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A More Sensitive Assay for Histone Deacetylase

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The new assay uses as substrate a peptide derived from the amino terminal domain of calf histone H4. The peptide contains all the lysines that are acetylated in H4 *in vivo* and these lysines are specifically labeled *in vitro* with acetic anhydride to a high specific activity. This substrate allows histone deacetylase activity to be measured economically and with high sensitivity either with pure enzyme or with crude extracts.

Histone deacetylase is a key enzyme in the control of histone acetate content (see, for example, Ref. (1,2)) and changes in histone acetate content have been closely associated with changes in chromatin structure and function (e.g., Ref. (3–5)). Several studies of histone deacetylase have been carried out (6–10) but purification and characterization have only been reported in detail for the calf thymus enzyme (6,11,12). The assay has normally used *in vivo* acetylated histones (10,12–15) or histones acetylated *in vitro* with acetyltransferase (16,17). The first method gives a substrate with relatively low specific activity and is expensive; the second method suffers from the limited availability of acetyltransferase. The residues involved are lysines and these can be acetylated chemically with acetic anhydride (18). This method has not been used in deacetylase studies because it is not specific for the *in vivo* acetylation sites. However, calf thymus deacetylase does not require a complete histone molecule as substrate. The N-terminal domain of histone H4, in particular, appears to be sufficient to provide a normal substrate for both acetyltransferase and deacetylase (16,19). The sequence of the N-terminal domain of histone H4 is Ac[1]-Ser–Gly–Arg–Gly–Lys–Gly–Gly–Lys–Gly–Leu–Gly–Lys–Gly–Gly–Ala–Lys–Arg–His–Arg–Me-Lys–Val–Leu–Arg. . . . The lysines occur at positions 5, 8, 12, and 16 and all of these may be acetylated or deacetylated *in vivo* (20,21). Peptide 1-23 can be prepared from commercially available histone H4 using digestion with acetic acid and separation of the peptide on a column of Sephadex G-50, as described by Lewis *et al.* (23). The peptide is readily prepared in large amounts and is stable when stored dry. We report the use of peptide 1-23 of histone H4, acetylated with acetic anhydride, as a substrate for histone deacetylase assays.

[1] Abbreviations used: Ac, acetyl; Me, methyl; PMSF, phenylmethylsulfonyl fluoride.

MATERIALS AND METHODS

Total calf thymus histone, type II, was from Sigma. [^{3}H]Acetic anhydride, 25 mCi, 6.17 Ci/mmol, was from Amersham. All chemicals used were reagent grade. Purified calf thymus deacetylase I (13) was kindly provided by L. S. Cousens.

Buffer A was 30 mM NaCl, 1 mM KCl, 5 mM $MgCl_2$, 0.1% (w/v) Triton X-100, 10 mM Tris·HCl, pH 7.1 (22). Buffer B was 10 mM NH_4Cl, 0.25 mM EDTA, 5 mM 2-mercaptoethanol, 20% (v/v) glycerol, 15 mM Tris·HCl, pH 7.9. Buffer C was 1 mM $MgCl_2$, 0.25 mM EDTA, 5 mM 2-mercaptoethanol, 20% (v/v) glycerol, 75 mM Tris·

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TABLE 1

DEACETYLASE ASSAY

	Counts per minute
Complete assay mixture	625
Minus peptide substrate	0
Minus deacetylase enzyme	158
With heated (2 min, 80°C) enzyme	169
Kept at 0°C	160

Note. Purified calf thymus deacetylase I (13) was incubated, after appropriate dilution, in the standard assay, as described under Materials and Methods, for 2 h at 30°C with the acetylated peptide.

HCl, pH 7.9. PMSF (phenylmethylsulfonyl fluoride) solution was 50 mM in isopropanol.

Preparation of acetylated peptide (H4, 1-23). Peptide 1-23 prepared from calf thymus histone H4 as described (23) was kindly provided by Dr. P. D. Cary. It was dissolved in 0.1 M sodium phosphate buffer, pH 7.5, at a concentration of 1 mg/ml. The acetylation reaction under nitrogen atmosphere was essentially done according to Riordan and Vallee (18), except that phosphate buffer was used to prevent acetate exchange (24). To 25-mCi-tritiated acetic anhydride (4 μmol) was added 1 mg of peptide (0.4 μmol) and the reaction mixture was incubated for 1 h in melting ice under frequent mixing. The total activity incorporated was approximately 120 μCi. Then 60 mg (600 μmol) nonradioactive acetic anhydride in 0.25 ml 0.1 M sodium phosphate buffer, pH 7.5, was added and the reaction was continued for 1 h. The reaction mixture was freeze-dried and then chromatographed over Sephadex G-25 Fine (2.5 × 150 cm), equilibrated with 0.5 M acetic acid to desorb free acetate from the acetylated peptide (25). The void volume was collected, freeze-dried, dissolved in sterile distilled water at a concentration of 0.48 mg/ml (52.5×10^6 cpm/ml), stored in small aliquots at −20°C, and used as substrate for the histone deacetylase assay (see below). The extent of acetylation of the peptide was determined after acid-urea gel electrophoresis according to Panyim and Chalkley (26) by staining and by fluorography, using the method of Chamberlain (27). This method includes the soaking of the gel in 10 vol of 1 M sodium salicylate, pH 6, followed by exposure of the dried gel at −76°C to Kodak X-Omat XR-5 film, preflashed as described (28).

Physarum polycephalum. Microplasmodia of *Physarum polycephalum,* strain M3c, were cultured as described (29). Nuclei were isolated essentially as described by Polman (22). Microplasmodia from 2 liters of culture were collected by centrifugation, washed with 800 ml of cold distilled water and with 400 ml of cold buffer A to which PMSF was freshly added to 1 mM. To the plasmodial pellet 5 vol of buffer A was added and 30-ml aliquots were homogenized in a 55-ml Potter–Elvehjem glass–Teflon homogenizer by 8 strokes up and down at 1000 rpm under cooling in ice. The homogenate was immediately diluted with 200 ml cold buffer A, centrifuged for 5 min at 65*g*, decanted from the slime pellet and filtered through two layers of Milkfilter (Kendall). The nuclear homogenate was spun for 10 min at 1500*g* and the nuclei were washed once or twice with fresh buffer A by repeated suction through a 2-in. 17-gauge needle and recentrifugation. The number of isolated nuclei was determined by hematocytometer counting of a nuclear suspension in buffer A. For the deacetylase assay nuclei were resuspended at a known concentration in buffer B to which PMSF was freshly added to 1 mM.

Nuclear extractions. Isolated nuclei were resuspended in buffer C containing 0.6 M KCl and stirred on ice for 40 min. The suspension was clarified by centrifugation for 10 min at 30,000*g*. The pellet was then extracted with buffer C containing 2 M KCl for 40 min on ice and clarified.

A forceful extraction of nuclei in buffer C was accomplished by sonication for 60 s on ice with a Branson sonifier with microtip at 60 W. The sonicate was clarified and the pellet was resonicated in buffer C containing

0.5 M KCl. This step was repeated with buffer C containing 1 M KCl (30).

All clarified supernatants and all pellets were extensively dialyzed at 4°C against buffer B and assayed for deacetylase activity.

Deacetylase assay. To 0.02 ml of a suspension of isolated nuclei in buffer B with 1 mM PMSF, or to 0.02 ml of a nuclear extract in this buffer, was added 0.02 ml of 0.1 M Tris · HCl buffer, pH 7.5, which contained also 0.5 μl tritiated acetylated peptide (H4, 1-23) and any additions as specified in text or legends. The reaction blanks contained enzyme preparations which were preheated for 2 min at 80 to 90°C. The mixture was incubated for 2 h at 30°C and the reaction was stopped in ice by the addition of 0.02 ml carrier histones (5 mg total calf thymus histones/ml) in 0.5 N HCl and 0.08 M acetic acid (13,16). Ethylacetate (0.3 ml) was added, the tubes were vortexed, equilibrated for 30 min at room temperature (22 to 23°C), and 0.2 ml upper phase was carefully removed by pipet. To this sample 2.5 ml of scintillation cocktail (type 3a70B purchased from Research Products Int. Corp.) was added and the radioactivity was determined. The extraction efficiency of acetic acid from the reaction mixture was 87%. The liberation of 1 pmol acetate resulted in a net increase of 68 cpm in 0.2 ml upper phase.

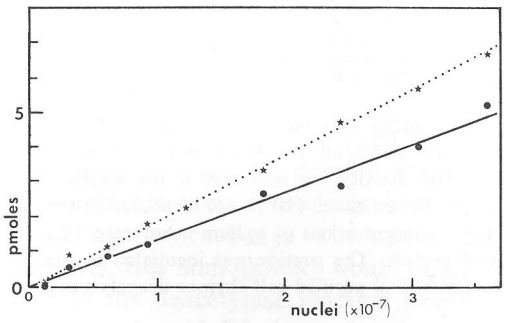

FIG. 1. Histone deacetylase activity in isolated nuclei. Nuclei from microplasmodia of *Physarum* were isolated and used in the standard deacetylase assay, as described under Materials and Methods. The amounts of acetate produced during incubations of 2 (●) and 4 h (★) were plotted versus the number of nuclei used in the 0.04 ml assay. The volume of 3.6×10^7 nuclei was measured to be 0.03 ml.

TABLE 2

EXTRACTION OF DEACETYLASE ACTIVITY FROM *Physarum* NUCLEI

	Deacetylase activity	
	cpm	%
Nuclei (10^9)	18,188	100
Initial 0.6 M KCl wash	2,888	16
Subsequent 2 M KCl wash	6,126	34
Final nuclear residue	9,538	52
Total	18,552	102
Initial sonicate	11,475	63
Subsequent 0.5 M KCl sonicate	3,152	17
Subsequent 1.0 M KCl sonicate	2,374	13
Final nuclear residue	1,599	9
Total	18,600	102

Note. The procedure for the extraction of 10^9 microplasmodial nuclei of *Physarum* is described under Materials and Methods.

RESULTS

Peptide 1-23 from calf histone H4 was acetylated with tritiated acetic anhydride. The acetylated peptide was analyzed by polyacrylamide gel electrophoresis in acetic acid-urea and gave five bands corresponding to 0 to 4 acetates per molecule. Fluorography showed that 93% of the radioactivity was in the acetylated bands, with mono- to tetraacetylated species containing 31, 23, 19, and 20% of the radioactivity, respectively. The mean specific activity was 295 Ci/mol peptide or 134 Ci/mol acetate.

This peptide was used as a substrate for purified histone deacetylase I and Table 1 shows that acetate was released specifically by the enzyme. The sensitivity of this assay was at least 100-fold higher than the assay used by L. Cousens (13) who kindly supplied us with the enzyme.

The peptide was also used as a substrate for histone deacetylase in crude extracts. For

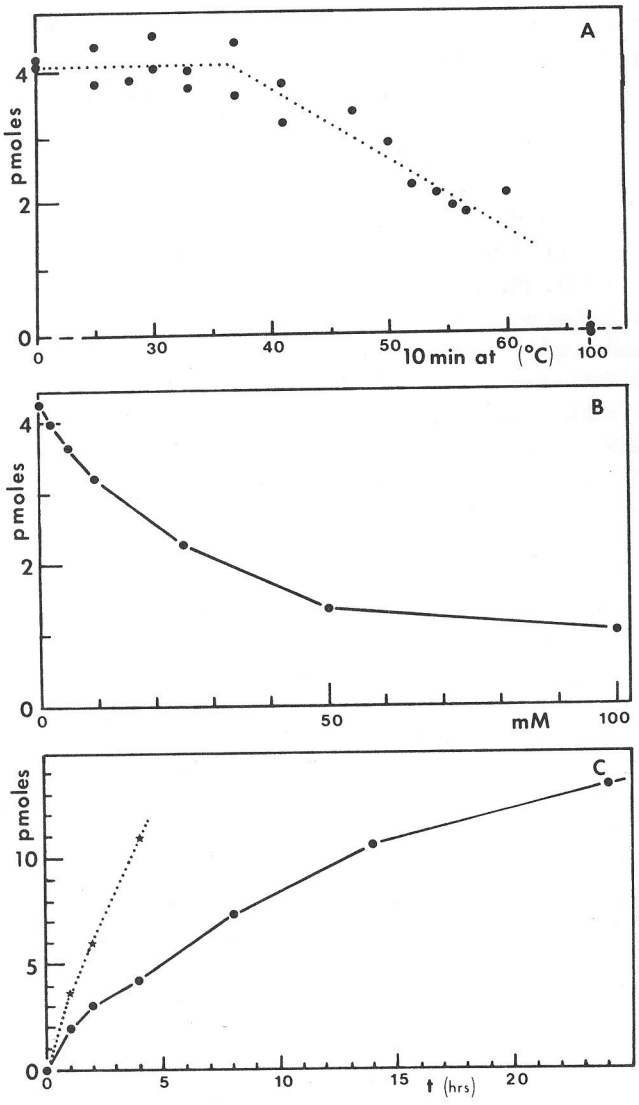

FIG. 2. (A) Temperature stability of nuclear deacetylase. Nuclei were incubated for 10 min at the indicated temperatures in buffer B at a concentration of 4×10^8 nuclei/ml (a different preparation from that used for Fig. 1). Subsequently the remaining deacetylase activity was measured in the standard assay. (B) Inhibition of nuclear deacetylase by butyrate. *Physarum* nuclei (10^7) were incubated in the standard deacetylase assay in the presence of the indicated concentrations of sodium *n*-butyrate. (C) Time curve of enzymatic deacetylation of the acetylated peptide. The peptide was incubated in the standard assay with 8×10^6 nuclei in buffer B (●) or with 0.15 μl purified calf thymus deacetylase I (13) (★) for the indicated length of time.

this purpose, we used nuclei isolated from microplasmodia of the true slime mold *P. polycephalum.* Figure 1 shows that the release of acetate increased linearly with the concentration of nuclei. The enzyme could be solubilized by sonicating the nuclei and Table 2 shows that the enzyme could be assayed, apparently quantitatively, either in intact nuclei or in a variety of extracts. Further purification of the enzyme has not been carried out but we have used this assay to measure histone deacetylase activity through

the naturally synchronous mitotic cycle in *Physarum* plasmodia (31).

As additional evidence to support the assay we determined the temperature dependence of the enzyme (Fig. 2A). The activity is also inhibited by sodium butyrate, although the *Physarum* enzyme is less sensitive than the calf thymus enzyme (Fig. 2B) (13). The time course of release of acetate was linear when purified calf thymus enzyme was used, at least up to 4 h at 30°C. With *Physarum* nuclei the rate of reaction dropped with time, although acetate was still being released after 14 h at 30° (Fig. 2C). The activity in nuclei showed a broad, pH optimum around pH 7.5 and mercaptoethanol or dithiothreitol was essential for activity.

DISCUSSION

Peptide 1-23 of histone H4, acetylated *in vitro*, has been used as a substrate for histone deacetylase. It is much cheaper than alternative substrates and provides very high specific activity while retaining the necessary specificity for lysines 5, 8, 12, and 16 in H4. The specific activity of the peptide described here was approximately 1.4×10^3 times the specific activity of the acetylated H4 used by Cousens *et al.* (13) so the new assay is more than 1000-fold more sensitive than that used by Cousens *et al.*

The particular preparation of acetylated peptide described in this report contained a mixture of acetate levels. If required, the peptide could be fully acetylated to give a homogeneous $(acetate)_4$-peptide or peptides with different acetate levels could be separated by ion-exchange chromatography. However, this homogeneity would be lost as soon as the deacetylase reaction began. In *Physarum* in mid-G2 phase of the cell cycle, the proportions of H4 species with 0 to 4 acetates are 15, 55, 23, 4, and 3%, respectively (4). If the acetates were uniformly labeled this would give counts in the proportions 0, 44, 37, 10, and 10% which are somewhat similar to the proportions used in the acetylated peptide although the amounts of label in the tri- and tetraacetylated species were higher in the peptide.

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