High-performance capillary electrophoresis of core histones and their acetylated modified derivatives

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By using high-performance capillary electrophoresis, we have successfully separated rat liver core histones into several subfractions. Inconvenient interactions of the highly basic proteins with the capillary wall were eliminated by a phosphate buffer system containing 0.03 % hydroxypropylmethylcellulose. Sample amounts of a few nanolitres were analysed within about 20 min. Multiacetylated histones H4 and H3 from induced Friend erythroleukaemic cells prepurified by h.p.l.c. were clearly separated into their non-acetylated and distinct acetylated forms. Our results illustrate that the application of capillary zone electrophoresis on its own or in combination with h.p.l.c. to the analysis of histones provides an important new alternative to traditional gel electrophoreses.

INTRODUCTION

Histones are basic protein components of eukaryotic chromatin and are of great importance with regard to the organization of chromatin structure. In the nuclei of all eukaryotic cells DNA is packed in basically the same way: two each of histones H2A, H2B, H3 and H4 form the fundamental chromatin subunit, the nucleosome, whereas histone H1 is associated with the chromosomal linker region. Four of these five histone classes each consist of several variants, differing slightly in their amino acid sequence [1,2]. Moreover, under various biological conditions histones are known to be post-synthetically modified, such as acetylated, phosphorylated, ADP-ribosylated etc. These modifications alter the local charge and structure of histones substantially and are thought to be important for regulatory functions. When analysing histones, it is apparent that the complexity and heterogeneity of these proteins make high demands on the analytical methods applied. Until now gel electrophoresis has been an important analytical tool. However, there are some drawbacks. In particular, the electrophoretic methods are labour-intensive and time-consuming. In addition to gel electrophoresis, several h.p.l.c. techniques have been developed recently [3–12]. In fact, some remarkable results have been obtained, though various analytical problems left unsolved.

A new method, high-performance capillary electrophoresis (h.p.c.e.), combining substantial advantages of conventional gel electrophoresis and h.p.l.c. techniques, was introduced by Jorgenson & Lukasz [13] and has opened new prospects on the separation of biomolecules. In h.p.c.e. compounds are resolved according to their ability to migrate in an electric field inside a fused-silica capillary. Nanolitres or less of sample can be separated, and in contrast with common gel electrophoresis fast, quantitative, fully automated and highly efficient analyses will be achieved. A rapidly growing number of applications attest to the unique capabilities of h.p.c.e.

The present study was undertaken to evaluate the potential utility of h.p.c.e. for the separation of histones and modified derivatives with particular emphasis on using open-tube untreated fused-silica capillary columns.

MATERIALS AND METHODS

Chemicals

Hydroxypropylmethylcellulose (HPM-cellulose) (viscosity of 2 % aq. solution at 25 °C 4000 cP) and trifluoroacetic acid were purchased from Sigma Chemie (Munich, Germany), ethylene glycol monomethyl ether was obtained from Aldrich (Steinheim, Germany) and all other chemicals used were from Merck (Darmstadt, Germany).

Cell line and culture conditions

Friend erythroleukaemic cells (line F4N) were grown in suspension culture in Eagle's minimum essential medium with Earle's balanced salt solution supplemented with 10 % (v/v) fetal-calf serum and amino acids as described elsewhere [14]. Penicillin (100 units/ml) and streptomycin (100 µg/ml) were added routinely. Cell cultures were incubated at 37 °C in 5 % CO₂ in air. In order to achieve hyperacetylated core histones 1.75 mM-sodium butyrate was added for 24 h to cells growing exponentially at a density of 2×10^5 cells/ml.

Preparation of core histones

Rat liver nuclei were prepared as described by Kurokawa & MacLeod [4]. H1 histones were removed by extraction with 5 % (w/v) HClO₄ for 1 h. HClO₄-insoluble material was centrifuged down at 900 g for 15 min and the resulting pellet thereupon extracted with 0.2 M-H₂SO₄. Insoluble material was removed by centrifugation at 12000 g for 10 min, and the core histones were obtained as described previously [9]. Whole histones from Friend erythroleukaemic cells (line F4N) were prepared as described previously [7].

H.p.l.c.

All h.p.l.c. experiments were performed on a Beckman h.p.l.c. gradient system with two 114M pumps and a 412A system controller. The eluate was monitored at 210 nm with a model 165 u.v.–visible-region detector. The histones were separated on a Nucleosil 300-5 C₄ column (4.6 mm internal diam. $\times$ 100 mm; 5 µm particle pore size; end-capped). The freeze-dried proteins

Abbreviations used: h.p.c.e., high-performance capillary electrophoresis; HPM-cellulose, hydroxymethylpropylcellulose; AUT/PAGE, acid/urea/Triton/PAGE.

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were dissolved in 0.1 % (v/v) trifluoroacetic acid and 30–100 μ g samples of histones were injected on to the column. The histones were chromatographed within 50 min at room temperature and at a constant flow of 0.5 ml/min with a multi-step acetonitrile gradient starting at 38 % (v/v) solvent A/62 % solvent B [solvent A, water containing 0.1 % (v/v) trifluoroacetic acid and 10 % (v/v) ethylene glycol monomethyl ether; solvent B, 10 % (v/v) ethylene glycol monomethyl ether/70 % (v/v) acetonitrile and 0.1 % (v/v) trifluoroacetic acid]. The concentration of solvent B was increased linearly in the following order: from 62 % (v/v) to 66 % (v/v) (during 30 min), from 66 % (v/v) to 68 % (v/v) (5 min), from 68 % (v/v) to 80 % (v/v) (5 min), and then maintained at 80 % (v/v) (10 min).

Friend-cell histones were chromatographed as described previously [7].

Gel electrophoresis

Core histone fractions from the h.p.l.c. runs were collected, mixed with 10 μ l of 2-mercaptoethanol, freeze-dried and stored at -20°C . The proteins were analysed by both SDS/PAGE according to the procedure of Laemmli [15] (0.1 % SDS/15 % polyacrylamide) and AUT (acid/urea/Triton)/PAGE as described by Zweidler [16] (0.9 M acetic acid/8 M-urea/0.37 % Triton X-100/12 % polyacrylamide). The gels were stained for 1 h with 0.1 % (w/v) Serva Blue R in 40 % (v/v) ethanol/5 % (v/v) acetic acid and destained overnight in 20 % (v/v) ethanol/5 % (v/v) acetic acid.

Capillary electrophoresis

H.p.c.e. was performed on a Beckman system P/ACE 2000 controlled by an AT386 computer. Data collection and post-run data analysis were carried out by using P/ACE and System Gold software (Beckman Instruments, Palo Alto, CA, U.S.A.). The capillary cartridge used was fitted with a 75 μ m-internal-diameter fused-silica capillary, 58.8 cm total length (50 cm to the detector). Protein samples (concentration 0.5 mg/ml) were injected by pressure for 3 s and on-column detection was performed by u.v. absorption at 200 nm. An untreated capillary was used in all experiments, but after every five to ten injections the capillary was rinsed with water, 0.1 M-NaOH, water, 0.5 M-H₂SO₄, water and finally with the running buffer. Washing was done for 2 min with each solvent. Runs were carried out in 110 mM-phosphate buffer, pH 2.0, containing 0.03 % (w/v) HPM-cellulose. Applied voltage and capillary temperature differed and are given in the Figure legends.

RESULTS AND DISCUSSION

Numerous examples of successful h.p.c.e. separations of small molecules can be cited [17–19]. However, applications of these techniques to the analysis of proteins are complicated because of their high tendency to stick to the wall of the capillary. This inconvenient effect is primarily caused by the attraction between positively charged proteins and the negative silanol groups of the capillary surface. Such interactions result in band broadening and tailing with significantly reduced separation efficiency and a non-linear relationship between measured absorption values and analyte concentration. A number of solutions for the elimination of these adsorption effects have been reported, among these being the use of buffer pH values higher than the pI values of the proteins [20], the use of low-pH buffer systems [21], the application of 'dynamic coating' agents, which weakly interact with the capillary surface and are generally added to the separation buffer [22], and the chemical modification of the silanol groups [13,23,24]. Finally, combinations of these systems have been described [25]. For the analysis of core histones we

tested several of these systems under various conditions. Since histones are among the most basic naturally occurring proteins known (pI 11–12), under all separation conditions with a pH below pI severe wall interactions of the positively charged histones should occur. Indeed, with an untreated capillary and different buffer systems with pH values above 4.5, no proteins could be detected. Surprisingly, even with coated capillaries in the pH range 2–8, no migration of histones was observed. A hydrophobic interaction with the capillary coating may possibly be responsible for this unexpected result. Furthermore, various buffer systems in the range pH 1–4 in the presence and in the absence of different buffer additives, namely SDS, ethylene glycol, formamide, HPM-cellulose and poly(ethylene glycol) of distinct M_r values, were tested. In addition, the influences of buffer and additive concentration, capillary length and temperature and voltage applied were investigated. Fig. 1 illustrates the separation of whole core histones under optimized conditions. The run was performed in a 110 mM-phosphate buffer, pH 2.0, containing 0.03 % HPM-cellulose. The individual peaks of the electrophoretogram were identified by running pure core histone fractions under the same h.p.c.e. conditions. In order to obtain these proteins rat liver core histones were separated by h.p.l.c. as shown in Fig. 2(a). The identity and purity of the isolated fractions were first checked by SDS/PAGE (Fig. 2b). In this gel system all proteins appeared to be remarkably pure. Histone variants and histone modifications, however, are best resolved by PAGE in the presence of non-ionic detergents [16]. Therefore the same fractions were analysed additionally by AUT/PAGE (Fig. 2c). The multiple bands visible in lanes 3 and 4 of Fig. 2(c) are non-acetylated and distinct acetylated derivatives of the corresponding histones, whereas fractions 5 and 6 (Fig. 2a) consist of a mixture of the histone variants H2A.1 and H2A.X (Fig. 2c, lanes 5 and 6). Fraction 7 shows two bands in the AUT/PAGE gel (Fig. 2c, lane 7): non-acetylated and monoacetylated histone H3.2 (not contaminated with histone H3.3). Fraction 8 (Fig. 2c, lane 8), however, consists of non-acetylated and distinct acetylated histone H3.3, possibly contaminated by histone H3.2.

The protein of fraction 1 (Fig. 2a) migrated slightly faster than histone H4 in the SDS/PAGE gel (Fig. 2b) and between histones H2A.2 and H3.2 in the AUT/PAGE gel (Fig. 2c). We therefore conclude that this protein, also detectable in the electrophoretogram of the h.p.c.e. run at about 17 min (not shown in Fig. 1), represents an impurity and not a histone protein. In

Fig. 1. Separation of rat liver core histones by h.p.c.e.

Conditions were as follows: 110 mM-phosphate buffer, pH 2.0, with 0.03 % HPM-cellulose; injection time 3 s; voltage applied 15 kV; temperature constant at 25°C ; detection at 200 nm; capillary length 50 cm, capillary diameter 75 μ m.

Fig. 2. Separation of core histones by reverse-phase h.p.l.c. and analysis of the subfractions by SDS/PAGE and AUT/PAGE

(a) Separation of core histones by reverse-phase h.p.l.c. In order to obtain pure histone fractions designated as peaks 1-8, 50 μ g protein samples were injected on to the h.p.l.c. column. Numbered fractions were freeze-dried and subjected to SDS/PAGE and AUT/PAGE and h.p.c.e. analysis. (b) SDS/PAGE of peaks 1-8 from the separation described in (a). Lane st, total core histones (15 μ g) from rat liver nuclei. (c) AUT/PAGE of peaks 1-8 from the separation described in (a). Lane st, total core histones (20 μ g) from rat liver nuclei.

order to confirm our assignments in Fig. 1, the pure histone fractions were added to the whole core histone standard. Separating these mixtures, we found a characteristic change of one or more peaks in the electrophoretogram. These procedures finally led to the following assignment of the nine peaks shown in Fig. 1: the fastest-migrating one contains two H3 histone variants, namely H3.2+H3.3, followed by histones H2A.1, H3.2+H3.3, H2A.2, H4, H2A.2+H3.2+H3.3, H2A.X, H4 and finally H2B. Three interesting facts should be noted in this context. (1) The migration order of core histone proteins in capillary electrophoresis is quite dissimilar to that observed in the different kinds of gel electrophoresis commonly used. (2) The core histones H2A, H2B, H3 and H4 are clearly separated from each other. Moreover, histone H2A is further resolved into its variants H2A.1, H2A.2 and H2A.X. It should be noted that the

Fig. 3. AUT/PAGE of histones from untreated and butyrate-treated Friend erythroleukaemic cells (line F4N)

AUT/PAGE of whole histones (30 μ g) from untreated (lane cst) and butyrate-treated (lane bst) Friend erythroleukaemic cells and of isolated histone fractions (3-5 μ g) from untreated (lane 1, histone H4; lane 2, histone H3) and butyrate-treated cells (lane 3, histone H4; lane 4, histone H3) respectively.

precise separation of histone H2A.1 from histone H2A.X has been a problematic part of core histone fractionation by the application of reverse-phase h.p.l.c. [9]. The H3 histone variants H3.2 and H3.3, however, show identical electrophoretic mobilities and are not resolved. Likewise it is impossible to separate histones H3.2 from H3.3 in AUT/PAGE, but they may be separated efficiently by h.p.l.c. with a shallow acetonitrile in aq. trifluoroacetic acid gradient (Fig. 2a) or by following a method previously described [4]. (3) Histones H3 and H4 are resolved into three and two peaks respectively. Since rat liver histones H3 and H4 are known to be post-synthetically acetylated to a larger extent, we were inclined to believe that the apparent peaks in the electrophoretogram (Fig. 1) might be non-acetylated and acetylated forms of the corresponding histones. Thus, for a further characterization of the peaks in question, Friend erythroleukaemic cells were cultured in the absence and in the presence of sodium butyrate for 24 h. Butyrate is known to be a potent inhibitor of histone deacetylase(s) and therefore causes a hyperacetylation of core histones [26]. Histones H3 and H4 were isolated by h.p.l.c. as described previously [7] and the fractions obtained were analysed by both AUT/PAGE (Fig. 3) and h.p.c.e. (Figs. 4 and 5). The same characteristic differences in the acetylation pattern of histones H4 (Fig. 4) and H3 (Fig. 5) of untreated (a) and butyrate-treated (b) F4N cells are discernible in both electrophoretic systems. The AUT/PAGE gels of histone fractions H4 and H3 respectively are also shown as insets in the electrophoretograms of the h.p.c.e. runs and confirm our above-mentioned assumption. As can be seen in Figs. 4 and 5, the non-acetylated histone protein migrates fastest followed by the acetylated derivatives clearly separated from each other. With regard to Fig. 5(b) it seems necessary to refer to the relative high background of this h.p.c.e. analysis, which is possibly caused by degradation or oxidation of histone H3, though 2-mercaptoethanol was present in the course of histone preparation, isolation and h.p.c.e. analysis.

Consequently, as a result of these investigations an unambiguous assignment of all of the peaks in Fig. 1 was possible. Thus the three peaks containing histones H3.3+H3.2 represent,

Fig. 4. Separation of multiacetylated histone H4 by h.p.c.e.

Separation of multiacetylated histone H4 of untreated (a) and butyrate-treated (b) Friend erythroleukaemic cells. The inset shows the corresponding electrophoretogram from the AUT/PAGE (Fig. 3); ac_0 , ac_1 , ac_2 , ac_3 and ac_4 : non-, mono-, di-, tri- and tetra-acetylated histone derivatives. Conditions were the same as for Fig. 1 except that the voltage applied was 12 kV and capillary temperature was 30 °C.

Fig. 5. Separation of multiacetylated histone H3 by h.p.c.e.

Separation of multiacetylated histone H3 of untreated (a) and butyrate-treated (b) Friend erythroleukaemic cells. Conditions and abbreviations used were the same as for Fig. 4.

of modified histone derivatives, provides an important alternative to the traditional gel electrophoreses.

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REFERENCES

- Urban, M. K., Franklin, S. G. & Zweidler, A. (1979) *Biochemistry* **18**, 3952–3960
- Cole, R. D. (1984) *Anal. Biochem.* **136**, 24–30
- Gurley, L. R., D'Anna, J. A., Blumenfeld, M., Valdez, J. G., Sebring, R. J., Donahue, P. R., Prentice, D. A. & Spall, W. D. (1984) *J. Chromatogr.* **297**, 147–165
- Kurokawa, M. & MacLeod, M. C. (1985) *Anal. Biochem.* **144**, 47–54
- Lindner, H., Helliger, W. & Puschendorf, B. (1986) *J. Chromatogr.* **357**, 301–310
- Lindner, H., Helliger, W. & Puschendorf, B. (1986) *Anal. Biochem.* **158**, 424–430
- Lindner, H., Helliger, W. & Puschendorf, B. (1988) *J. Chromatogr.* **450**, 309–316
- Helliger, W., Lindner, H., Hauptlorenz, S. & Puschendorf, B. (1988) *Biochem. J.* **255**, 23–27
- Lindner, H., Wesierska-Gadek, J., Helliger, W., Puschendorf, B. & Sauermann, G. (1989) *J. Chromatogr.* **472**, 243–249
- Lindner, H., Helliger, W. & Puschendorf, B. (1990) *Biochem. J.* **269**, 359–363
- Lindner, H. & Helliger, W. (1990) *Chromatographia* **30**, 518–522

12. Marvin, K. W., Yau, P. & Bradbury, E. M. (1990) *J. Biol. Chem.* **265**, 19839–19847
13. Jorgenson, J. W. & Lukacs, K. D. (1983) *Science* **222**, 266–272
14. Krieg, C. J., Ostertag, W., Clarks, N., Pragnell, I. B., Swetly, P., Roesler, G. & Weimann, B. J. (1978) *Exp. Cell Res.* **116**, 21–29
15. Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685
16. Zweidler, A. (1978) *Methods Cell Biol.* **17**, 223–233
17. Tsuda, T., Nomura, K. & Nakagawa, G. (1982) *J. Chromatogr.* **248**, 241–247
18. Gassmann, E., Kuo, J. E. & Zare, R. N. (1985) *Science* **230**, 813–814
19. Tsuda, T., Nomura, K. & Nakawaga, G. (1983) *J. Chromatogr.* **264**, 385–392
20. Lauer, H. H. & McManigill, D. (1986) *Anal. Chem.* **58**, 166–170
21. Grossman, P. D., Colburn, J. C., Lauer, H. H., Nielsen, R. G., Riggan, R. M., Sittampalam, G. S. & Rickard, E. C. (1989) *Anal. Chem.* **61**, 1186–1194
22. Hjerten, S., Valtcheva, L., Elenbring, K. & Eaker, D. (1989) *J. Liq. Chromatogr.* **12**, 2471–2499
23. Swedberg, S. A. (1990) *Anal. Biochem.* **185**, 51–56
24. Hjerten, S. (1985) *J. Chromatogr.* **347**, 191–198
25. McCormick, R. M. (1988) *Anal. Chem.* **60**, 2322–2328
26. Candido, E. P., Reeves, R. & Davie, J. R. (1978) *Cell* **14**, 105–113
27. Wiktorowicz, J. E. & Colburn, J. C. (1990) *Electrophoresis* **11**, 769–773

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