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IDENTIFICATION OF ADP-RIBOSYLATED HISTONES BY THE COMBINED USE OF HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY AND ELECTROPHORESIS

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SUMMARY

Reversed-phase high-performance liquid chromatography (HPLC) was employed for analysing mono- and oligo(ADP-ribosyl)ated histones. Under the chromatographic conditions described, the ADP-ribosylated histones showed similar retention times to the unmodified histones, although the molecular weight and the charge of the proteins are significantly altered by their modification. The simultaneous elution of unmodified and labelled modified histones was detected by two types of gel electrophoresis and by autoradiography. In addition, the HPLC fractions did not display overlapping ladders of the multiply modified histones, as is commonly seen in one-dimensional electrophoretic analyses of unfractionated material. Hence individual bands could be unambiguously assigned. After *in vitro* labelling of isolated rat liver nuclei, the following ADP-ribosylated and unmodified histones were identified by HPLC and gel electrophoresis: histone H1°, four histone H1 subfractions, histone H2A.1, histone H2A.2, oxidized histone H2A.2, histone H2A.X, histone H2A.Z, histone H2B, three histone H3 variants and histone H4.

INTRODUCTION

Under various biological conditions, histone molecules of eukaryotic cells are known to be modified in reactions catalysed by the DNA-dependent enzyme nuclear ADP-ribosyltransferase (E.C. 2.4.2.30). Although all main histone species have been reported to be capable of modification by covalent linkage to mono- or poly(ADP-

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ribose) residues, knowledge about the modification of histone variants is scarce (for reviews, see refs. 1 and 2).

ADP-ribosylated histones are usually identified by one-dimensional acrylamide gel electrophoreses[3–5] of the labelled compounds. As poly(ADP-ribose) chains of different lengths may be attached to the histone molecules, overlapping of histones and modified histones often excludes the identification of the individual components.

In this study, the behaviour of ADP-ribosylated histones during reversed-phase high-performance liquid chromatography (RP-HPLC)[6–8] was investigated. Experimental conditions were established that allowed the concomitant elution of the highly negatively charged ADP-ribosyl derivatives of individual histones or histone variants and their respective unmodified counterparts. Hence the individual ADP-ribosylated histone species can rapidly be detected and fractionated by RP-HPLC, prior to further analysis by gel electrophoresis. This permits the unambiguous assignment of the derivatives without the shortcomings outlined above. Moreover, the conditions were improved for the HPLC separation of some histone variants.

EXPERIMENTAL

HPLC-grade acetonitrile (grade S) and water were obtained from Rathburn Chemicals (Walkerburn, U.K.) and trifluoroacetic acid (TFA) was purchased from Sigma (Munich, F.R.G.).

Preparation of labelled ADP-ribosylated histones

Isolated rat liver nuclei[9] ($5 \cdot 10^8$ nuclei/ml) were incubated with 0.5 m*M* [adenylate-^{32}P]NAD (New England Nuclear) in the presence of 10 μg/ml DNAse I in medium A [100 m*M* Tris–HCl (pH 8.0), 10 m*M* magnesium chloride, 1 m*M* dithiothreitol (DTT), 1 m*M* phenylmethylsulphonyl fluoride (PMSF)] at 25°C for 30 min. The labelled nuclei were washed twice with medium A containing 5 m*M* unlabelled NAD at 4°C. The histones were then extracted with 0.2 *M* sulphuric acid at 4°C and precipitated by addition of 5 volumes of ethanol and overnight storage at −20°C. The precipitate of the acid-soluble proteins was washed twice with ethanol and vacuum dried. ADP-ribosylated histones were separated from unmodified histones by boronate column chromatography as previously described[10].

HPLC of histones

The Beckman HPLC system consisted of two Model 114M pumps, a 421A system controller and a Model 165 variable-wavelength UV–VIS detector. The absorbance of the effluent was measured at 210 nm. The detection signal was recorded by a Shimatzu C-R3A integrator. Histone separations were performed on a Bio-Rad RP-304 C_4 (5-μm silica, 33-nm pore size) column (250 mm × 4.6 mm I.D.). The histone preparations were dissolved in 0.1% TFA. After equilibration of the column with the initial chromatographic solvent, 50 μl of histone solution, containing 225 μg acid-soluble nuclear protein extract, were injected. The histones were chromatographed within 45 min at room temperature and at a flow-rate of 1.3 ml/min. In Fig. 1 a multi-step gradient starting at 74% A–26% B (solvent A, 0.1% TFA in water; solvent B, 0.1% TFA in acetonitrile) was used. Successively the concentration of

solvent B was increased linearly from 26 to 31% B (during 5 min), 31 to 33% (10 min), 33 to 38% (5 min), then maintained at 38% (16 min), and increased from 38 to 55% B (15 min). Fractions of 0.65 ml of the HPLC effluent were collected using a fraction collector. To determine the radioactivity of the ^{32}P-labelled proteins, 0.2-ml aliquots were counted in a Beckman liquid scintillation spectrometer.

Electrophoretic analysis of histones

The peak HPLC fractions were appropriately pooled and the solvent vacuum evaporated by the use of a Sera-Vac centrifuge. The samples were analysed in two electrophoretic systems. Sodium dodecyl sulphate (SDS) gel electrophoresis on 15% polyacrylamide slab gels was performed as described by Laemmli[4], except that 4 *M* urea was additionally included in the gel buffer and 6 *M* urea in the sample buffer. These gels were stained with Coomassie Blue R. Alternatively, the samples were electrophoresed on 12% polyacrylamide slab gels (AUT gels) in 0.9 *M* acetic acid–7.5 *M* urea–6 m*M* Triton X-100 as described by Zweidler[5]. The proteins were then detected by amido black staining. The gels were dried and the radioactive compounds detected by autoradiography.

RESULTS AND DISCUSSION

In order to prepare labelled ADP-ribosylated histones, isolated nuclei were incubated with [^{32}P]NAD. As the chromatin-associated nuclear ADP-ribosyltransferase is stimulated by DNA strand breaks, DNase was added to the incubation mixture. Under these conditions approximately 1% of the total histones was ADP-ribosylated. By transfer of the ^{32}P-labelled ADP-ribose moiety of NAD, the acceptor molecules were modified with a single ADP-ribose moiety or a chain of covalently linked residues (for reviews, see refs. 1 and 2). On analysis of the acid extract in the subsequent HPLC experiments, the modified and unmodified histones could easily be differentiated. In the effluent fractions the unmodified histones were detected by their UV absorption and the ADP-ribosylated histones by their radioactivity.

It was our aim to develop an HPLC technique permitting not only good fractionation of individual histones, but also the concomitant collection of specific histones together with their respective ADP-ribosylated derivatives. Preliminary experiments based on our previously developed method[6–8] revealed that shallow gradients gave good fractionation of the unmodified histones. However, this was associated with long elution times, low sensitivity and inconvenient tailing of the ^{32}P-labeled ADP-ribosylated histones. On the other hand, gradients permitting very short elution times gave unsatisfactory separations of the histones. A gradient allowing optimal fractionation of rat liver histones is shown in Fig. 1.

For the identification of the histones in the pooled peak HPLC fractions, two electrophoretic gel systems were used. The AUT gel system had the advantage of satisfactorily separating histone H2A from histone H2B, and of resolving variants of the histones H2A and H3. In both the SDS gel (Fig. 2) and the AUT gel (Fig. 3) systems, the unmodified histones were detected by staining (lane a) and the [^{32}P]ADP-ribosylated histones by autoradiography (lane b). Owing to the different specific activities, various exposure times had to be used in these qualitative analyses of the modified proteins.

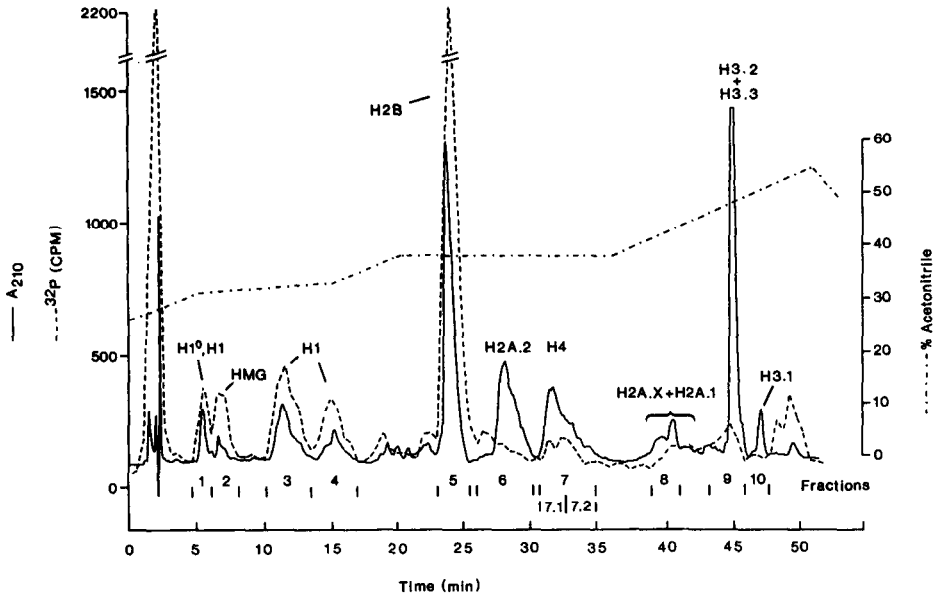

Fig. 1. Fractionation of [^{32}P]ADP-ribosylated and unmodified histones by RP-HPLC. Application of acid-soluble nuclear proteins (225 μg). Other conditions as in Experimental.

According to their position in the HPLC trace (Fig. 1) and their electrophoretic resolution (Figs. 2 and 3), the fractions contained the following proteins: fraction 1, histone H1° and a histone H1 subfraction; fraction 2, probably HMG1 and HMG2 proteins; fraction 3, a histone H1 subfraction; fraction 4, two different histone H1 subfractions; fraction 5, histone H2B; fraction 6, histone H2A.2 and, with increasing mobilities in the AUT gel and lower intensity, probably histone H2A.Z, and oxidized histone H2A.2 (Fig. 3, lane 6a); fraction 7, histone H4; fraction 8, histone H2A.X and histone H2A.1, both discernible in the UV recording of the column effluent and in the gels, histone H2A.X situated at the position of histone H3 in the SDS gel, but above

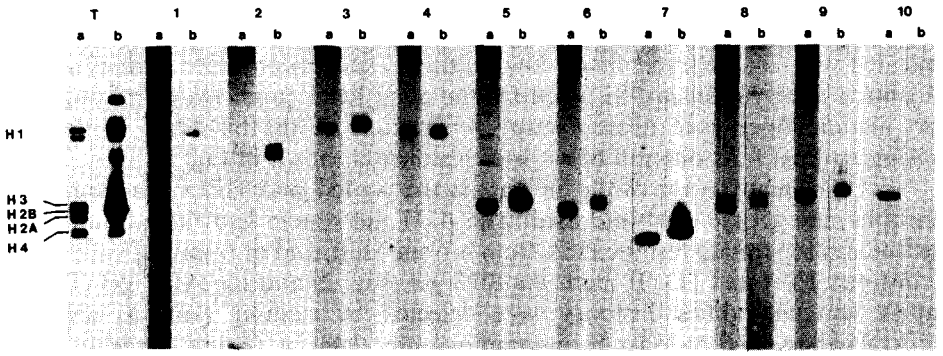

Fig. 2. SDS gel electrophoresis of HPLC fractions. 1–10, Number of effluent fractions, as designated in Fig. 1; T, acid-soluble nuclear proteins applied to the HPLC column. Lanes: a, staining; b, autoradiography.

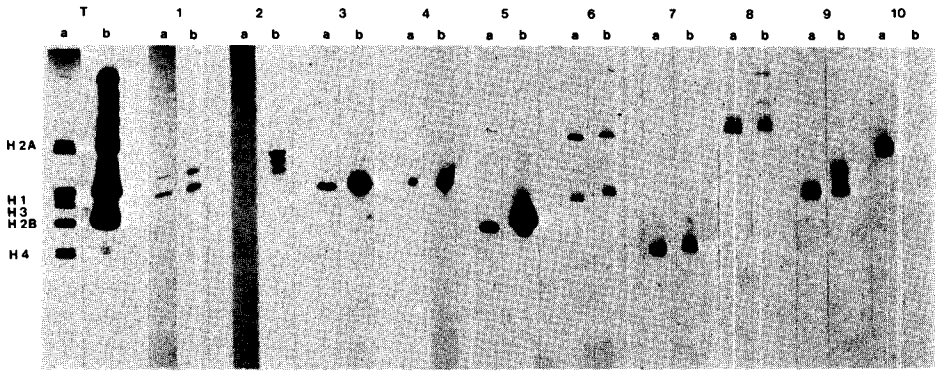

Fig. 3. AUT gel electrophoresis of HPLC fractions. Details as in Fig. 2.

histone H2A.1 in the AUT gel (Fig. 3, lane 8a); fraction 9, a histone H3 subfraction, consisting of the histone H3.2 and histone H3.3 variants; and fracton 10, the histone H3.1 variant.

Additionally, ADP-ribosylated forms of most the above-mentioned proteins were observed. Owing to their low concentrations, the modified proteins were only detectable by autoradiography (Figs. 2 and 3, lanes b). Especially in the SDS gel, the electrophoretic mobilities of the modified proteins were significantly decreased, apparently in accordance with the degree of ADP-ribosylation. This is best illustrated by the case of the fast-moving histone H4 and its derivatives. In accordance with the molecular weight increase, the apparent mono(ADP-ribosyl)ated histone H4 is clearly discernible at a position slightly above that of the unmodified histone (Fig. 2). In addition, a ladder is seen of histone H4 molecules which had been poly(ADP-ribosyl)-ated to different extents, whereas the other histone proteins seem to be mono(ADP-ribosyl)ated.

Moreover, Figs. 1–3 demonstrate that the ADP-ribosylated histones or histone variants were recovered in the same fractions as their unmodified counterparts. No cross-contamination of molecules collected in different HPLC fractions was observed. The recovery of histones and radioactivity was above 85%. Further, Fig. 1 shows that the elution of ADP-ribosylated histones is slightly retarded in comparison with that of the unmodified histones. In essence, however, the introduction of negatively charged ADP-ribosyl residues in the histone molecules and the concomitant molecular weight increase of about 550 Da per monomer unit, did not strongly affect the retention of histones in the HPLC column. Control experiments revealed that the labelled material was sensitive to snake venom phosphodiesterase digestion (not shown).

When material enriched by boronate chromatography was fractionated by means of HPLC, oligo(ADP-ribosyl)ated histones could be detected in all fractions in the autoradiography of the subsequent SDS gel electrophoresis (Fig. 4). Fig. 4 especially demonstrates the advantages of the described method. The autoradiography does not show overlapping zones of modified proteins as observed by the customarily used direct electrophoretic analysis of samples unfractionated by HPLC (Fig. 4, lane T). For example, the ladders of modified histone H2A.2 and H4 molecules clearly depict the modification of the molecules by one to six and one to four,

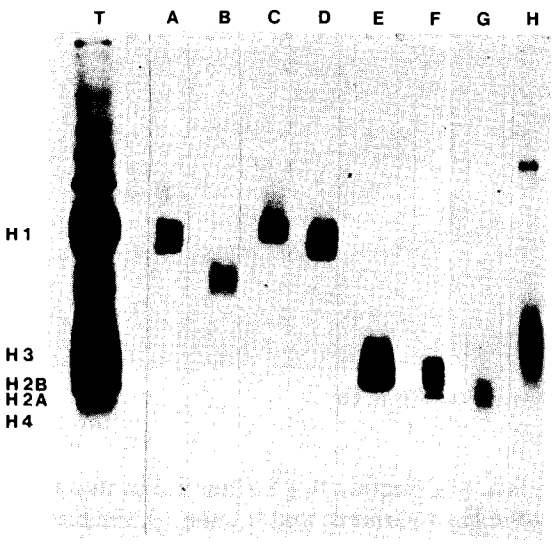

Fig. 4. Analysis of [^{32}P]ADP-ribosylated histones enriched by boronate column chromatography. SDS gel electrophoresis. T, Boronate-enriched material applied to the HPLC column. Collected effluent fractions (corresponding designation in Fig. 1 in parentheses): A, histones H1° and H1 (1); B, HMG proteins (2); C, histone H1 (3), D, histone H1 (4); E, histone H2B (5); F, histone H2A.2 (6); G, histone H4 (7); H, histones H3 (9 + 10).

respectively, ADP-ribose residues. In our experience, the appearence of either ladders or broad bands depends on the combination of certain experimental conditions, such as the degree of labelling, the protein concentration in gel electrophoresis and the exposure time in autoradiography.

In conclusion, using the described HPLC technique ADP-ribosylated histones can be rapidly fractionated for analytical and preparative purposes. Applied solely or in combination with gel electrophoresis, the method will prove useful in studies of ADP-ribosylation reactions that occur under various biological conditions (for reviews, see refs. 1 and 2). The present data illustrate the resolution of several histone H1 subfractions, distinct histone H2A and H3 variants and their respective multiple ADP-ribosyl derivatives.

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