding capacity to DNA and thus might be involved in alterations of the nucleosome structure.

In recent years we have focused our interest on the identification of the enzymes involved in histone acetylation and have isolated and partially purified histone-specific acetyltransferases from several mammalian tissues (Gallwitz, 1971; Gallwitz & Sures, 1972; Sures & Gallwitz, 1975). Histone acetylating enzymes have also been described from other laboratories (Bondy et al., 1970; Cano & Pestaña, 1976; Horiuchi & Fujimoto, 1972; Libby, 1978; Lue et al., 1973; Nohara et al., 1968; Noland et al., 1971). Since these enzymes seem to occur in relatively low amounts in the tissues examined, it proved to be difficult to purify and characterize them extensively and to use them for further studies. We therefore decided to isolate the histone acetyltransferases from calf thymus from which large-scale preparations seemed feasible. In addition, the primary sequences of the calf thymus histones which serve as enzyme substrates are known. Recently, we could identify the acetylation sites occurring in these histones in vivo by sequence analysis (unpublished experiments).

Here we describe the isolation, partial purification, and characterization of three histone-specific acetyltransferases from calf thymus. The existence of at least three histone-specific acetyltransferases in calf thymus favors the assumption that, if these enzymes have in fact different substrate specificities, those highly specific modification reactions might be regulated independently, thus leading to numerous differently acetylated histone proteins.

Basically, there are two possibilities of how the enzymes may function: either each of them specifically modifies all possible lysine residues within only one histone or each of the enzymes acetylates different sites within different histones. If indeed only three histone acetyltransferases exist, it seems likely that they recognize certain structural features of their protein substrates, perhaps in the immediate neighborhood of the lysyl residues to be modified. In this case more than one histone should be acetylated by one of the enzymes.

In a recent study Thwaits et al. (1976a,b) have found that, using a crude mixture of rat liver acetyltransferases, multiple sites were acetylated in the N-terminal regions of calf thymus histones H3 and H4 in vitro.

To determine the substrate specificity of the different calf thymus acetyltransferases, we have compared the kinetics of acetylation of purified histones H2a, H2b, H3, and H4 using these enzymes and, in a more qualitative way, the extent of

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acetate transfer to the different histones using as substrate a mixture of total calf thymus histones. In addition, we compared the tryptic fingerprint patterns of the histones H2a, H2b, H3, and H4 acetylated in vitro with either histone acetyltransferase A or B. The results suggest that the three enzymes modify all four histones but to a different extent and at different sites. Histone acetyltransferases A and C also acetylate histone H1 which is not acetylated in vivo, whereas enzyme B nearly exclusively uses histones H4 and H2a as its substrates.

## Materials and Methods

*Materials.* [1-$^{14}$C]Acetylcoenzyme A (sp act. 56 mCi/mmol) was obtained from New England Nuclear Corp. Poly-L-lysine ($M_r$ 140 000), poly-L-arginine ($M_r$ 65 000), poly-L-serine, protamine chloride (grade V), protamine sulfate (grade I), and DCC[1]-treated trypsin were purchased from Sigma Chemical Co. Bovine serum albumin, hemoglobin, *N*-ethylmaleimide, iodoacetamide, and *p*-(chloromercuri)-benzoate were from Serva Entwicklungslabor, Heidelberg; ribonuclease A was from Worthington; $N^{\epsilon}$-acetyllysine was from Cyclo Chemical, Division of Travenol Laboratories; DEAE-cellulose, phosphocellulose, and DEAE-Sephadex A-50 were from Whatman; hydroxylapatite (Bio-Gel HT) and Bio-Gel P-10 were from Bio-Rad Laboratories. All other chemicals were from Merck.

*Enzyme Preparation.* Histone acetyltransferases were extracted from calf thymus which was obtained 15–30 min after the death of the animals and frozen immediately in liquid nitrogen. The tissue was thawed in approximately 10 volumes of buffer A [75 mM Tris-HCl, pH 7.9, 1 mM $MgCl_2$, 0.25 mM EDTA, 5 mM 2-mercaptoethanol, 1 M $(NH_4)_2SO_4$, and 20% (v/v) glycerol] and homogenized in the cold by using an Ultraturrax homogenizer. An equal volume of buffer A was added to the viscous homogenate before filtering it successively through two and six layers of gauze. The homogenate was then sonicated in 50-mL aliquots (50 × 2 s) by using a Branson sonifer (20 kHz; 125 W), followed by the addition of $(NH_4)_2SO_4$ to a final concentration of 3.5 M, and stirred in the cold for at least 2 h. Precipitated protein was pelleted by centrifugation for 90 min at 40000*g* and dissolved in about 10 volumes of buffer B [15 mM Tris-HCl, pH 7.9, 10 mM $NH_4Cl$, 0.25 mM EDTA, 5 mM 2-mercaptoethanol, and 20% (v/v) glycerol].

Before applying the proteins to DEAE-cellulose, we removed $(NH_4)_2SO_4$ by Sephadex G-25 column chromatography or by dialysis for 15 h against 2 × 500 volumes of buffer B. With either method a significant amount of protein, including the majority of histones, precipitated out of solution and was removed by centrifugation.

*Assay of Histone Acetyltransferase Activity.* Histone acetyltransferase activity was measured by determining the amount of [$^{14}$C]acetate incorporated into hot trichloroacetic acid insoluble material after incubating calf thymus histones with the enzyme. The incubation mixture contained the following in a final volume of 110 μL: 50 mM Tris-HCl, pH 8, 0.1 M KCl, 10 mM $NH_4Cl$, 0.2 mM EDTA, 3.5 mM 2-mercaptoethanol, 14% glycerol, 0.01 μCi of [1-$^{14}$C]acetyl-CoA, 10 μg of acceptor protein, and varying amounts of enzyme protein.

Incubations were performed in open glass tubes at 37 °C for 20 min and terminated by pipetting 100 μL of the incubation mixture onto filter paper disks (Schleicher & Schuell 2043b) which were then immersed in ice-cold 25% trichloroacetic acid. After 10–20 min the filter paper disks were heated to 95 °C for 5 min, washed once with 25% trichloroacetic acid, ethanol, ethanol–ether, 1:1 (v/v), and ether, and dried. The dried filters were counted in a liquid scintillation counter using a toluene-based scintillation fluid.

Units of enzyme activity are expressed as nanomoles of acetate incorporated into 10 μg of histone at 37 °C and 20 min of incubation under the conditions described above.

*Isolation of Histones.* Histones were extracted from either calf thymus nuclei or chromatin. Frozen calf thymus was thawed in about 10 volumes of buffer C (0.25 M sucrose, 10 mM $MgCl_2$, 25 mM KCl, 50 mM Tris-HCl, pH 7.5, and 50 mM $NaHSO_3$), minced with scissors, and homogenized by using a Potter Elvehjem homogenizer. The homogenate was filtered through six layers of gauze and centrifuged for 5 min at 1000*g*. The nuclear pellet was resuspended twice in buffer C and centrifuged as before, followed by five washings with 0.14 M NaCl containing 50 mM $NaHSO_3$. Nuclei were suspended in 10 volumes of 0.25 N HCl, homogenized, and stirred for 2 h in the cold. After centrifugation at 27000*g* for 10 min and dialysis of the supernatant against 300 volumes of 0.025 N HCl for 12 h, histones were precipitated with 10 volumes of acetone at −20 °C for 24 h.

Chromatin was prepared by homogenizing the washed nuclei twice in 50 volumes of a EDTA–Tris solution (20 mM EDTA, 10 mM Tris-HCl, pH 8.5, and 50 mM $NaHSO_3$), followed by centrifugation for 10 min at 12000*g*. The sediment was suspended in 50 volumes of glass-distilled water, homogenized for 2 min with full speed by using an Ultraturrax homogenizer, and pelleted for 10 min at 27000*g*. The viscous chromatin was suspended in about 100 volumes of distilled water, and histones were extracted by adding ice-cold 2 N $H_2SO_4$ to a final concentration of 0.4 N and stirring for 60 min in the cold. After centrifugation of the extract at 27000*g* for 10 min, the histones were precipitated from the supernatant with 4 volumes of ethanol at −20 °C for 24 h. Histones were further purified on CM-cellulose following the procedure described by Bailey & Dixon (1973). The acid-soluble proteins were dissolved at a concentration of 10 mg/mL in 0.225 M LiCl–50 mM $CH_3COOLi$, pH 5.1, and adsorbed to CM-cellulose (1 g of cellulose per 20 mg of protein) equilibrated with the same medium. Nonhistone proteins were eluted with 20 column volumes of LiCl–$CH_3COOLi$ buffer. $Li^+$ ions were then removed by washing the column with 25 mM $NH_4HCO_3$–50 mM $CH_3COONH_4$, pH 6.5, and the histones were eluted from the column with 0.2 N HCl. They were precipitated overnight with 10 volumes of acetone at −20 °C, collected by centrifugation, and dried under vacuum.

The labeled histones were dissolved in 0.01 N HCl (10 mg/mL) and applied to a 2.5 × 150 cm Bio-Gel P-10 column equilibrated with 0.01 N HCl. The 4.5-mL fractions were collected and the radioactivity of 50-μL aliquots was measured in 5 mL of Bray's scintillation fluid (Bray, 1960). The different histones were pooled, lyophilized, and rechromatographed on the same column. Individual nonradioactively labeled histones were prepared in the same way. The purity of the histones was checked by polyacrylamide gel electrophoresis.

*Preparation of Calf Thymocytes for Histone Acetylation.* Calf thymus was obtained at the slaughterhouse about 10–15 min after the death of the animals. Twenty grams of thymus tissue freed from fat and fascia was immediately transferred to 500 mL of ice-cold Eagle's minimum essential spinner medium and minced under the medium with sharp scissors.

[1] Abbreviations used: CM, carboxymethyl; DCC, diphenylcarbamyl chloride; DEAE, diethylaminoethyl; EDTA, (ethylenedinitrilo)tetraacetic acid.

Cells were gently squeezed out of the tissue under the medium with a spatula. The suspension was gently pressed twice through four layers of gauze, and the cells were pelleted by centrifugation for 5 min at 100*g*. Thymocytes were suspended in 10 mL of Eagle's minimum essential spinner medium and incubated with 500 μCi of [1-$^{14}$C]acetate in a 100-mL Erlenmeyer flask at 22 °C for 75 min with gently shaking. The cells were then chilled in an ice bath, and for the extraction of histones nuclei were prepared as described above.

*Acetylation of Histones with Calf Thymus Histone Acetyltransferases.* Two hundred micrograms each of the purified calf thymus histone fractions was incubated with histone acetyltransferase A or B at 37 °C for 20 min in a final volume of 2.5 mL containing 50 mM Tris-HCl, pH 8.0, 10 mM $NH_4Cl$, 0.1 M KCl, 0.175 mM EDTA, 3.5 mM 2-mercaptoethanol, 14% (v/v) glycerol, and 0.25 μCi of [1-$^{14}$C]acetyl-CoA. The incubation mixtures were then chilled in ice and an equal volume of cold 0.5 N HCl was added dropwise under stirring. Acid extraction was performed for 90 min at +4 °C. After centrifugation at 40000*g* for 10 min, acid-soluble proteins were dialyzed for 15–20 h against 0.1 N HCl. Proteins were lyophilized and subjected to tryptic digestion.

To measure the incorporation of acetate into histones, we performed incubations in a volume of 110 μL under the conditins described above.

*Analysis of Tryptic Peptides.* For tryptic digestion histones were dissolved at a concentration of 1 mg/mL in 0.1 M $NH_4HCO_3$, pH 8.0, and incubated at 37 °C with DCC-treated trypsin at an enzyme/histone ratio of 1:20 (w/w). After 2 h of digestion an equal amount of trypsin was added and digestion was continued for an additional 2 h. The hydrolysate was then chilled in ice, acidified with acetic acid, and centrifuged for 10 min at 4000*g*, and the soluble material was lyophilized.

Two to five microliters of tryptic peptides (3000–15 000 cpm) was spotted on Whatman 3 MM paper (34 × 57 cm) for fingerprint analysis. High-voltage electrophoresis was carried out in the first dimension for 2.5 h (17 000 V/75 mA) at pH 1.9 using 87 mL of acetic acid and 25 mL of formic acid per L of water. Ascending chromatography in pyridine–1-butanol–glacial acetic acid–water (15:10:3:12) was performed for 12 h in the second dimension. Dansylated lysine was added to the tryptic digest as a marker.

*Polyacrylamide Gel Electrophoresis.* For slab gel electrophoresis histones were dissolved in 50 mM Tris-HCl, pH 6.8, 1% sodium dodecyl sulfate, 1% 2-mercaptoethanol, and 10% glycerol, heated for 1 min at 90 °C, and separated on 15% polyacrylamide gels containing 0.1% sodium dodecyl sulfate (Laemmli, 1970). Radioactivity was detected by fluorography (Laskey & Mills, 1975).

*Identification of Labeled Amino Acids.* Purified histones were acetylated with the respective acetyltransferases and isolated from the incubation mixture with 0.25 N HCl as described previously (Gallwitz & Sures, 1972). Acetylated proteins were digested with trypsin for 5 h at 37 °C in 0.1 M ammonium bicarbonate, pH 8.0, at a substrate/enzyme ratio of 25:1 (w/w), followed by digestion with Pronase for 20 h at 37 °C at a substrate/enzyme ratio of 10:1. The digests were lyophilized and dissolved in a small volume of water after removing any insoluble material by centrifugation. Ascending paper chromatography (Schleicher & Schuell 2034b) was performed in pyridine–1-butanol–glacial acetic acid–water (15:10:3:12 by volume) with authentic $N^{\epsilon}$-acetyllysine as a marker. The dried chromatogram was cut into 0.5-cm strips for counting the radioactivity.

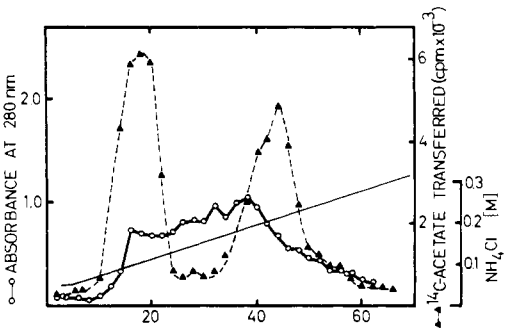


FIGURE 1: DEAE-cellulose chromatography of histone acetyltransferases. $(NH_4)_2SO_4$-precipitated, desalted proteins of the first homogenate from 50 g of calf thymus tissue were adsorbed onto DEAE-cellulose (3 × 12 cm) and eluted with a linear 0.01–0.35 M $NH_4Cl$ gradient (450 mL) at a flow rate of 50 mL/h. Enzyme activity (▲); optical density at 280 nm (O).

*Other Methods.* Proteins were measured either by the method of Lowry et al. (Lowry et al., 1951) or by turbidity at 400 nm in 1.1 M trichloroacetic acid.

## Results

*Extraction and Purification of Histone Acetyltransferases.* The purification of histone acetyltransferases was monitored by assaying the enzyme activity using total calf thymus histones and [1-$^{14}$C]acetyl-CoA as substrates.

A homogenate of whole calf thymus, usually 50 g in 600 mL of buffer A, was prepared by using an Ultraturrax homogenizer. The viscous homogenate was sonicated under cooling, and the proteins were then precipitated with 3.5 M $(NH_4)_2SO_4$, leaving most of the nucleic acids in the supernatant. The precipitated proteins were desalted on Sephadex G-25 or by dialysis and applied to a DEAE-cellulose column (Figure 1). The column was developed with a linear 10–350 mM $NH_4Cl$ gradient at pH 7.9. Two prominent peaks of histone acetyltransferase activity, designated as A and B, were observed eluting at about 0.1 and 0.22 M $NH_4Cl$, respectively.

*Purification of Histone Acetyltransferase A.* Fractions containing enzyme activity A were pooled from several chromatographic runs on DEAE-cellulose, and the proteins were concentrated by $(NH_4)_2SO_4$ precipitation at 55% saturation. Precipitated proteins were dissolved in buffer D [10 mM $NaH_2PO_4/Na_2HPO_4$, pH 6.8, 0.25 mM EDTA, 5 mM 2-mercaptoethanol, and 10% (v/v) glycerol] and dialyzed against the same buffer for 10–15 h. After dialysis the soluble proteins were adsorbed to hydroxylapatite at pH 6.8 in buffer D at 10 °C and eluted with a linear 10–600 mM sodium phosphate gradient. As seen in Figure 2, histone acetyltransferase A eluted at a sodium phosphate concentration of about 0.3 M. The fractions having enzyme activity were dialyzed against buffer B for about 12 h and applied to a phosphocellulose column equilibrated with the same buffer. The column was developed with a linear 10–600 mM $NH_4Cl$ gradient (Figure 3). The enzyme eluted at a $NH_4Cl$ concentration of about 0.23 M. It should be mentioned that phosphocellulose chromatography always resulted in a significant loss of enzyme activity. In a last purification step, which was often omitted to avoid further inactivation, histone acetyltransferase A was chromatographed on DEAE-Sephadex A-50 from which the enzyme eluted at a $NH_4Cl$ concentration of 0.18 M, well behind the main protein peak. The most active fractions were collected, concentrated by ultrafiltration using a Diaflo PM-30 membrane or in Minicon cells, type B-15, and stored in liquid nitrogen.

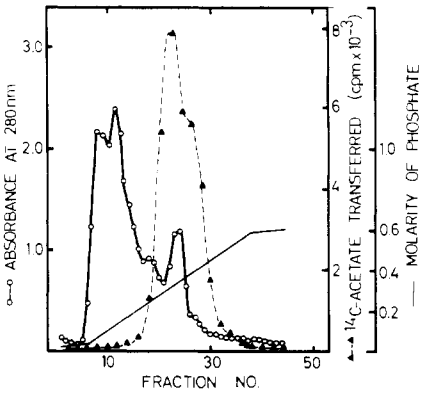


FIGURE 2: Hydroxylapatite chromatography of histone acetyltransferase A. Fractions containing enzyme activity A were pooled after DEAE-cellulose chromatography, precipitated, dialyzed, and applied to a hydroxylapatite column (volume 4 mL). Adsorbed proteins were eluted with a linear 0.01–0.6 M sodium phosphate buffer gradient (150 mL) at 10 °C and a flow rate of 50 mL/h. Enzyme activity (▲); optical density at 280 nm (O).

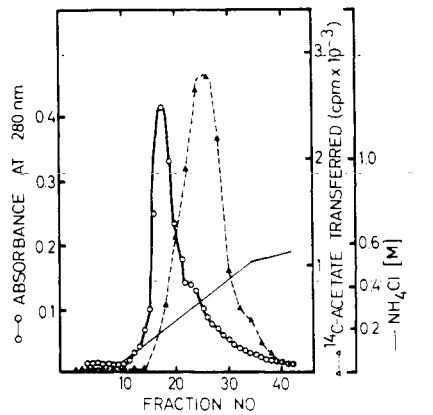


FIGURE 3: Phosphocellulose chromatography of histone acetyltransferase A. Enzymatically active fractions were pooled after hydroxylapatite chromatography, dialyzed against buffer B, and applied to a phosphocellulose column (volume 7 mL). Elution was with a linear 0.01–0.6 M $NH_4Cl$ gradient (180 mL) at a flow rate of 40 mL/h. Enzyme activity (▲); optical density at 280 nm (O).

Table I: Purification of Histone Acetyltransferase A

| step | units/mg of protein[a] | yield (%) | purification (x-fold) |
|---|---|---|---|
| $(NH_4)_2SO_4$-precipitated protein | 0.61 | 100[b] | 1 |
| DEAE-cellulose | 1.91 | 43 | 3.1 |
| hydroxylapatite | 5.59 | 15.4 | 9.2 |
| phosphocellulose | 13.64 | 6.6 | 22.4 |

[a] Enzyme units are defined as nanomoles of acetate incorporated into 10 μg of histones (total volume of 110 μL) in 20 min at 37 °C. [b] First step at which the enzyme activity could be measured after removal of endogenous histones. Activity comprises all three enzymes A–C.

A purification scheme for histone acetyltransferase A is given in Table I. The overall purification of enzyme A seems surprisingly low although the enzyme was well separated from the bulk of protein in all steps of purification (see Figures 1–3). It was, however, impossible to measure acetyltransferase activity in the first tissue homogenate because of the high $(NH_4)_2SO_4$ concentration necessary to solubilize the enzymes. Enzyme activity could only be determined after the first $(NH_4)_2SO_4$ precipitation, which removed most of the nucleic acids, and subsequent dialysis, which resulted in the precipitation of some proteins including most of the histones. In addition, the purification of enzyme A by chromatography on DEAE-cellulose is an underestimation since in the protein mixture applied to the ion exchanger acetyltransferases B and C were also measured. Despite that, it is obvious that histone acetyltransferase A was inactivated to a significant extent during the different steps of purification.

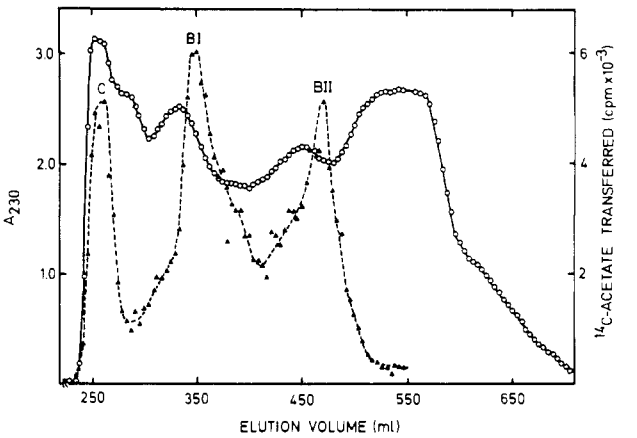


FIGURE 4: Elution profile of histone acetyltransferase B after gel filtration on Sephadex G-200. The proteins after DEAE-cellulose chromatography corresponding to enzyme activity B were pooled, precipitated with ammonium sulfate, dialyzed, and rechromatographed on DEAE-cellulose. Enzymatically active fractions were concentrated in Minicon cells (type B-15) and passed over Sephadex G-200 (2.5 × 210 cm) at a flow rate of 5 mL/h. Enzyme activity (▲); optical density at 280 nm (O).

Table II: Purification of Histone Acetyltransferase B

| step | units/mg of protein[a] | yield (%) | purification (x-fold) |
|---|---|---|---|
| $(NH_4)_2SO_4$-precipitated protein | 0.35 | 100[b] | 1 |
| DEAE-cellulose | 0.84 | 58.7[c] | 2.3 |
| Amicon filtration (B-15) | 2.88 | 22.4[c] | 8.2 |
| Sephadex G-200 | 83.8 | 6.8 | 239.4 |

[a] Enzyme units are defined in Table I. [b] First step at which the enzyme activity could be measured. [c] Enzymes B and C measured together.

*Purification of Histone Acetyltransferase B.* The enzyme was rechromatographed on DEAE-cellulose after precipitation with $(NH_4)_2SO_4$ to 55% saturation to further purify histone acetyltransferase B. Fractions containing enzyme activity were pooled and concentrated in Minicon cells, type B-15. This concentration led to a fourfold enrichment of the enzyme activity. The concentrated protein solution was then passed over a 2.5 × 210 cm Sephadex G-200 column equilibrated with buffer B containing 0.3 M KCl. As shown in Figure 4, three peaks of enzyme activity were usually resolved, one eluting with the void volume (enzyme C) and two others (designated B I and B II) varying in peak heights. The proteins in the enzymatically active fractions were concentrated in Minicon B-15 cells, and, after incubation for 3 h at room temperature in the presence of 0.1 M 2-mercaptoethanol, they were rechromatographed on a Sephadex G-200 superfine column equilibrated with buffer B containing 0.3 M KCl. Whereas the enzyme activities B I and B II eluted now at an identical position relative to marker proteins, all of the enzyme activity C was again found in the exclusion volume. It therefore appears that enzyme activity B I is an aggregation product of B II and that activity C is an acetyltransferase different from enzymes B and A.

The purification of histone acetyltransferase B is summarized in Table II. As already discussed for enzyme A, the

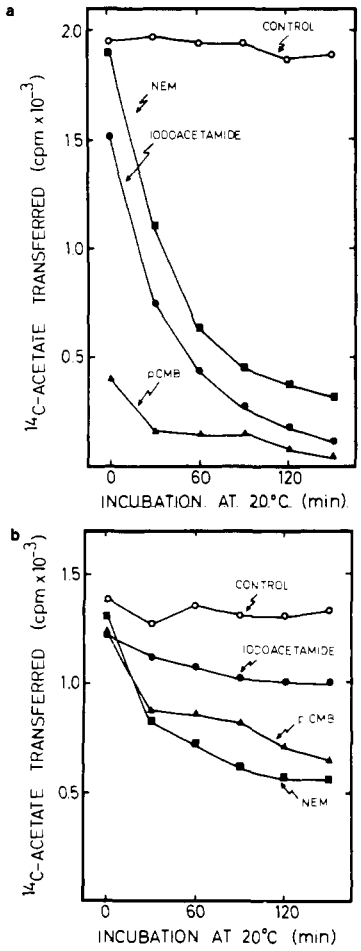


FIGURE 5: Effect of *N*-ethylmaleimide (NEM), iodoacetamide, and *p*-(chloromercuri)benzoate (pCMB) on histone acetyltransferases A and B. Enzyme A (a) or B (b), dialyzed against 15 mM Tris-HCl, pH 7.9, and 20% glycerol for 10 h, was incubated for 150 min with NEM ($1.25 \times 10^{-3}$ M), iodoacetamide ($1.25 \times 10^{-4}$ M), and pCMB ($1.25 \times 10^{-4}$ M), respectively. Aliquots were removed every 30 min, dialyzed against buffer B for 4 h, and assayed for enzyme activity as described under Materials and Methods.

purification of acetyltransferase B is an underestimation.

*Properties of the Enzymes.* The acetylation of total calf thymus histones was linear for about 20–25 min at 37 °C using either enzyme A or B. Both enzymes had a broad pH optimum: maximal acetylation of all histones was found in the range between pH 7.6 and pH 8.4.

The molecular weight of histone acetyltransferase A was determined by gel filtration on Sephadex G-150 superfine. The enzyme migrated as a globular protein with a molecular weight of about 120 000. According to gel filtration on Sephadex G-200, the molecular weight of histone acetyltransferase B was about 98 000. The molecular weight of enzyme C was very likely higher than 200 000 since after repeated chromatographic runs on Sephadex G-200 the activity was exclusively found in the void volume of the column. The inhibitory effects of *p*-(chloromercuri)benzoate, *N*-ethylmaleimide, and iodoacetamide on the activity of enzymes A and B are presented in parts a and b of Figure 5. At pH 7.9 and a protein concentration of 100 μg/mL, *p*-(chloromercuri)benzoate at a concentration of $1.25 \times 10^{-4}$ M inhibited histone acetyltransferase A by more than 90% after a preincubation time of 30 min at 25 °C. A comparable inhibitory effect of *N*-ethylmaleimide and iodoacetamide was observed at reagent concentrations of $1.25 \times 10^{-3}$ M and a preincubation time of 150 min at 25 °C.

Under the same experimental conditions, histone acetyltransferase B at a protein concentration of 70 μg/mL was inhibited only by about 50% after a preincubation time of 150 min with *N*-ethylmaleimide and *p*-(chloromercuri)benzoate. Iodoacetamide had only a marginal inhibitory effect on the activity of enzyme B (Figure 5b).

*Substrate Specificity.* The substrate specificity of acetyltransferase A and B was tested by using different basic and acidic proteins as well as the homopolymer poly-L-lysine as acetate acceptors. Only histones and poly-L-lysine were acetylated, the latter being a better substrate for acetyltransferase A. The arginine-rich protamines were not acetylated by the two enzymes.

Histones complexed with DNA in the form of mononucleosomes were also tried as substrate for the isolated enzymes. The uptake of acetate, however, was very low compared to histones free of DNA, and H3 was nearly exclusively acetylated either in the presence or in the absence of added enzyme (data not shown).

Histones acetylated with [$^{14}$C]acetyl-CoA by using the different acetyltransferases were reextracted from the incubation mixture and digested with trypsin and Pronase to identify the acetylation products. The resulting amino acids were then separated by ascending paper chromatography using $N^{\epsilon}$-acetyllysine as a marker. The radioactivity of all histones acetylated in vitro comigrated with $N^{\epsilon}$-acetyllysine.

In order to determine the substrate specificity of these enzymes for different histones, we studied the kinetics of acetylation by incubating purified individual calf thymus histones H2a, H2b, H3, and H4 with enzymes A, B, and C. A more detailed analysis was carried out by comparing tryptic fingerprints of these in vitro acetylated histones with the pattern of tryptic peptides derived from histones acetylated in vivo. It should be noted that the acetyltransferases used did not contain any histone deacetylase activity.

In a first experiment total calf thymus histones were incubated with [$^{14}$C]acetyl-CoA and each of the different enzymes. After 30 min at 37 °C the incubation mixtures were treated with dodecyl sulfate and 2-mercaptoethanol and directly loaded onto a 15% dodecyl sulfate–polyacrylamide slab gel. The fluorogram of one such gel is shown in Figure 6. It can be seen that in the presence of all five histones acetyltransferase B nearly exclusively modifies histone H4 (slot 5). Enzymes A and C, however, transfer acetyl groups to all five histones, although to a varying extent. Enzymes A and C also preferentially acetylate histone H4 and they both use histone H1 as a substrate, which is not acetylated in vivo. However, both enzymes show a slightly different specificity for histone H3 (slots 3 and 6) which is obviously a better substrate for acetyltransferase A.

Substrate saturation curves resulting from the incubation of the three acetyltransferases with increasing amounts of each of the purified calf thymus histones H2a, H2b, H3, and H4 are shown in Figures 7–9. The results clearly demonstrate that at constant amount of enzyme protein the degree of acetylation of different histones varies significantly, depending on the concentration of the histones.

Acetyltransferase B preferentially uses histone H4 as a substrate. Histone H2a is also efficiently modified by this enzyme which, on the other hand, hardly acetylates histones H3 and H2b (Figure 7).

A more complex result was obtained by using acetyltransferase A (Figure 8). At low histone concentrations (up to 100 μg/mL) histone H4 is more extensively labeled than H2a which becomes a better substrate at higher concentrations

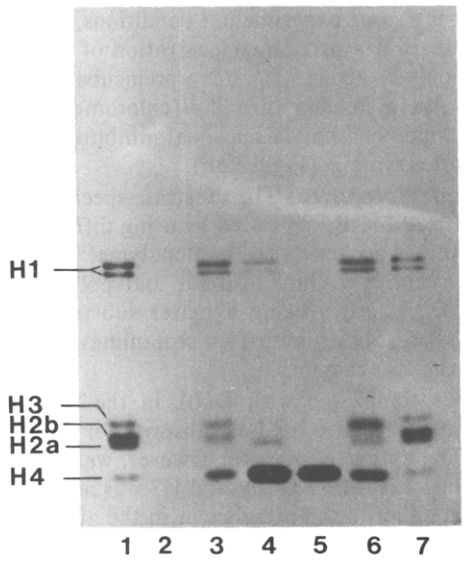


FIGURE 6: 15% polyacrylamide–dodecyl sulfate gel electrophoresis of calf thymus histones modified in vitro with acetyltransferases A, B, and C. Incubation conditions, gel electrophoresis procedures, and fluorography are described under Materials and Methods. The autoradiogram shows the following: [$^{14}$C]lysine-labeled marker histones from HeLa cells (slots 1 and 7); total calf thymus histones acetylated with either acetyltransferase C (slot 3), acetyltransferase B before (slot 4) and after (slot 5) Sephadex G-200 chromatography, and acetyltransferase A (slot 6); histones incubated under the same conditions in the absence of enzymes (slot 2).

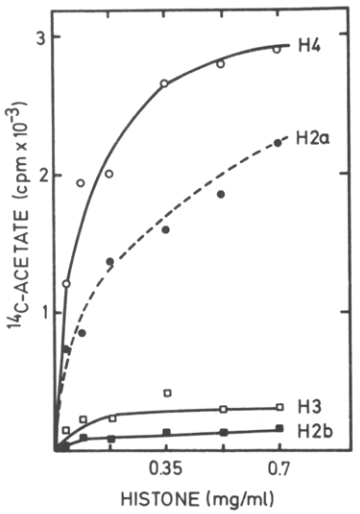


FIGURE 7: Acetylation of histones by acetyltransferase B. Increasing amounts of histones were incubated with a constant amount of enzyme for 20 min at 37 °C under the conditions described under Materials and Methods.

(more than 400 $\mu$g/mL). Again, histones H3 and H2b are the less preferred substrates though the acetylation of these histones by enzyme A is higher than that observed for enzyme B.

Histone acetyltransferase C (Figure 9) acetylates all four histones nearly equally well at low histone concentrations, whereas at concentrations higher than 350 $\mu$g/mL histones H2a and H2b are more effectively modified than histones H3 and H4.

The unusual shape of the curves obtained after incubation of acetyltransferase A with histone H3 (Figure 8) and acetyltransferse C with histones H3 and H4 (Figure 9) could be explained by the fact that these histones interact with the enzyme proteins (or proteins contaminating the enzyme preparations) which results in precipitation of enzyme–histone

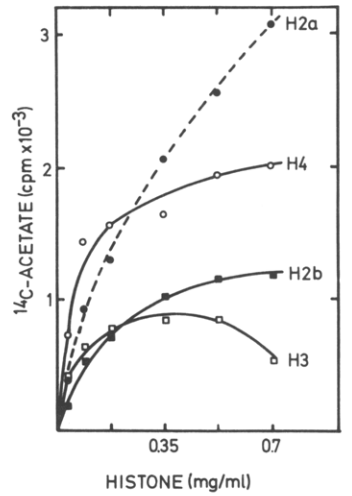


FIGURE 8: Acetylation of histones by acetyltransferase A. For details see Figure 7.

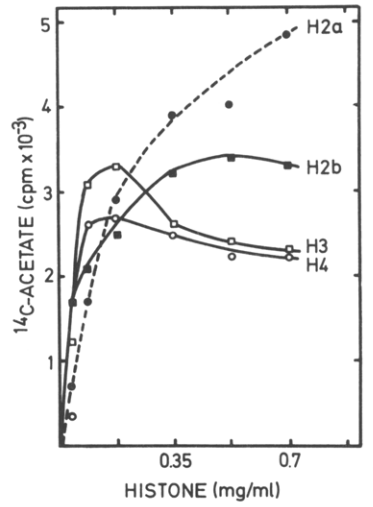


FIGURE 9: Acetylation of histones by acetyltransferase C. For details see Figure 7.

complexes during the incubation. In fact, a varying degree of turbidity could be observed in all incubation mixtures.

*Comparison of in Vivo and in Vitro Acetylation Sites by Fingerprint Analysis.* From the experiments described above it seems likely that the three histone acetyltransferases retain at least some of their substrate specificity in vitro. These results prompted us to examine the specificity of the enzymes in more detail. Histones which had been acetylated in vitro with either enzyme A or B were digested with trypsin and the resulting peptides were subjected to fingerprint analysis. The fingerprint patterns were compared to those derived from tryptic digestions of histones which had been acetylated in vivo by incubating calf thymocytes in suspension culture with [$^{14}$C]acetate.

Using this labeling procedure, we have recently identified the acetylation sites of the four calf thymus histones H2a, H2b, H3, and H4 by sequence analysis (unpublished experiments). This investigation revealed that lysine residues 9, 14, 18, and 23 of histone H3 are acetylated. In addition, a minor species of H3 must exist in calf thymus which instead of threonine has a lysine residue in position 3 which can be modified by acetylation. Histone H4 has four acetylation sites (lysine residues 5, 8, 12, and 16), H2b can also be acetylated at four sites (lysine residues 12, 15, 20, and 23), and histone H2a has one major acetylation site in position 5 and a minor one in position 9.

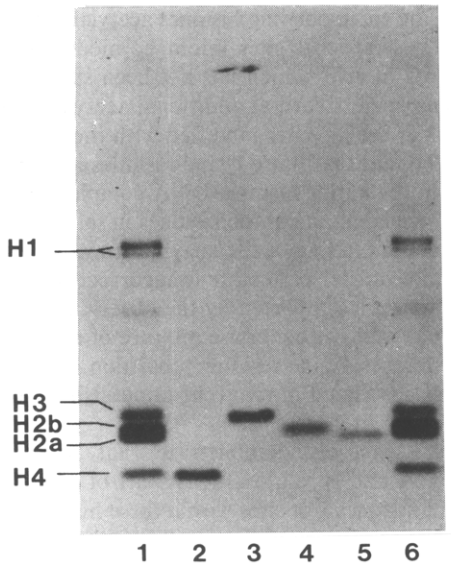


FIGURE 10: 15% polyacrylamide–dodecyl sulfate gel electrophoresis of purified calf thymus histone fractions acetylated in vivo. The histones were isolated by chromatography on Bio-Gel P-10 as described under Materials and Methods. The fluorogram shows [$^{14}$C]lysine-labeled marker histones from HeLa cells (slots 1 and 6) and histone H4 (slot 2), histone H3 (slot 3), histone H2b (slot 4), and histone H2a (slot 5) modified in vivo with [1-$^{14}$C]acetate.

Since the different lysine residues which are subject to acetylation can be modified independently from each other, multiple acetylpeptides can be expected to result from tryptic digestion. Histone H4, for instance, if acetylated at all four possible lysine residues, could maximally give 10 tryptic peptides. The number of acetylated peptides can in addition be influenced by other modifications such as methylation and phosphorylation which occur in the N-terminal regions of the histones.

When radioactive tryptic fingerprints from in vivo and in vitro acetylated histones are compared, one has to realize that the purified histone fractions, which were used as substrates for the acetyltransferases in vitro, were already modified to a certain extent. It seems very unlikely, however, that in a random population of molecules all of the histones show the same acetylation pattern, so that, theoretically, each of the lysine residues in question should still be available for in vitro modification. The radioactive intensity of a certain peptide spot therefore does not reflect the actual preference of an enzyme for a specific acetylation site. Enzymatically deacetylated histones which are more useful for these studies were not available at the time the experiments were carried out.

The purity of the four in vivo acetylated histones used for tryptic digestion was demonstrated by dodecyl sulfate–polyacrylamide gel electrophoresis as shown in Figure 10.

We found that in vivo acetylated calf thymus histone H4 gave five labeled tryptic peptides (Figure 11a; see paragraph at end of paper regarding supplementary material). When purified histone H4 was acetylated in vitro with acetyltransferase B which, as shown above, nearly exclusively modifies H4, three of these five acetylpeptides were detected [Figure 11c (supplementary material)]. On the other hand, acetylation of histone H4 with enzyme A gave quite a different and rather complex fingerprint pattern with eight labeled peptides [Figure 11b (supplementary material)]. From these eight peptides only three faintly labeled ones were found in the same position as the peptides resulting from in vivo acetylation. This observation indicates that other additional lysine residues than those modified in vivo become acetylated by acetyltransferase A in vitro.

The tryptic fingerprint of in vivo acetylated histone H3 showed 10 labeled peptides [Figure 12a (supplementary material)]. The pattern of acetylpeptides derived from digestion of histone H3 acetylated by enzyme A was quite similar in that all of its six labeled peptides were also found in the in vivo pattern [Figure 12b (supplementary material)]. In contrast, histone H3 acetylated with acetyltransferase B gave four acetylpeptides from which the most intensively labeled one was not found in the in vivo pattern [Figure 12c (supplementary material)].

A striking difference was observed between the peptide patterns of histone H2a acetylated with enzyme A or B. In vivo labeled histone H2a showed one major acetylpeptide in addition to two very faintly labeled ones [Figure 13a (supplementary material)]. Modification of histone H2a with acetyltransferase B resulted in the same predominantly labeled peptide and four additional [$^{14}$C]acetyl-labeled peptides which were labeled to a much lesser extent [Figure 13c (supplementary material)]. The tryptic pattern observed for H2a acetylated with enzyme A was rather complex in showing 10 labeled spots in addition to the predominant radioactive peptide found after in vitro acetylation [Figure 13b (supplementary material)]. Acetyltransferase A therefore seems to modify several other lysine residues in histone H2a in vitro. These findings make us believe that in vivo acetyltransferase B modifies the major acetylation site, lysine residue 5, in calf thymus histone H2a.

The fingerprint pattern of in vivo acetylated histone H2b showed 14 labeled peptides [Figure 14a (supplementary material)]. This large number of peptides observed after tryptic digestion could result from partial phosphorylation of the two serine residues in the N-terminal region (positions 6 and 14) of histone H2b which would add to the complexity of the fingerprint due to the known four acetylation sites. It is also possible that this histone was not fully digested with trypsin because of three Lys-Lys residues in the region where the enzymatic acetylation takes place. Nevertheless, eight of the labeled peptides derived from in vivo acetylated histone H2b were also found after modification of this histone with acetyltransferase A [Figure 14b (supplementary material)]. In contrast, only three weakly labeled peptides were detected in the fingerprint of histone H2b with enzyme B [Figure 14c (supplementary material)]. We are therefore inclined to think that most of the lysine residues in histone H2b, which are subject to acetylation, are modified in vivo by acetyltransferase A rather than enzyme B.

## Discussion

Though the functional significance of histone acetylation has not been clearly proven to date, these specific modifications could provide a physical basis for structural alterations occurring in nucleosomes. The existence of multiple enzymes catalyzing those modifications might reflect the complexity of steps which are necessary to induce structural and/or functional changes within the chromatin.

The paper describes the separation and partial purification of three histone-specific acetyltransferases from calf thymus, two of which (enzymes A and B) have been further characterized.

We already introduced this nomenclature for similar enzymes isolated from rat thymus (Gallwitz & Sures, 1972) and liver (Gallwitz, 1973) nuclei. The histone acetylating enzymes bind to DEAE-cellulose at neutral pH, and enzyme A is eluted at a lower salt concentration than enzyme B. These two

enzymes are further clearly distinguishable by their substrate specificity (enzyme B shows a clear preference for histone H4 acetylation), and, in addition, histone acetyltransferase A is much more tightly bound to chromatin than enzyme B, the latter being predominantly found in the cytoplasm (our unpublished observation). These criteria hold for histone acetyltransferases A and B from rat liver and thymus as well as from calf thymus. We therefore suggest to use this nomenclature until these enzymes are completely characterized.

The purification of both histone acetyltransferases from calf thymus turned to be quite difficult. Histone acetyltransferases seem to be present in only low amounts within the cell which made it necessary to work with large quantities of starting material to obtain sufficient material for further purification. Enzyme A was well separable from the bulk of protein on different ion-exchange columns; however, a quite significant loss of activity was observed during each step of purification. Though the peak of enzyme activity did not coincide with the main protein peak eluting from the final DEAE-Sephadex column, no additional purification step was carried out.

All attempts to further purify acetyltransferase B by chromatography on phosphocellulose, CM-cellulose, or hydroxylapatite failed. Either enzyme activity B as well as the bulk of protein did not bind to the ion-exchange materials or no sufficient separation could be achieved. Sephadex G-200 gel filtration, however, resulted in the separation of three enzyme activities C, B I, and B II. The B I activity is an unstable aggregation product of histone acetyltransferase B (B II). This could be shown by rechromatography on Sephadex G-200 of all three enzyme activities after treatment with 2-mercaptoethanol. Enzyme C still eluted with the void volume whereas the B I activity was now eluting at the same position as B II.

Histone acetyltransferases A and B are acidic proteins, as revealed by their elution properties on DEAE-cellulose, with IEP's at about pH 5.5 (data not shown). From all the proteins tested both enzymes specifically acetylate histones, though acetylation of the homopolymer poly-L-lysine was observed.

Treatment of both enzymes with SH-blocking reagents prior to incubations with histones and acetyl-CoA resulted in a significant loss of enzyme activity. Whereas acetyltransferase A was nearly completely inhibited by those reagents, the activity of enzyme B was affected to about 50%. These results indicate a possible involvement of SH groups in the acetate transfer from acetyl-CoA to histones which has previously been shown for the histone acetyltransferase B from rat thymus nuclei (Gallwitz & Sures, 1972). Although a preferential use of different histones by the two enzymes could be shown, the question of whether only one enzyme is involved in the acetylation of all accessible lysine residues within one histone is still not answered. Our data do not rule out the possibility that both enzymes A and B might recognize different lysine residues within one histone, thus acting together. Such a mechanism has been discussed because of similar sequences surrounding acetylated lysine residues within different histones (Candido & Dixon, 1972). Those sequences, however, do not seem to be the only recognition sites for the acetyltransferases. Other parts of the histones show identical sequences which are not subject to modification.

The unexpected acetylation of histone H1, which is not a substrate in vivo, as well as the acetylation of poly-L-lysine by acetaltransferase A could, however, be explained by the ability of the enzyme to recognize lysine-rich sequences in vivo.

Except for histone H2a, not all of the peptides derived from in vivo labeled histones could be detected in the fingerprint patterns of the corresponding histones acetylated with enzyme A or B. These specific sites might be modified by acetyltransferase C in vivo, which has not been studied so far.

The occurrence of several additional acetylpeptides in the fingerprints of the histones modified with the enzymes could be due to the fact that more lysine residues are accessible in vitro than in the native histone–DNA complex. In addition, the altered conformation of the histones in solution could lead to an unspecific binding of the enzymes to its substrates, resulting in the transfer of acetate to incorrect lysine residues. This assumption is supported by the observation of Thwaits et al. (1976a) that, with a crude mixture of rat liver nuclear acetyltransferases, lysine residue in position 4 of histone H3, which is not acetylated in vivo (our unpublished results), becomes modified in vitro.

Our results clearly demonstrate that histone acetyltransferases A and B retain at least part of their specificity for different histones—or sites within these histones—in vitro. However, the in vitro modification by both enzymes of additional lysine residues not acetylated in vivo underlines the importance for a specific substrate conformation which is required for the enzymes to specifically acetylate certain lysine residues within the nucleosomal histones.

It should be noted that, though we were not able to purify the different enzymes to homogeneity, the presented procedure yields histone-specific acetyltransferases active and pure enough for further studies. In combination with the use of histone-specific deacetylases (Vidali et al., 1972) which we could partially purify from calf thymus recently (unpublished experiments), more detailed investigations of the interaction between deacetylated and acetylated histones and DNA become feasible.

## Acknowledgments

We are indebted to the late Dorothea Meyer zu Stieghorst for expert technical assistance.

## Supplementary Material Available

Figures 11–14 showing the tryptic fingerprints derived from histones H4, H3, H2a, and H2b (8 pages). Ordering information is given on any current masthead page.

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# Equilibrium Substrate Binding Studies of the Malic Enzyme of Pigeon Liver. Equivalence of Nucleotide Sites and Anticooperativity Associated with the Binding of L-Malate to the Enzyme-Manganese(II)-Reduced Nicotinamide Adenine Dinucleotide Phosphate Ternary Complex†

Terry A. Pry‡ and Robert Y. Hsu*

ABSTRACT: Malic enzyme (ME) from pigeon liver is a tetrameric protein containing apparently identical subunits. In the present study, equilibrium dialysis and fluorescence titration techniques are employed to determine the binding parameters of nucleotide cofactors, malate, and the inhibitor oxalate. ME binds $NADP^+$ or NADPH at four independent and equivalent sites with dissociation constants of 1.33 $\mu$M (pH 7.5, 4 °C) and 0.29 $\mu$M (pH 7.0, 5 °C), respectively, showing "all-of-the-sites" reactivity. The affinity of both nucleotides decreases with increasing temperature, yielding $\Delta H_{dissociation}$ values of 11.4 kcal/mol for E-$NADP^+$ and 8.9 kcal/mol for E-NADPH, thus implicating the involvement of polar forces in the binding process. The affinity of $NADP^+$ is independent of pH between 6.1 and 8.4, whereas that of NADPH is highly pH dependent and decreases ~63-fold from pH 6.0 to pH 8.0. The pH profile suggests the participation of a protonated enzyme group(s) (p$K$ = 7.2-7.5) in NADPH binding, probably a histidine residue. The affinity of $NADP^+$ is enhanced ca. twofold by pyruvate, in the presence of $Mn^{2+}$ (50-100 $\mu$M) saturating only two "tight" metal sites [Hsu, R. Y., Mildvan, A. S., Chang, G. G., & Fung, C. H. (1976) *J. Biol. Chem. 251*, 6574]. Binding of $Mn^{2+}$ at weak metal sites ($K_D \simeq 0.9$ mM) prevents this change. Malate binds free ME or binary E-$Mn^{2+}$ and E-$NADP^+$ (H) complexes weakly with dissociation constants of ≥2 mM. The affinity is significantly increased by $Mn^{2+}$ and NADPH in the ternary E-$Mn^{2+}$-NADPH complex, yielding two "tight" ($K_D$ = 22-30 $\mu$M) and two "weak" ($K_D$ = 250-400 $\mu$M) malate sites per enzyme tetramer as the result of either preexisting nonidentity or negative cooperativity between initially identical sites. The transition-state inhibitor oxalate binds ME tightly ($K_D$ = 65 $\mu$M) at the two tight malate sites, showing "half-of-the-sites" stoichiometry. The binding parameters are unaffected by $Mn^{2+}$, whereas the affinity of this inhibitor is enhanced 3.5-fold by saturation with NADPH. Further evidence for the half-of-the-sites reactivity of the affinity label bromopyruvate [Pry, T. A., & Hsu, R. Y. (1978) *Biochemistry 17*, 4024] is obtained by sequential modification of the four putatively identical SH groups of ME with bromopyruvate, 5,5′-dithiobis(2-nitrobenzoic acid), and $K^{14}CN$. The modified enzyme has a structure of $E_4(S\text{-pyr})_2(S\text{-}^{14}CN)_2$ and is "inactive" in the reaction with malate. In contrast, the $E(S\text{-}^{14}CN)_4$ derivative prepared in the absence of bromopyruvate is completely active. The oxidative decarboxylase reaction is inhibited by high concentrations (≥0.3 mM) of malate in the presence of tightly bound $Mn^{2+}$. Direct binding studies show a parallel increase in the affinity of NADPH, confirming our previous notion [Reynolds, C. H., Hsu, R. Y., Matthews, B., Pry, T. A., & Dalziel, K. (1978) *Arch. Biochem. Biophys. 189*, 309] that malate inhibits the rate-limiting NADPH release step.

Pigeon liver malic enzyme [L-malate:$NADP^+$ oxidoreductase (decarboxylating), EC 1.1.1.40] is a tetramer composed of identical or nearly identical subunits (Nevaldine et al., 1974). The substrates $NADP^+$ and malate exhibit typical Michaelis-Menten (i.e., noncooperative) kinetic behavior in the oxidative decarboxylase reaction at a constant level of $Mg^{2+}$ (Hsu et al., 1967). The initial velocity and product inhibition patterns are consistent with a sequential, ordered kinetic mechanism with $NADP^+$ adding first, followed by malate, and the release of $CO_2$, pyruvate, and NADPH as products. More recently, the possibility of anticooperativity or nonidentical active sites is suggested by the following observations: (a) metal binding studies indicating the presence of two "tight" and two to four "weak" $Mn^{2+}$ sites per enzyme tetramer (Hsu et al., 1976); (b) the apparent kinetic negative cooperativity of $Mn^{2+}$, potentiated by the substrate malate (Hsu et al., 1976); (c) a transient burst of enzyme-bound NADPH which equals approximately half of the active-site concentration

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