Properties of chicken erythrocyte histone deacetylase associated with the nuclear matrix

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Histone H2B is deacetylated more rapidly than H3 and H4 in chicken immature erythrocytes. Histone deacetylase from chicken immature erythrocytes was partially purified, and the histone specificities of the multiple histone deacetylase forms were determined. Ion-exchange (Q-Sepharose) and gel-exclusion (Superdex 200) chromatography of extracts from erythrocyte nuclei showed two forms (HD1 and HD2) of histone deacetylase. HD1, with a molecular mass of about 55 kDa, preferred free H3–H4 relative to H2A–H2B, while HD2, with a molecular mass of approx. 220 kDa, had a slight preference for H3–H4. HD1 and HD2 differed in pH- and ionic-strength-dependence. HD2 dissociated into HD1 when treated with 1.6 M NaCl or when applied to a Q-Sepharose column. The enzymic properties of nuclear-matrix-bound histone deacetylase showed a striking

difference from that of HD1 and HD2, particularly in its strong preference for H2A–H2B. Treatment of the nuclear matrix with 1.6 M NaCl and 1% 2-mercaptoethanol solubilized histone deacetylase, which chromatographed as 400 and 220 kDa forms on a Superdex 200 column. The solubilized enzyme retained its histone preference for H2A–H2B. Chromatography of the nuclear-matrix-derived enzyme on Q-Sepharose yielded one peak of enzyme activity with chromatographic properties and histone specificities similar to those of HD1. These results provide support for the active form of the enzyme *in situ* being a high-molecular-mass complex associated with proteins that are components of the nuclear matrix. Substrate preference of the enzyme is governed by the proteins associated with the histone deacetylase.

INTRODUCTION

The core histones (H2A, H2B, H3 and H4) are subject to reversible post-translational acetylation of ϵ -amino groups of specific lysine residues within their N-terminal domains [1]. Histone acetylation can remodel the structure of chromatin by subtle changes in nucleosome structure, changing the ability of histone H1 to condense chromatin structure, and altering the capacity of nucleosomes to bind transcription factors [2,3]. Dynamically acetylated histones are associated with transcriptionally active DNA ([2] and references cited therein). Experiments with the histone deacetylase inhibitors butyrate and trichostatin A have demonstrated the importance of reversible acetylation in transcription, the cell cycle and differentiation [4].

Histone acetylation is a dynamic process that is governed by two enzyme activities: histone acetyltransferases and deacetylases. In vertebrates, histone acetyltransferase and histone deacetylase (HD) are associated with the internal nuclear matrix [3,5,6]. Nuclear matrix is the structure remaining after nuclei are digested with nuclease and extracted with buffers of intermediate and/or high ionic strength, and this structure consists of residual nucleoli, internal nuclear matrix and nuclear pore lamina. The nuclear matrix is involved in several nuclear processes, including transcription, replication and RNA processing [7–10]. Further, transcriptionally active, but not repressed, chromatin is attached to the nuclear matrix [2]. We proposed that histone acetyltransferase and HD have a role in mediating a dynamic attachment between transcriptionally active chromatin and the nuclear matrix [2].

Nuclear matrices isolated from chicken liver, trout liver and trout hepatocellular carcinoma retain 80% or greater of the nuclear HD activity. However, approx. 50% of the nuclear HD activity is associated with the chicken immature erythrocyte nuclear matrix. By monitoring HD activity in nuclear-matrix

preparations isolated under various conditions, we have presented evidence that the stability of the chicken immature erythrocyte nuclear matrix is considerably less than that of chicken and trout liver nuclear matrices [6].

Histone acetyltransferases are generally divided into two groups: nuclear A-type enzymes and cytoplasmic B-type enzymes. Nuclear A-type enzymes are capable of acetylating all free core histones as well as nucleosomal histones, while B-type enzymes acetylate H4 and H3 [11,12]. The various A-type enzymes have different substrate preferences [13–15]. Multiple forms of HDs have been found in plants, animals, yeast and the slime mould, *Physarum* [13,15–18]. The HD forms, which are nuclear enzymes, often differ in ionic-strength-dependence, inhibition by butyrate, optimum pH and substrate specificity [13,15,19].

Analysis of histone deacetylation rates in chicken immature erythrocytes demonstrated a multiplicity of deacetylation rates for the core histones [20]. The highly acetylated isoforms (e.g., tetraacetylated) isoforms of H4 were rapidly deacetylated, while the mono- and di-acetylated H4 isoforms were deacetylated slowly. H2B was more rapidly deacetylated than H3 and H4 [20,21].

In the present study we show that approx. 90% of the chicken erythrocyte HD activity is present in the nucleus. However, the extent of enzyme leakage from the nuclei was dependent on the buffers used to isolate the erythrocyte nuclei. HD released into the cytoplasmic fraction and the enzyme retained by the erythrocyte nuclear matrix were characterized. Two HDs (HD1 and HD2) with distinctive enzymatic properties were found in the cytoplasmic fraction. The molecular mass of HD2 was approx. 4-fold greater than that of HD1. At high ionic strength, HD2 dissociated to HD1. HD2 and HD1 differed in pH-dependence and histone-specificity. Nuclear-matrix-bound HD had different properties and histone-specificity (e.g., a pronounced preference

Abbreviation used: HD, histone deacetylase.

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for H2A and H2B) from that of HD1 and HD2. The solubilized nuclear-matrix HD existed as a high-molecular-mass complex. Chromatography of this complex on Q-Sepharose generated an HD form with properties similar to those of HD1. The results of this study suggest that the association of HD with a component(s) of the nuclear matrix alters its enzyme properties and provide evidence that the nuclear-matrix form of the enzyme is the active HD *in situ*.

MATERIAL AND METHODS

Preparation of cytoplasmic fraction and nuclei

Adult chicken mature and immature erythrocytes were collected from adult White Leghorn chickens as described in [22] and were homogenized in RSB buffer [10 mM Tris/HCl (pH 7.5)/10 mM NaCl/3 mM MgCl₂] with 0.25 % Nonidet P40 and 1 mM PMSF. After centrifugation at 1475 *g* in a SS34 rotor for 10 min at 4 °C, the supernatant was further centrifuged at 12100 *g* in a SS34 rotor for 10 min. This supernatant, the cytoplasmic fraction, was applied to a Q-Sepharose (Pharmacia) column or Superdex 200 HR 10/30 gel-filtration column (Pharmacia). The pellet from the first centrifugation was washed three to five times in RSB buffer with 1 mM PMSF to yield nuclei. In determining the amount of HD released from the nuclei, the enzyme activity in each of the supernatants was measured. For some studies, erythrocytes were homogenized in RSB buffer containing 1 M hexylene glycol.

Preparation of nuclear matrix, nuclear matrix extract and nuclear extract

The procedure was essentially the same as described in [3] with the following modifications. The DNA concentration of nuclei in digestion buffer was 40 *A*₂₆₀ units/ml, and DNase I (Sigma D5025 or D4263) was added to a final concentration of 200 µg/ml. After stepwise extraction with 0.25 M (NH₄)₂SO₄ and 2 M NaCl, the pellet (nuclear matrix) was resuspended in 10 mM Tris/HCl, pH 7.5, with 1 mM PMSF, and dialysed against the same buffer overnight at 4 °C. In some cases the nuclear matrix pellet was further extracted twice with 1.6 M NaCl and 1 % (v/v) 2-mercaptoethanol. The soluble fraction collected by centrifugation was then dialysed against 10 mM Tris/HCl, pH 7.5, overnight, centrifuged at 12100 *g* in an SS34 rotor for 10 min, and the supernatant (nuclear-matrix extract) was subjected to chromatographic analysis. To extract HD from nuclei, nuclei (40 *A*₂₆₀/ml) in RSB buffer were digested with DNase I (200 µg/ml) for 1 h at 0 °C and then the solution was made 0.45 M in NaCl. Following centrifugation at 12100 *g* for 10 min (SS34 rotor), the supernatant was made 1.7 M in (NH₄)₂SO₄. The precipitate was collected and dissolved in RSB buffer. Greater than 95 % of the HD activity in the 0.45 M NaCl extract was precipitated in 1.7 M (NH₄)₂SO₄.

Chromatographic separation of HD1 and HD2

The Q-Sepharose column was packed at a volume of 7 ml and connected to LKB FPLC system (Pharmacia). A 30 ml portion of cytoplasmic fraction was typically loaded on to the column. The column was first washed with 30 ml of RSB buffer, then bound proteins were eluted by a linear gradient of 0–20 % buffer B (RSB buffer/2 M NaCl) in 80 ml, followed by a linear gradient from 20 % buffer B to 40 % buffer B in 30 ml, and finally reaching 100 % buffer B in 10 ml elution buffer. Typically, 1.5 ml fractions were collected, and 50 µl was assayed for HD activity. For gel-filtration chromatography, 0.1–0.5 ml of sample was loaded on a Superdex 200 column (column volume 24 ml). The

elution buffer used depended on the enzyme source. With the cytoplasmic fraction (dissociation study), 10 mM Tris/HCl (pH 7.5)/1.6 M NaCl was used, while for the cytoplasmic fraction (including HD1 and HD2 fractions from Q-Sepharose column) and nuclear-matrix extract, 25 mM sodium phosphate/citric acid buffer, pH 7.0, or 10 mM Tris/HCl, pH 7.5, was the elution buffer. Fractions were of volume 0.5 ml, and the flow rate was controlled by FPLC at 0.5 ml/min. A gel-filtration molecular-size calibration kit (Sigma) was used to calculate the molecular size of HD1 and HD2.

Preparation of substrate for HD assay

The labelling of adult immature erythrocyte with sodium [³H]acetate (ICN Biomedicals; 27 Ci/mmol) was as described [5], except the cells were incubated with label for 60 min in the presence of 10 mM sodium butyrate. To isolate labelled histones, nuclei were extracted with 0.2 M H₂SO₄ as described in [5]. Histones H2A–H2B or H3–H4 labelled to high specific radioactivities were isolated from chromatin that was soluble in 90 or 100 mM NaCl (S₉₀ or S₁₀₀) [23]. Fraction S₉₀ was then applied to a hydroxyapatite (Bio-Rad HTP) column at a ratio of 1 mg of DNA to 0.25 g of hydroxyapatite. After washing the column with 0.3 M NaCl in 0.1 M potassium phosphate, pH 6.7, a linear NaCl gradient from 0.3 to 2 M was run through the hydroxyapatite column using the FPLC system to separate H2A–H2B dimers from (H3–H4)₂ tetramers. Fractions containing H2A–H2B or H3–H4 were pooled. An aliquot of these two fractions was resolved on 15 % polyacrylamide AUT gel [acetic acid/6.7 M urea/0.375 % (w/v) Triton X-100] to ensure the purity of the preparation. Fluorography of the same AUT gel was performed as described in [23]. H2A–H2B and H3–H4 were dialysed against water and concentrated by freeze-drying. For preparation of chromatin substrate for the HD assay, the salt-soluble chromatin fraction S₁₀₀ was isolated as described above. The chromatin fragments were treated with CM-Sephadex to remove any free histones.

HD enzyme assay

In most cases (including the NaCl and sodium butyrate inhibition assay), the HD assay was done with chicken erythrocyte labelled histones (150 000 d.p.m., 180 µg) in 25 mM sodium phosphate/citric acid buffer, pH 7.0, at 37 °C for 15–30 min. When fractions from the gel-filtration column were assayed, the incubation time was extended to 60 min to detect HD activities. Total reaction volume was 0.3 ml. The enzyme's pH optimum was determined in 50 mM Mops with a pH range of 5.5–8.0 in the presence of 80 mM NaCl. After incubation, enzyme reaction was stopped by 0.12 M/0.72 M (final concn.) of acetic acid/HCl, and 2 vol. of ethyl acetate were mixed with reaction solution. The samples were centrifuged at 9000 *g* for 1 min; a 0.5 vol. of added ethyl acetate was then counted for radioactivity in a liquid-scintillation counter to determine the amount of liberated label.

RESULTS

Chromatographic separation and characterization of HD forms HD1 and HD2

The permeability properties of chicken erythrocyte nuclei leads to the selective leakage of specific proteins into the cytoplasmic fraction during isolation [24]. Chicken immature erythrocyte nuclei isolated in RSB buffer retained 67 % of the total HD activity. In contrast, trout liver nuclei had 86 % of the total HD activity. Previous studies have shown that the presence of hexylene glycol prevents nuclear proteins (e.g., high-mobility-

Figure 1 Separation of HD forms by ion-exchange chromatography

The cytoplasmic fraction, which contained nuclear-derived HD, isolated with RSB buffer/0.25% (v/v) Nonidet P40 was applied to a Q-Sepharose column and eluted as described in the Materials and methods section. Fractions (1.5 ml each) were collected. Protein concentration was monitored with a Pharmacia LKB Uvicord SII instrument at 280 nm. HD activity was determined using radiolabelled histones as substrate. The peaks of activity were eluted at 0.32 and 0.52 M NaCl.

Figure 2 Gel-exclusion chromatography of HD forms HD1 and HD2

(A) A 0.5 ml portion from fractions of the pooled first HD activity peak (HD1) from the Q-Sepharose column (Figure 1) was analysed by Superdex 200 column (Pharmacia) chromatography with 25 mM sodium phosphate/citric acid buffer. A_{280} and HD activities were determined as described in the legend to Figure 1. (B) The fractions containing the second peak of HD activity (HD2) from the Q-Sepharose column were concentrated about 4-fold before 0.5 ml was loaded on to the Superdex 200 column. Fractions (0.5 ml) were collected.

group proteins) from leaking out of nuclei [25–27]. When 1 M hexylene glycol was added to RSB buffer, leakage of HD activity could be reduced to about 10% of the total HD activity. As with other nuclear enzymes, hexylene glycol did not inhibit HD activity [25].

Two peaks of HD activity were observed when the cytoplasmic extract (RSB buffer) isolated from immature erythroid cells was loaded on to a Q-Sepharose column (Figure 1). Fractions containing HD activity were pooled, yielding fractions HD1 (first peak) and HD2 (second peak). The observed activities of the two HD forms were not an accurate reflection of their relative activities, as the ionic strength at which the second peak of HD activity (HD2) was eluted repressed enzyme activity (see Figure 5B below). However, when HD1 and HD2 were dialysed against 10 mM Tris/HCl, pH 7.5, the activity of HD1 containing fraction was 3–4-fold greater than that of the column fraction with HD2.

Figure 3 Dissociation of the high-molecular-mass form of HD

(A) The cytoplasmic fraction (0.5 ml) was applied directly to a Superdex 200 column and eluted with 25 mM sodium phosphate/citric acid buffer as described in the Materials and methods section. (B) The cytoplasmic fraction was treated with 1.6 M NaCl and 1% (v/v) 2-mercaptoethanol, and 0.5 ml of the sample was applied to the Superdex 200 column, equilibrated with 10 mM Tris/HCl buffer (pH 7.5)/1.6 M NaCl. Fractions (0.5 ml) were collected. A 40 μ l sample from each fraction was assayed in 10 mM Tris/HCl (pH 7.5)/210 mM NaCl (final concn.) at 37 °C for 1 h. Similar results were obtained when column fractions were assayed in 10 mM Tris/HCl (pH 7.5)/105 mM NaCl.

HD1 and HD2 were further fractionated on a Superdex 200 column (Figure 2). The majority of HD1 activity was eluted with a molecular mass of approx. 55 kDa (Figure 2A). A minor amount of HD activity was eluted with a high molecular mass. In contrast, the majority of fraction HD2 activity was eluted with a molecular mass of about 220 kDa (Figure 2B). A small amount of fraction HD2 activity was eluted as a lower-molecular-mass species. These results suggested that the cytoplasmic HD exists as high- and low-molecular-mass forms, with the low-molecular-mass HD1 form predominating. Following ion-exchange and gel-filtration chromatography, the HD specific activity was increased approx. 100-fold relative to the cytoplasmic fraction.

Dissociation of high-molecular-mass form of HD to the low-molecular-mass form

When the cytoplasmic fraction was applied directly to a Superdex 200 column, two peaks of HD activity were observed, with the high-molecular-mass (220 kDa) form of HD predominating (Figure 3A). The activity of the high-molecular-mass HD form was approx. 3–4-fold higher than that of the low-molecular-mass form. This result was contrary to what was expected from the results of cytoplasmic HD fractionation on the Q-Sepharose column. Separating the HD forms on a Q-Sepharose column shifted the relative abundance of high- and low-molecular-mass forms of HD.

To investigate whether the high-molecular-mass HD form, HD2, dissociated into the low-molecular-mass form (HD1), the cytoplasmic extract was treated with 1.6 M NaCl and 1% (v/v) 2-mercaptoethanol and run through a Superdex 200 column. Figure 3B shows that this treatment resulted in dissociation of high-molecular-mass HD2 form, with the activity of the low-molecular-mass HD1 being 1.5-fold higher than that of the high-molecular-mass HD2 form. Pooled fractions containing HD2 from chromatographic separation of the cytoplasmic fraction through a Superdex 200 column (Figure 3A) were also treated with 1.6 M NaCl and 1% 2-mercaptoethanol prior to a second chromatographic run on the gel-exclusion column. The high-molecular-mass HD2 form diminished, while the low-molecular-mass HD1 form increased in abundance (results not shown). The addition of high salt and its subsequent removal did not selectively inhibit the activity of HD1 or HD2. Treatment of the cytoplasmic extract with 1.6 M NaCl, but not 1% 2-

Figure 4 pH-dependence of HD1, HD2 and nuclear-matrix HD

HD1 and HD2 were obtained from the cytoplasmic fraction by Q-Sepharose column chromatography (see Figure 1). Nuclear matrix was isolated as described in the Materials and methods section. The enzyme fractions were dialysed against 10 mM Tris/HCl, pH 7.5. HD1, HD2 and nuclear-matrix HD were analysed in the enzyme assay at various pH values. The results are means $\pm$ S.D. for three separate experiments for HD1 (●), HD2 (■) and nuclear-matrix histone deacetylase (▲).

Figure 5 Concentration-dependent effect of ionic strength on histone deacetylase forms

(A) A range of NaCl concentrations (0–500 mM) were included in the HD assay buffered with 10 mM Tris/HCl, pH 7.5. Maximal HD was designated as 100% activity for the enzyme, and activities at other salt concentrations were calculated as the percentage of activity at optimum NaCl concentration. (B) Different concentrations of NaCl were added to the HD assay buffered with 25 mM sodium phosphate/citric acid. HD activity without addition of NaCl was designated as 100% activity for the enzyme, and the activities with NaCl were compared and calculated as the percentage of activity in the absence of NaCl. HD1 (●), HD2 (■) and nuclear-matrix HD (▲).

mercaptoethanol, alone was sufficient for dissociation to occur (results not shown).

Properties of nuclear-matrix HD and HD forms HD1 and HD2

The optimum pH of HD forms HD1, HD2, and nuclear-matrix-associated enzyme was determined. Figure 4 shows that HD1 had a distinct pH optimum of 7.5. The pH optima for HD2 and nuclear-matrix HD were less pronounced, with HD2 and nuclear-matrix forms having pH optima of 6.5–7.0 and 7.0–7.5 respectively.

The optimum ionic strength for the three HD forms was buffer-dependent. With Tris/HCl as the buffer, 50 mM was the optimum NaCl concentration for HD1 and HD2, and 100 mM was optimal for the nuclear-matrix form (Figure 5A). Elevating the ionic strength higher than 100 mM suppressed activity of the HD forms to various extents. At 0.5 M NaCl nuclear-matrix HD, HD2 and HD1 had 75, 30 and 5% of their maximal activities respectively. In phosphate buffer, the presence of NaCl reduced the activities of the three forms of HD (Figure 5B). Increasing the ionic strength affected HD1 activity much more than that of HD2 or the nuclear-matrix HD.

Butyrate is a non-competitive inhibitor of HDs. The three forms of HD were inhibited by butyrate, with HD2 being the most sensitive (results not shown). The concentrations of sodium butyrate to achieve 50% inhibition of HD1, HD2 and nuclear-matrix HD were 45, 21 and 80 μ M respectively. Trichostatin A, a specific non-competitive inhibitor of HD, at 10 μ M inhibited 90% or more of the HD1, HD2 and nuclear-matrix HD activity (results not shown).

Histone specificities of HD1, HD2 and nuclear-matrix HD

The histone octamer consists of two H2A–H2B dimers and a (H3–H4)₂ tetramer. H2A–H2B dimers and (H3–H4)₂ tetramers labelled with [³H]acetate were isolated from chromatin fraction soluble in 90 mM NaCl by hydroxyapatite dissociation chromatography [23] (inset to Figure 6A). Using total histone as substrate, the amount of each enzyme preparation required to release 2000 d.p.m. of [³H]acetate in 15 min was first determined. The assay was repeated with the adjusted volume of each enzyme preparation. Figure 6A shows that the three HD preparations had equivalent activities with a fixed amount of total histone. Using equivalent HD activities, the preferences of HD1, HD2 and nuclear-matrix HD for H2A–H2B and/or H3–H4 were determined. Figure 6B shows that HD1 had a preference for H3–H4. With H3–H4 or H2A–H2B as substrates the activity of HD1 reached a plateau at about 30 μ g. HD2 had a slight preference for H3–H4, and the enzyme activity increased linearly with greater amounts of substrate (Figure 6C). The histone specificity of unfractionated cytoplasmic HD was similar to that of HD2 (results not shown). Figure 6D shows that nuclear-matrix HD had a strong preference for H2A–H2B. The activity of HD increased linearly with H2A–H2B or H3–H4 as substrates up to 50 μ g.

To determine whether one of the histone pairs was selectively deacetylated, the H2A–H2B or H3–H4 histone substrates from a 45 min incubation with each HD preparation were electrophoretically resolved on AUT 15%-polyacrylamide gels. The fluorogram was scanned, and the relative amount of label in each histone was calculated. The H3–H4 substrate had a value of 1.01 ± 0.06 (H3/H4; $n = 3$), while the H2A–H2B had a relative signal of 0.54 ± 0.09 (H2A/H2B; $n = 5$). Following incubation with HD1, the H3/H4 and H2A/H2B ratios were 0.40 ± 0.05 ($n = 3$) and 0.56 ± 0.07 ($n = 4$) respectively. With HD2 as the HD, the H3/H4 and H2A/H2B ratios were 0.88 ± 0.04 ($n = 3$) and 0.53 ± 0.11 ($n = 4$) respectively. Incubation with nuclear-matrix HD resulted in H3/H4 and H2A/H2B ratios of 0.74 ± 0.04 ($n = 3$) and 0.56 ± 0.18 ($n = 4$) respectively. Thus cytoplasmic HD1 had a strong preference for H3, while HD2 and nuclear-matrix HD had a slight preference for H3 versus H4. Further, the results suggest that H2A and H2B were processed equivalently by the three HD forms.

We also tested the ability of the three HD forms to deacetylate histones in nucleosomes. Salt-soluble oligonucleosomes (S_{100}) isolated from chicken immature erythrocytes that were incubated

Figure 6 Histone specificities of HD1, HD2 and nuclear-matrix HD

(A) Fractions containing HD1 (●), HD2 (■) and nuclear-matrix HD (▲) were incubated with labelled histone (180 μg, 160 000 d.p.m) at 37 °C for various times. Inset to (A): 4 μg of S_{90} core histones (lane 1), H2A–H2B (lane 2) and H3–H4 were resolved by AUT PAGE as described in the Materials and methods section. The gel was stained in Coomassie Blue. Inset to (B): fluorogram of the gel shown in the inset to (A). 1, 2, 3, and 4 correspond to the mono-, di-, tri- and tetra-acetylated species of histone H4 respectively. (B, C, D) Enzyme volumes that had been standardized with total labelled histones were incubated with various amounts of either labelled H2A–H2B (●, 1915 d.p.m./μg) or H3–H4 (■, 2546 d.p.m./μg). (B) HD1; (C) HD2; (D) nuclear-matrix HD.

Figure 7 Characterization of nuclear-matrix HD

(A) Nuclear-matrix extract was prepared and analysed on a Superdex 200 column as described in the Materials and methods section. Fractions (0.5 ml) were collected. A_{280} measurements and enzyme assays were done as described in the legend to Figure 2. (B) HD activity in the nuclear-matrix extract was chromatographed on a Q-Sepharose column and assayed as described in Figure 1. A_{280} and HD activity are shown.

with sodium [3 H]acetate were used as substrate. Nuclear-matrix HD, but neither HD1 nor HD2, deacetylated histones in oligo-nucleosomes (results not shown).

Characterization of solubilized nuclear-matrix and nuclear HD

Nuclear matrices were treated with 1.6 M NaCl and 1% (v/v) 2-mercaptoethanol. Such treatment of nuclear matrices solubilizes internal nuclear-matrix components [28]. The solubilized nuclear-matrix enzyme deacetylated H2A, H2B, H3 and H4, with a preference for H2A and H2B (results not shown). The nuclear-

Figure 8 Histone specificity of nuclear-matrix-extracted HD following chromatography on Q-Sepharose

Pooled fractions 38–42 from the Q-Sepharose column shown in Figure 7(B) were desalted and assayed for HD activity. The volume of enzyme required to release 2000 d.p.m. from total labelled histones (180 μg) in 15 min was used to determine histone specificity for H2A–H2B (●) or H3–H4 (■) as described in the legend to Figure 6.

(Figure 7A). The majority of the nuclear-matrix-derived HD activity was eluted as a high-molecular-mass complex of at least 400 kDa, and a shoulder of HD activity with a molecular mass of 220 kDa. The nuclear-matrix extract was chromatographed on a Q-Sepharose column. Figure 7B shows that a single peak

of HD activity was detected. The chromatographic behaviour of nuclear-matrix solubilized HD activity was similar to that of HD1 (compare Figure 7B with Figure 1). Figure 8 shows that the HD activity deacetylated H2A–H2B and H3–H4, with a preference for H3–H4. Thus the substrate preference of the Q-Sepharose-column-fractionated nuclear-matrix-extracted HD activity was similar to that of HD1.

HD extracted with 0.45 M NaCl from DNase I-digested nuclei was also analysed. The solubilized enzyme was precipitated with 1.7 M $(\text{NH}_4)_2\text{SO}_4$ and then resuspended in RSB buffer. Similar to the nuclear-matrix-solubilized enzyme, the nuclear-extracted HD deacetylated all four core histones, with a preference for H2A–H2B. On a Superdex 200 column the majority (98%) of HD activity in this fraction chromatographed with a molecular mass greater than 400 kDa. The remainder of the activity had a molecular mass of 55 kDa. Chromatography of the nuclear-extracted enzyme on a Q-Sepharose column resulted in its dissociation to a low-molecular-mass form (results not shown).

DISCUSSION

The permeability properties of the chicken erythrocyte nuclei leads to the leakage of transcription factors and HD into the cytoplasmic fraction. This leakage was lessened by the addition of hexylene glycol to the RSB buffer, reducing the loss of HD by approx. 20%. The percentages of HD activity that we observed to be present in the cytoplasmic fraction with and without hexylene glycol never approached the levels reported by others [18] (about 80%). Taking into account the loss of HD activity into the cytoplasmic fraction, revised values of 35 and 73% of the total HD activity are found in the chicken immature erythrocyte and trout liver nuclear matrices respectively [6]. The lower amount of HD activity associated with the chicken immature erythrocyte nuclear matrix is most certainly a reflection of the instability of the erythrocyte nuclear matrix [6].

Solubilization of the nuclear matrix-bound HD activity was achieved by incubating erythrocyte nuclear matrices with 1.6 M NaCl and 2-mercaptoethanol. Similar conditions were used to release NF1 activity from chicken erythrocyte nuclear matrices [28]. Nuclear matrices treated in this way appear as empty shells of nuclear pore-lamina complexes (H. Y. Chen, J.-M. Sun, M. J. Hendzel, J. B. Rattner and J. R. Davie, unpublished work), showing that the internal nuclear matrix has been disrupted. Gel-exclusion chromatography of the nuclear matrix- and nuclear-solubilized HD activities revealed several HD forms, with the high-molecular-mass (at least 400 kDa) HD form predominating. These results are similar to those obtained by Hay and Candido [29,30] who found that HD from HeLa cells exists as a high-molecular-mass complex with a protein composition analogous to that of nuclear-matrix preparations.

The cytoplasmic HD exists primarily as a 220 kDa complex. Our results and those of others argue that this HD form was originally in the nucleus and leaked out of the nuclei during isolation [18]. Treatment of the cytoplasmic HD with 1.6 M NaCl resulted in the dissociation of the high-molecular-mass 220 kDa HD complex to a 55 kDa HD1 form. Interestingly, the solubilized nuclear matrix HD remained as high-molecular-mass complexes (of about 400 kDa and 220 kDa) under the same conditions. This suggests that the solubilized nuclear-matrix HD is associated with proteins that stabilize the complex from dissociation into the low-molecular-mass 55 kDa HD1 form under these conditions.

Recently, Sanchez del Pino et al. [31] reported that, at low ionic strength, yeast HD was isolated as a high-molecular-mass complex (500 kDa). Elevating the ionic strength with 500 mM

NH_4Cl or NaCl resulted in the dissociation of the HD complex to a low-molecular-mass form of 150 kDa. Both the 500 kDa and 150 kDa HD forms chromatographed as a single peak of activity with 0.25 M NH_4Cl from DEAE-Sepharose. Similarly, the chicken erythrocyte high-molecular-mass HD complex from nuclear matrix or cytoplasmic fraction was eluted primarily as a single peak of activity at 0.32 M NaCl from Q-Sepharose. These observations suggest that chromatography on ion-exchange columns leads to dissociation of the high-molecular-mass HD complex. Also proteins associated with solubilized high-molecular-mass nuclear-matrix-derived HD complexes are unable to prevent dissociation of the complex when applied to the ion-exchange column.

The yeast 150 kDa HD form had a greater sensitivity to trichostatin A inhibition than the 500 kDa HD form, but the free-histone specificity of these two HD forms was similar. In contrast, the chicken erythrocyte 220 and 55 kDa HD forms had similar butyrate- and trichostatin A-sensitivities, but different histone preferences and ionic-strength-sensitivities. Multiple forms of HD from plants, animals, yeast and *Physarum* have been detected by ion-exchange chromatography [13,15,17], and these HD forms differ in their abilities to deacetylate the four core histones. Our results and those of Sanchez del Pino et al. [31] suggest that the multiple HD forms may have originated from a high-molecular-mass HD complex.

In our analysis of free histone specificity we observed that enzyme's histone preference was dependent on the concentration of histone and enzyme. For example, at low concentrations of histone, the nuclear-matrix HD had a slight preference for H3–H4. As the concentration of the substrate was increased, H2A–H2B was definitely the preferred histone pair. Studies analysing the histone-specificity of different HD forms and putative phosphorylated HD forms have failed to mention whether the enzyme/substrate ratio was considered in the evaluation of histone preference [15,19,31].

In chicken immature erythrocytes the acetylated isoform of H2A, acetylated isoforms of H2B, and the highly acetylated isoforms of H4 are rapidly acetylated and deacetylated. Further, H2B is deacetylated more rapidly than H3 or H4 in these cells [20,21]. Chicken erythrocyte nuclear skeletons (fraction P_E) isolated by extracting micrococcal-nuclease-digested nuclei with EDTA contain 80–90% of the nuclear HD and histone acetyltransferase activities and less than 10% of the nuclear DNA [3]. This residual nuclear fraction catalyses reversible acetylation using as substrate the chromatin fragments attached to the insoluble nuclear structure. The nuclear-skeleton-associated HD had a preference for nucleosomal H2B. Correspondingly, the nuclear-matrix HD had a preference for the free histones H2A and H2B. The similarity in deacetylation rates of the core histones *in situ* with the histone-specificity properties of the nuclear-matrix-bound HD suggests that the high-molecular-mass nuclear-matrix form of the enzyme is one of the active HD forms *in vivo*. To achieve the nucleosomal histone specificity observed *in situ*, our results suggest that HD is in a complex with proteins that are components of the nuclear matrix. It is likely that HD is in a dynamic equilibrium with components of the nuclear matrix and chromatin. Changes in histone specificity as dictated by the proteins associated with HD may be of physiological significance.

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