ISOLATION AND CHARACTERISATION OF SUBFRACTIONS OF NUCLEAR PROTEIN H1°

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1. Introduction

In 1969 Panyim and Chalkley [1] reported the occurrence of a 'new histone' which has become known as H1°, because it bears some similarity to H1 (in amino acid composition, for example). There has been little further characterisation of H1° except for the observation that upon electrophoresis in acid–urea systems the putative H1° may appear as two bands running close together [2,3]. These bands have been named H1° and H1°° but since no other electrophoretic system was used and no protein isolation or amino acid analyses performed, the possibility exists that one of these bands was a contaminant or product of protein degradation.

H1° is an interesting protein because its presence seems to correlate with a lack of DNA synthesis [1,4] and it has been proposed that it may act as a DNA synthesis repressor [4]. We have therefore been studying H1° and here report fractionation of H1° and more thorough characterisation than has been performed previously.

2. Experimental

Nuclei were prepared from fresh calf or ox liver at 4°C and in the presence of protease inhibitor phenylmethylsulphonyl fluoride (0.5 mM). Nuclei were freed of cytoplasm by centrifugation through sucrose (2.4 M), $MgCl_2$ (10 mM) and Tris–HCl (10 mM, pH 7.5), at 30 000 × *g* for 1 h. Freshly-prepared nuclear pellets were extracted with $HClO_4$ (5%, w/v) and H1° precipitated together with H1 by addition of acetone (3.5 vol.) [5].

H1 and H1° were fractionated by chromatography on Bio-Rex 70 ion-exchange resin [1]. Elution was with sodium phosphate buffer (0.1 M, pH 6.8) and a shallow gradient of guanidinium chloride (GuCl, as shown in fig.1), at a rate of two 5 ml fractions/h. The elution of protein was monitored at 220 nm, and the salt gradient was monitored by refractive index.

A Rank Hilger Chromospek amino acid analyser was used to analyse protein fractions after their hydrolysis in HCl (6 M), 110°C, 18 h. For N-terminal amino acid identification, proteins were dansylated [6], and after hydrolysis were subjected to:

(a) One dimensional chromatography on thin-layer cellulose plates in a mixture of butanol/pyridine/acetic acid/water (30:20:6:24, by vol.);
(b) Two dimensional chromatography on thin-layer polyamide plates in the 3 solvent systems of Hartley's scheme 2 [6] followed by further development in the second direction by a mixture of pyridine acetate (0.1 M, pH 4.4)/ethanol (3:1, v/v) [7], then by Na_3PO_4 (0.05 M)/ethanol (3:1, v/v) [6].

Treatment of proteins with cyanogen bromide was as in [8].

Digestion of proteins by trypsin was in ammonium bicarbonate solution (0.2 M, pH 8.2) with a 50-fold excess of substrate (w/w) for 40 h at 37°C. Peptide mapping was performed on thin-layer cellulose plates, with development in the first dimension by high-voltage electrophoresis at pH 3.5 (2 kV, 20 min) using a Shandon flat plate apparatus, and in the second dimension by ascending chromatography overnight in a mixture of butanol/acetic acid/water/pyridine (30:72:288:240, by vol.). Spots on the chromatogram were visualised after staining with ninhydrin or with phenanthrenequinone, which causes arginine-containing peptides to fluoresce under ultraviolet irradiation [9].

Electrophoresis was performed on 15% polyacrylamide gels, in either sodium dodecylsulphate (SDS, 0.1% w/v) and Tris–HCl (0.375 M, pH 8.8) [10], or

acetic acid (0.9 M) [11] and urea (1.5 M). Routinely, gels were stained with Coomassie brilliant blue R250 (0.1, w/v) in methanol/acetic acid/water (50:10:40, by vol.), followed by destaining in methanol/acetic acid/water (10:10:80, by vol.).

3. Results and discussion

Ion-exchange chromatography separated H1 from H1°, as in [1], with H1° emerging at 10.4–11% (w/v) GuCl as shown in fig.1. The shallow salt gradient used also split H1° into fractions, which have been designated H1°a, H1°b and H1°c, in order of elution.

The different forms of H1° were separated on acid–urea (1.5 M) gels of size 0.6 × 25 cm by prolonged electrophoresis (200 V, 20 h, room temp.) (See fig.2). Their mobilities in this system increased in the following order (determined by running pairs of samples on single gels):

$$H1°a < H1°b << H1°c$$

Upon SDS–gel electrophoresis H1° was found to run just ahead of the H1 bands. H1°a and H1°b ran coincidentally but H1°c ran slightly faster (see fig.2).

The relative amounts of H1° subfractions were estimated from sizes of peaks in elution profiles and in scans of acid–urea gels which had been stained quantitatively with Procion Navy (by the method in [12]). Results were, however, inconsistent. Furthermore, preparations made by an alternative method were found by chromatography and gel electrophoresis to contain only H1°a and H1°b, with no H1°c. By the alternative method (which allows very little time for protein degradation) whole, fresh, ox liver was extracted directly and at 4°C with $HClO_4$ (5%, w/v), and this extract made 18% (w/v) with respect to Cl_3COOH, to selectively precipitate various proteins including H1 and H1°. This precipitate was then dissolved in water, made 5% (w/v) $HClO_4$, and H1 and H1° precipitated together by addition of HCl and acetone (3.5 vol. [5]).

The ratio total H1°/total H1 in preparations made by either method was 0.14 in every case. This figure agrees with that of 0.13 which has been obtained for calf liver H1° by scanning amido black-stained gels

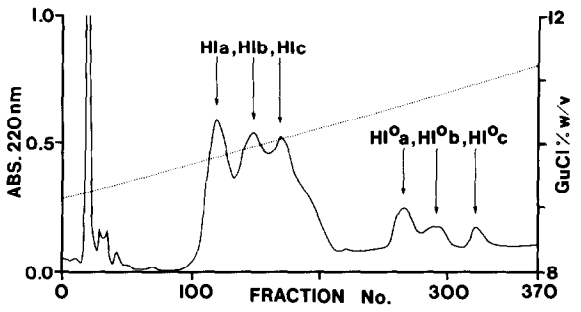

Fig.1. Profile of elution of ox liver nuclear H1 and H1° fractions from a Bio-Rex 70 ion-exchange column, with a GuCl gradient (...) in sodium phosphate (0.1 M, pH 6.8). Peaks are labelled according to the protein they contain.

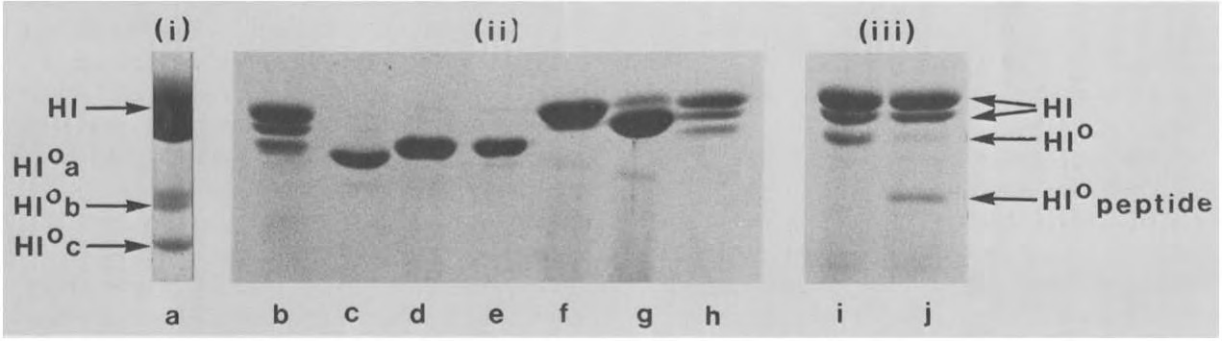

Fig.2. Polyacrylamide gel electrophoresis of H1 and H1° fractions, and of H1° peptide. Direction of band movement downwards. Gels stained by Coomassie brilliant blue R250. (i) Acid–urea (1.5 M) gel electrophoresis for 20 h. Sample: (a) H1 and H1° fractions. Relative mobilities as indicated. (ii) SDS–gel electrophoresis. Samples: (b,h) combined H1 and H1° fractions; (c,d) partially purified H1 fractions, from peaks H1a and H1b (respectively) of fig.1; (e,f,g) H1° fractions H1°a, H1°b and H1°c (respectively). (iii) SDS–gel electrophoresis. Samples: (i) combined H1 and H1°, treated with formic acid, 40 h; (j) combined H1 and H1° treated with cyanogen bromide in formic acid, 40 h. Mobilities of H1, H1° and H1° peptide as indicated.

Table 1
Total amino acid analyses of H1a and of H1° fractions

	H1a	H1°a	H1°b	H1°c
Asp (A)	1.8	3.3	3.2	2.8
Thr	5.7	6.7	6.6	6.0
Ser	6.2	9.3	9.4	9.4
Glu (A)	3.8	5.4	5.3	5.2
Pro	8.5	7.8	7.2	8.1
Gly	6.2	4.4	4.0	5.7
Ala	23.8	14.2	13.6	13.8
Val	5.1	5.7	5.7	6.2
Cys	0.0	0.0	0.0	0.0
Met	0.0	0.4	0.4	Trace
Ile	0.9	2.6	2.5	2.7
Leu	4.1	2.3	2.0	2.3
Tyr	0.5	1.3	1.5	1.3
Phe	0.6	1.2	1.3	1.2
Lys	30.3	31.1	32.7	30.5
His (B)	0.0	1.0	1.0	0.9
Arg	2.0	3.1	3.2	3.4
Acidics (A)	5.6	8.7	8.5	8.0
Basics (B)	32.3	35.2	36.9	34.8
B/A	5.8	4.0	4.3	4.3
Lys/Arg	15.1	10.0	10.2	9.0

Expressed as mol% with no correction for hydrolytic loss

[13]. The data suggest that H1°c is an artefact which arises during preparation of nuclei despite the presence of protease inhibitor at 4°C, and that H1°c derives from H1°a and/or H1°b (since the amount of combined H1° fractions is constant). The data also confirm that H1° is concentrated in the nucleus.

All H1° fractions were subjected to further analysis. The total amino acid contents of these proteins were very similar, whether from calf or ox. Table 1 compares these analyses with that of a partially-purified H1 subfraction (H1a, the earliest-eluting peak of H1) from the same source.

Whilst no H1 fraction was found to contain methionine, analyses of H1° fractions showed traces of it. The figures given in table 1 are for true methionine (in its reduced form) and so are underestimates because during protein isolation, methionine is oxidized to some extent. The resulting sulphone which behaves differently from reduced methionine upon chromatography during amino acid analysis, was not estimated. The presence of methionine in H1° was confirmed by cleavage of purified H1° fractions (including H1°c) by cyanogen bromide. Controls of similarly-treated H1 fractions, and of H1° fractions in formic acid (but no cyanogen bromide) showed no cleavage. The methionine residue(s) is probably near one end of the H1° molecule, for cleavage at this residue generates a peptide which has a mobility, upon SDS–gel electrophoresis, only slightly greater than that of intact H1° (see fig.2 (iii)). The minor peptide(s) is insufficiently stained to see or is lost from the gel.

If H1° has just one methionine residue, it may be estimated that H1° is ~200 residues long, or about the same size as the H1 molecule. The similar mobilities of H1 and H1° on SDS–gel electrophoresis lend support to this approximation.

While the amino acid analyses of H1° in table 1 generally agree with that in [1], the discovery of methionine is noteworthy, for its presence and reaction with cyanogen bromide can facilitate primary sequence determination, and also aid identification.

In none of the H1° fractions could any dansylated, free, N-terminal amino acid be identified, despite exhaustive chromatography. It is therefore concluded that the N-terminal residue is blocked in each H1° fraction.

Tryptic peptide maps of the different H1° fractions were identical to each other, whether from calf or ox, when stained by phenanthrenequinone. When chromatograms were stained by ninhydrin the different fractions were very similar, showing only slight quantitative differences in several very minor spots. The significance of these differences is unknown. Similarly, tryptic peptide maps of partially purified H1 fractions were identical. Figure 3 shows typical peptide maps, of H1a and of H1°a, and it may be seen that H1° is characteristically different from H1 from the same source. It may be added that the two-dimensional tryptic peptide maps of ox liver H1° are no more like those of ox liver H1 than are those of chicken erythrocyte H5 (data not shown).

These results show that the H1° fractions a–c are very similar proteins, and so how H1°c derives from H1°a and/or H1°b is unclear. Preparations containing H1°c show no evidence on gel electrophoresis or chromatography of any (further) proteolytic degradation, but the possibility remains that H1°c arises by cleavage of a very small, specific peptide(s) from the C-terminal end of the parent molecule, in spite of the presence of a protease inhibitor at 4°C. The slightly faster mobility of H1°c on SDS–gel electrophoresis might support this possibility. An alternative explanation may lie in side chain modification of H1°, which can incorporate label in vivo

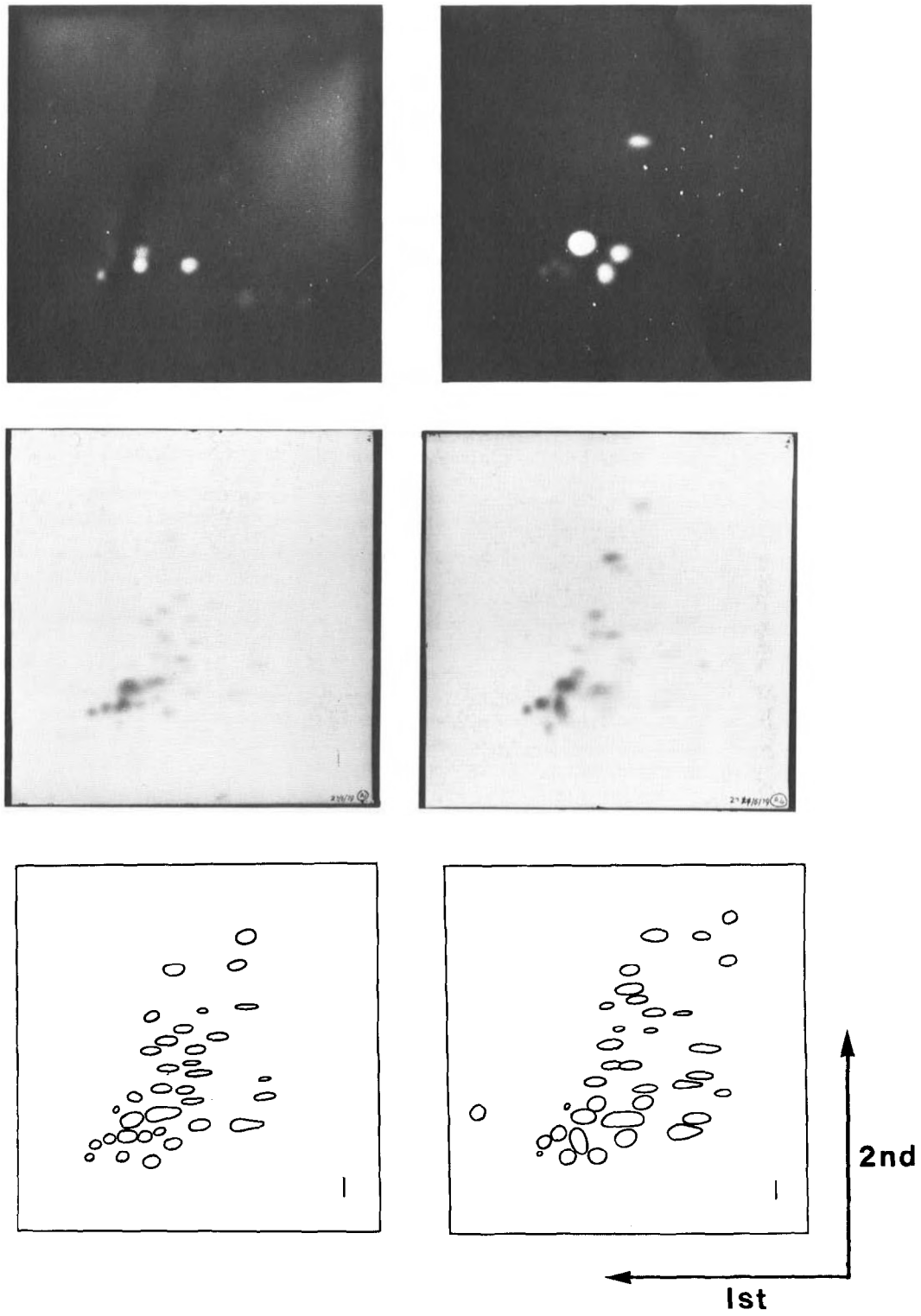

Fig.3. Two-dimensional tryptic peptide maps of H1a (on the left) and of H1°a (on the right). First dimension: high-voltage electrophoresis. Second dimension: ascending chromatography. From top to bottom: plates stained by pheneanthrenequinone; plates stained with ninhydrin; traces of ninhydrin-stained plates, showing positions of minor spots.

when injected as methionine, acetate or phosphorus [4]. More basic forms of H1°, (which run faster on acid–urea gel electrophoresis) could be generated, for example, by action of a phosphatase during nucleus isolation. Loss of adenosine diphosphoribosyl moieties by non-enzymic means is not the cause of the artefact, for conditions which rapidly bring about such a loss from proteins (namely 0.1 M NaOH, 37°C, for ⩾10 min [14]) do not affect the electrophoretic behaviour of H1° (data not shown). Deamidation of H1° in the acid conditions used in preparation of proteins is also not responsible for the artefact, for deamidation generates a more acidic protein, not a more basic one.

In what way(s) the two real forms of H1° (a,b) which exist in cattle liver differ is not yet known; they may be differently modified forms of the same protein (though not by adenosine diphosphoribosylation). It is possible that H1°a and H1°b correspond to the two bands noted in [2], but it is not clear which forms of H1° found here correspond to H1° and H1°° in [3].

The results presented here show that H1° (as one protein or as a family of subfractions) is readily distinguishable from H1. Therefore, if H1° is to be considered as an H1 subfraction, it ought to be regarded as one which is markedly different from other H1 subfractions, and as one which might therefore have a different function.

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