Resistance of chromatin superstructure to tryptic digestion modulated by conjugated polyacrylamide

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Soluble polyacrylamide conjugates have been used to modulate tryptic digestion of chromatin. Digestion of histones H_1/H_5 and H_3 are mutually dependent and relatively independent of that of the core histones which are not significantly digested until 50–60% of H_3 is degraded. H_1/H_5 and H_3 are most exposed, H_3 behaves as a 'non-core' histone, its destruction appears to be the critical factor in the collapse of the chromatin superstructure during tryptic digestions. The digestion kinetics are explained by proposing that the initial sites of attack are in cavities much larger than the diameter of trypsin (4–5 nm). Procedures for the preparation of soluble polyacrylamide and its conjugation to trypsin are described.

Chromatin *Nucleoprotein* *Histone* *Trypsin* *Erythrocyte* *Liver nuclei*

1. INTRODUCTION

The structure of chromatin at the level of the nucleosome is fairly well understood, but many properties of the higher levels of chromatin organisation are still unclear (reviews in [1,2]). Studies with proteases have established the relative susceptibilities of basic nucleosomal proteins and indicated a peculiarly exposed behaviour of histone H_1 in the nucleosome [3–10]. Passive probes have indicated the presence of two broad classes of cavities in intact nuclei [11,12].

Here, we have used free trypsin as a small probe and trypsin conjugated to soluble polyacrylamide (PA–trypsin) as a large probe with the same active centre to examine the effect of chromatin superstructure on histone exposure.

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Abbreviations: PA–trypsin, soluble polyacrylamide–trypsin conjugate; trypsin–TPCK, L-(tosylamido-2-phenyl)ethyl chloromethyl-treated trypsin; TEMED, *N,NN',N*-tetramethylethylbenediamine; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid; M_r, relative molecular mass.

2. EXPERIMENTAL

2.1. *Preparation of nuclei*

Nuclei were prepared from rat liver [13] or chicken erythrocytes [12].

2.2. *Preparation of heterogeneous soluble polyacrylamide*

A 100 ml solution of acrylamide (20 mmol), *N',N*-methyl bisacrylamide (3.5 mmol), allylamine (1.3 mmol), TEMED (0.86 mmol), K_2HPO_4 (10 mmol) (pH 8.0) was heated (80–90°C) under N_2. Ammonium persulphate solution (100 ml, 0.1%) was added at 100 ml/h and the resulting mixture removed at 200 ml/h into a receiving vessel containing 1 ml of 2-mercaptoethanol as a reaction terminator. The product was reduced in volume and dialysed free of low-M_r materials.

2.3. *Conjugation of soluble polyacrylamide and trypsin*

Trypsin–TPCK (10 mg) and soluble polyacrylamide (26 mg) each in 1 ml of Ca–borate–glycol buffer [13] were dialysed against this buffer at 4°C overnight. To the soluble polyacrylamide, 0.3 ml

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neutralized 25% glutaraldehyde was added and incubated at 37°C for 2.5 h under N_2. The polyacrylamide–glutaraldehyde complexes were separated from free glutaraldehyde on a Sephadex G-50 column in 0.5 mM Na-acetate, 10 mM NaCl (pH 5.5); added to the trypsin and incubated for 4 h at 20°C under N_2. The conjugation reaction was terminated with ethanolamine-acetate/cyanoborohydride [13], reduced with dithiothreitol and the high-M_r species collected from the void volume of an ultragel ACA-34 agarose gel filtration column.

2.4. *Digestions*

Incubations (0.3 ml) consisted of nuclei (0.5–1.0 mg DNA) free trypsin or PA–trypsin in Hepes-A buffer [13] plus 0.5 mM $CaCl_2$. With rat liver nuclei, EGTA at a 2–3 molar excess over the total Ca^{2+} concentration was added to inhibit the $(Ca^{2+} + Mg^{2+})$-dependent endonuclease. For each set of digestions a characteristic reference time (100% SC) corresponding to structural collapse was determined; i.e., the time from the initiation of digestion to the point when the digestion mixture changed abruptly from a turbid suspension to a clear solution containing, usually, a single aggregated clump.

Incubations were terminated at times up to 100% SC by adding 0.01 ml *p*-aminobenzamidine (250 mM in Hepes-A buffer). The insoluble material was collected by brief high-speed centrifugation, resuspended in 2 mM EDTA (0.2 ml) and brought to 1.5% SDS.

2.5. *Gel electrophoresis*

Samples were reduced in hot, alkaline, SDS and run on both 20% polyacrylamide gels [14] and 18–22.5% linear polyacrylamide gradients [15]. The appropriate Coomassie blue stained bands were extracted with 99% formic acid and the dye quantified at 640 nm. The data are all expressed relative to the corresponding bands in the original undigested sample.

3. RESULTS

3.1. *H_3 is an index of structural integrity*

Histone digestions were non-linear. Since it is highly probable that during the latter stages of in-

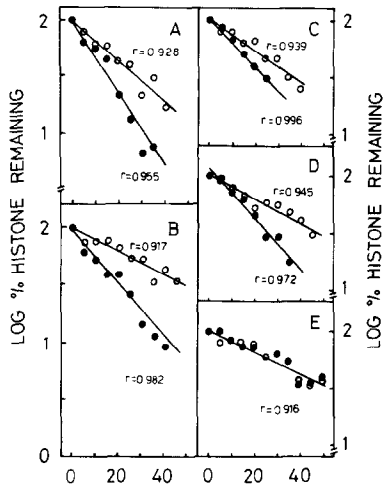

Fig.1. The rate of digestion of various histones as a function of % SC. The lines are fitted for digestions with free trypsin (●) or PA–trypsin (○) with coefficients of determination (*r*) indicated. The ratios of μg DNA:μg tryptic activity were 3500:1 with rat liver nuclei and free trypsin, 1500:1 with avian erythrocyte nuclei and free trypsin, and 25:1 with either nuclei type and PA–trypsin. The logarithms of the % remaining at various % SC are shown for rat liver H_1 (A), H_3 (B), chicken erythrocyte H_1 (C), H_5 (D) and H_3 (E).

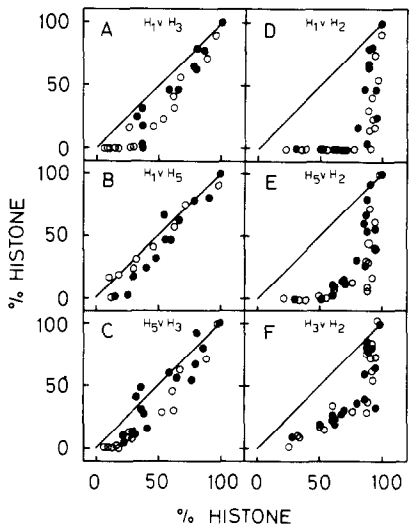

Fig.2. Paired digestion plots for various chicken erythrocyte histones. The levels of histones, as a % of the initial amount remaining after the digestions with free trypsin (●) and PA–trypsin (○) are plotted for [H_1 *vs* H_3] (A), [H_1 *vs* H_5] (B), [H_5 *vs* H_3] (C), [H_1 *vs* H_2] (D), [H_5 *vs* H_2] (E) and [H_3 *vs* H_2] (F). The solid lines (45°) represent the expectation for strictly concerted digestion of the histone pairs.

cubation there are considerable structural perturbations resulting from the earlier phases of digestion, we only examined the early stages of the time course (0–45% SC). Furthermore, as the end-point of these digestions is 100% SC, the initial rates of digestion in fig.1 are not relative to time but relative to the progression of structural collapse.

The rate of digestion of H_1, H_5 and rat liver H_3 is slower, relative to structural collapse, with PA–trypsin (40–50%) than that with free trypsin (fig.1). The corresponding rate of digestion of chicken erythrocyte H_3 is apparently the same with both probes (fig.1E); i.e., the rate of digestion of chicken H_3 is linearly related to the degree of structural collapse elicited with either probe.

In all cases, at full structural collapse, the typical limit digest pattern of resistant degradation products [3] is reached in the presence or absence of polyacrylamide (not shown).

3.2. *H_3 tends to co-digest with H_1/H_5*

Histones H_{2A} and H_{2B} were aggregated as 'H_2' and used as an index of the destruction of the nucleosome core. The digestions of the histones H_1, H_5, H_3 and H_2 have been replotted in pairs to give a clear indication of the degree of co-digestion (fig.2,3). Chicken erythrocyte H_1, H_5 and H_3 all co-digested with each other but not with H_2 (fig.2).

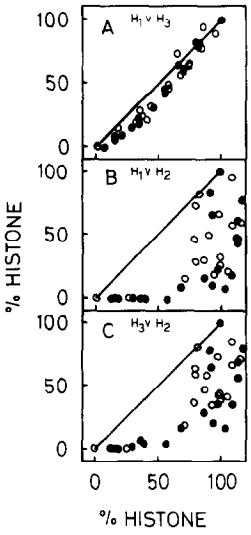

Fig.3. Paired digestions for histones H_1, H_3 and H_2 from rat liver nuclei. The data are presented as in fig.2, and are plotted for [H_1 *vs* H_3] (A), [H_1 *vs* H_2] (B) and [H_3 *vs* H_2] (C).

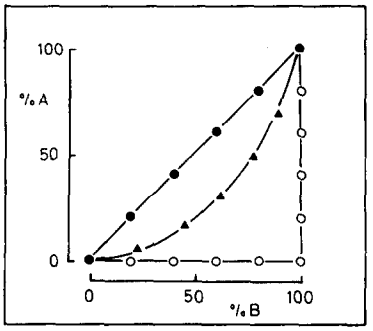

Fig.4. A representation of three broad types of kinetics expected when a two zone structure is attacked. Type I response (●) represents concerted digestions, type II (▲) independent digestion and type III (○) sequential digestion (see section 4).

Similarly, rat liver H_1 and H_3 co-digested but neither with H_2 (fig.3).

4. DISCUSSION

4.1. *Histone H_1/H_5 and H_3 fall into a similar class with respect to exposure*

The co-digestion data presented in fig.2 and 3 are most easily interpreted by considering a two stage attack. Three extreme types of digestion kinetics can be expected (summarised in fig.4); type I which represents concerted digestion of the two 'regions' (A,B) observed when either the two zones are mutually protective or when B is intrinsically more susceptible than A but A is shielding B in some way; type II which represents relatively independent digestion, where A and B have comparable susceptibilities and are being attacked independently and type III where A is completely digested before B is attacked; i.e., sequential digestion (fig.4). With chicken erythrocyte nuclei, type I kinetics are seen with [H_1 *vs* H_3], [H_1 *vs* H_5] and [H_5 *vs* H_3] (fig.2) and with rat liver nuclei, [H_1 *vs* H_3] (fig.3). This suggests these three histones are mutually protective or one is strongly protecting the others. In either case, the suggestion is that these histones have a close organizational relation and our data cannot determine whether H_1/H_5 or H_3 is the most exposed protein in absolute terms. The digestion of chicken [H_3 *vs* H_2] appears to be type II kinetics, [H_5 *vs* H_2] is in-between types II and III kinetics, whereas [H_1 *vs* H_2] is clearly a type III digestion (fig.2).

The digestion of rat liver [H_1 *vs* H_2] and [H_3 *vs* H_2] are also type II (fig.3).

We believe these data are best explained by proposing that H_1 and H_3 are intimately associated; a conclusion in agreement with some cross-linking experiments [16].

4.2. *Collapse of higher order structure correlates with H_3 destruction better than H_1, H_5 or H_2*

The conjugation of trypsin to a large inert ballast reduces the rate of degradation of chicken H_1 and H_5 to about 50% of that by free trypsin (fig.1C,D). Despite these changes the rate of degradation of H_3 is unchanged relative to the degree of structural collapse (% SC) (fig.1E). This is either an unexpected coincidence or it indicates that the degradation of H_3 is the primary cause of structural collapse. A study involving chymotrypsin digestion of isolated chromatin also suggested a crucial role for H_3 in maintaining the rigid supercoil characteristics of chromatin [17]. In agreement with [3,7,8,17] we confirmed that tryptic digestion of H_3 is quite rapid compared to that of the other core histones (fig.1); the digestion of the core as typified by H_2 has a definite lag, and does not commence until 50–60% of H_3 is degraded irrespective of the probe or nuclei type used (fig.2,3).

The rate of digestion of H_3 in the rat liver nucleus, unlike that in the chicken erythrocyte nucleus is dependent on the presence of polyacrylamide ballast on the trypsin (fig.1B). This probably reflects the more heterogeneous nature of rat liver chromatin relative to chicken erythrocyte chromatin.

4.3. *The ballast effect*

Both chicken erythrocyte and rat liver nuclei behave as if they have two broad classes of cavities: one class ~4–5 nm wide; the other ~10–15 min wide [11,12]. Free trypsin is 4.2 nm in diameter whereas our PA–trypsin probe has a diameter of ~10–30 nm as estimated on Sepharose 2B (not shown).

The difference in probe size results in a requirement for more tryptic activity when ballast is present but causes quite small quantitative differences in the relative susceptibilities of the histones. This would seem to imply that when the most exposed protein(s) on the external surfaces of the cavities are eventually degraded by either probe to some critical point, local structural collapses occur, proceeding perhaps in a cascade fashion.

Although increasing the diameter of trypsin by 3–5-fold with polyacrylamide did have observable effects on the degradation kinetics, it did not materially change the order of events early in digestion. We believe that this indicates that the initial sites of tryptic attack are in cavities larger than 4–5 nm in both rat liver and chicken erythrocyte nuclei. This places useful restrictions on models of solenoidal chromatin structures.

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