Analytical Study of the Degradation of Nucleohistone during Calf Thymus Chromatin Autolysis

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The mechanism of nucleohistone degradation in calf thymus chromatin during autolysis of the latter involves a release of lysine-rich histone fractions, followed by degradation of the thus-exposed DNA portions; the latter portions are richest in adenine-thymine sequences. After this degradation process, residual nucleohistone fragments of low molecular weight (10^5) remain. Their chemical composition is special, in that the DNA contains a larger proportion of guanine-cytosine sequences than does the initial DNA and it is combined with arginine-rich histones.

In previous work on the controlled autolysis of calf kidney Vendrely, Alfert, Matsudaira & Knobloch (1958) studied the biochemical behaviour of nuclear chromatin when incubated in a temperature of 37°C in aseptic conditions. It appeared that during the pycnosis that occurred in the nuclei (at least during the first hours of autolysis) the nucleohistone complex underwent a depolymerization; its molecular weight decreased, although some characteristic properties of the native macromolecule remained (i.e. solubility at high and low ionic strength and insolubility in 0.14M-sodium chloride). The association between DNA and histone seemed, therefore, to be preserved in the degradation products thus formed.

This natural fragmentation process of the nucleohistone complex during autolysis appeared to be a less violent method for obtaining oligonucleotide-histone complexes of low molecular weight that conserve their native association; it thus seemed more favourable than the usual procedures such as ultrasonication (Mazen & Champagne, 1968), progressive precipitation after denaturation by chloroform (Chargaff, Crampton & Lipshitz, 1953; Crampton, Lipshitz & Chargaff, 1954; Lucy & Butler, 1955), and column fractionation (Bakay, Kirschner & Toennies, 1960).

The chemical study of such nucleohistone fragments might clarify certain relations between the nucleotide sequence of DNA and the peptide composition of the associated histone fractions. Thus, if we could determine an affinity of the various histone fractions for certain DNA regions with a specific base sequence, we might later be able to better understand the mechanism by which certain genes are repressed, while other genes act to initiate the synthesis of mRNA. In this

way, we decided to study the behaviour of nucleohistones during their degradation in the tissue by aseptic autolysis.

A preliminary study has already been presented (Vanderplancke, 1964).

MATERIALS AND METHODS

Crude chromatin. We chose to use calf thymus as experimental material, since it is a tissue rich in nuclear matter, and its nucleohistone is already well known. All the experiments were performed at 4°C. Fresh fat-free thymus (100g) was blended in 400ml of a 0.14M-NaCl solution containing 0.15% sodium citrate. The sediment was recovered by centrifuging at 3000g for 10min and washed three times by homogenization and subsequent centrifugation, at successively decreasing speeds (2500, 2000 and 1500 g). A final rinse was done in 0.14M-NaCl solution without citrate, followed by centrifugation at 1000 g. The residual pellet was dispersed in 200ml of 0.14M-NaCl, at pH 7, saturated with chloroform (0.6%, v/v) to ensure asepsis. The suspension was then equally divided among several flasks, each one intended for an incubation of different duration. This suspension contained washed chromatin that was essentially nucleohistone [Murray (1969) used this preparation as crude nucleohistone] but also includes a few non-histone proteins (acidic proteins and the enzymic proteins responsible for the autolysis).

Experimental autolysis and extraction of nucleohistone products. The chromatin suspension was incubated at 37°C. At the end of incubation, the autolysate was rapidly cooled in ice-water to stop the enzymic action. Since nucleohistone was the only material suitable for our purpose, we had to eliminate most of the non-nucleohistone chromatin content. This was done by the classical procedure of nucleohistone isolation at low ionic strength. Combard-Vanderplancke & Vendrely (1968) discussed this method as a means of keeping the nucleohistone products in their initial complex state. The autolysate was dialysed for 24h against several changes of distilled water to

Scheme 1. Method for the fractionation of calf thymus nucleohistone.

solubilize the nucleohistones. The pellet containing most of the non-nucleohistone substances was separated by centrifugation in a Spinco centrifuge (60 min, 73 000 g, $r_{\max}$); the supernatant contained the nucleohistone degradation products and possibly a very few non-histone proteins as shown below.

We usually incubated concentrated material; in the case of very short-duration autolyses (<2h), when the native structure of the nucleohistone complex had not yet been affected, the dialysis residue was very viscous and gel-like and had to be diluted with cold water and briefly homogenized by stirring gently.

Fractionation of the nucleohistone degradation products. From the aqueous solution of degraded nucleohistone, three fractions were consistently separated: fraction I appeared as fibres, varying in length as a function of the incubation time, by precipitation with NaCl to 0.14M concentration; fraction II precipitated as a powder on the addition of acetic acid to the supernatant of fraction I, bringing it to pH 4.6; fraction III was the residue, soluble in 0.14M-NaCl and at pH 4.6, that was produced by freeze-drying the supernatant from fraction II. This method for the fractionation of nucleohistone degradation products is summarized in Scheme 1.

Analyses showed that fractions I and II were true nucleohistone complexes, since no uridine was detectable (and thus no RNA); in addition, the entire protein content was extractable by 0.125M- H_2SO_4 and contained no tryptophan and only traces of cystine (less than 0.1%). Therefore, only DNA and histone were present. As a more reliable proof, when purified nucleohistone was incubated instead of chromatin, we recovered after degradation by

its intrinsic low enzymic activity a nucleohistone fraction insoluble in 0.14M-NaCl and identical with the fraction I obtained with chromatin autolysis; then a nucleohistone fraction identical with fraction II was precipitated at pH 4.6.

Thus, if non-nucleohistone products were present in the solution after dialysis, they were recovered in fraction III. Our conclusions concerning nucleohistone structure, are drawn from the study of nucleohistone fractions I and II.

Analyses. Nitrogen (N) was measured by the method of Kjeldahl, phosphorus (P) by that of Briggs (Briggs, 1922). Histones were extracted in the way described by Vendrely & Picaud (1968). Amino acids were measured by the Moore and Stein technique, on a Beckman amino acid analyser. The base distribution in DNA was determined from the data of Vischer & Chargaff (1948), after separation by paper chromatography (Wyatt, 1951). The electrophoretic study of nucleohistone fractions was performed on starch gel, or on agarose-Sephadex G-200 gel (Vendrely, Coirault & Vanderplancke, 1964) in KH_2PO_4 - Na_2HPO_4 buffer, pH 6.86, at a voltage of 4.4 V/cm. Starch gel and HCl, pH 2.2, were used for electrophoresis of isolated whole histone, and of the different histone fractions.

RESULTS

Kinetics of autolysis. Fractions I, II and III were collected after progressive time-periods of autolysis. The curves in Fig. 1 show the changes in weight of these three calf thymus nucleohistone fractions against time.

Fig. 1. Kinetics of calf thymus nucleohistone autolysis. Nucleoprotein (100mg) from calf thymus was incubated at 37°C in a 0.14M-NaCl solution added with chloroform. Progressive autolysis occurred, and the degradation products were collected in three fractions: fraction I (□—□), fraction II (●—●) and fraction III (×—×). The results are plotted as the dry wt. (mg) of each nucleohistone fraction against incubation time (h).

Fraction I, insoluble in 0.14M-sodium chloride, was initially the only existing one. After a gradual decrease for 2h, fraction I then decreased rapidly and was no longer recoverable after 6–7h of autolysis.

Inversely, fraction II (soluble in 0.14M-sodium chloride and precipitable at pH 4.6) only appeared clearly after 2h. It then increased rapidly, to reach a maximum after 6–7h of autolysis. After this time, it decreased slowly, and became relatively stable at 48h, at which time it amounted to about 65% of the initial material.

Thus, two phenomena are observed: during the first hours of autolysis, the change in the amount of fraction II is opposite to that of fraction I; this suggests that degradation of fraction I gives rise to fraction II. After 6–7h autolysis, fraction II obviously undergoes a rather slow and limited degradation (its amount is constant between 48 and 120h).

Fraction III appeared suddenly after 3–4h of autolysis, before fraction II begins its degradation; therefore, at this stage the acid-soluble products come directly from degradation of fraction I. Later on, acid-soluble products resulting from the degradation of fraction II accumulate in fraction III up to a plateau corresponding to the limitation of fraction II degradation. It is noteworthy that the total amount of acid-soluble products was not recovered in fraction III; a loss of some products occurs during the dialysis effected after autolysis (about 70% of acid-soluble products were thus diffusible after a 48h autolysis).

These results with calf thymus were also found with chromatin from other cellular specimens and from various species of animals (calf liver; rabbit lung, liver and kidney; rat thymus, kidney and spleen; chick and duck erythrocytes). Indeed, from these various autolysed chromatins three fractions were isolated by the procedure used for calf thymus; fractions I and II, which consisted of nucleohistones, were analytically identical with those produced from calf thymus. Thus the process of autolysis is the same in every case; only the speed of degradation varies.

Two successive stages in the general course of nucleohistone autolysis are thus demonstrated. The first stage is marked by the rapid and simultaneous appearance of fractions II and III, at the expense of fraction I. The second stage includes the slow and limited degradation of fraction II, whose products become acid-soluble and thus accumulate in fraction III.

To gain a better understanding of the autolytic process, we proceeded to study the recovered nucleohistone fractions.

Study of the nucleohistone fraction I. Analyses of fraction I, recovered after 4h of autolysis, are summarized in Table 1. As described above, this fraction contains only nucleohistone. The N/P ratio of 3.5 shows that this fraction represents a DNA-histone complex slightly impoverished in protein. Indeed, the analysis of amino acids in fraction I reveals the loss of lysine and histidine residues, and the electrophoretic pattern of the whole histone extracted from this fraction (Fig. 2) shows that the degradation mainly affects the lysine-rich histone portions, thus exposing the primitively combined DNA parts.

The molecular weight of fraction I is still elevated, which partly explains its maintained insolubility in 0.14M-sodium chloride and the constant physico-chemical properties of its DNA. The hyperchromicity (35.5%) and 'melting' curve ($T_m = 85^\circ\text{C}$) of the DNA isolated from fraction I are those of a native DNA. It is known that these native properties are maintained in polymerized DNA, whose molecular weight is at least 10^6 and which retain their native double-paired structure. This implies that it is the case for DNA fraction I, and that the decrease of the molecular weight of nucleohistone at this stage results from simultaneous splitting at the same level on both strands of the DNA portions exposed after the demonstrated release of lysine-rich histones (diplomatic scission).

Base composition of DNA fraction I is not altered if compared with a control nucleohistone.

Study of nucleohistone fraction II. Although, regardless of the incubation time, this fraction consists only of nucleohistone, the proportion of histone to DNA varies with the incubation time.

Table 1. *Analysis of the nucleohistone fraction I obtained after 4h autolysis of calf thymus*

Analysed nucleohistone-complex	N/P ratio	DNA base composition A+T/G+C	Basic amino acids (mol/100 mol of total amino acid)		
			Lysine	Arginine	Histidine
Total control nucleohistone	3.9	1.42	15.00	12.50	2.47
Fraction I	3.5	1.43	13.77	12.19	1.74

Fig. 2. Starch-gel electrophoresis pattern of fraction I whole histone. (a) Control whole histone from calf thymus; (b) whole histone extracted from calf thymus nucleohistone fraction I after 4h autolysis. Electrophoresis was carried out on starch gel in HCl, pH 2.2 and 2 mg of each histone was applied to the gel.

When this fraction emerges the products are similar to those of fraction I (the N/P ratio is 3.5 after 4h autolysis, corresponding to the impoverishment in histone), but there is then a return to the normal DNA/histone weight ratio, indicating the degradation of the exposed DNA (the N/P ratio rises again to 3.9 after 48h, which is the saturated-complex value).

Chemical composition (Table 2) and electrophoresis (Fig. 3) of fraction II whole histone obtained after 48h autolysis showed that arginine-rich histones (F3 and F2a) were not affected, whereas the lysine-rich histones were degraded. The same results have been observed after isolation of the different histone fractions from nucleohistone fraction II by method II described by Johns (1964). Thus, no supplementary degradation of histones occurred during subsequent autolysis at pH 7.

These results strongly suggested that the first stage of autolytic degradation, at pH 7, rapidly and mainly affected the lysine-rich histone fractions of nucleohistone complexes; consecutively the nucleo-

histone breakdown products become progressively soluble in 0.14M-sodium chloride (Butler & Johns, 1964), especially with the decrease in molecular weight due to diplotomic splits at the DNA level, thus giving rise to fraction II. Some DNA portions were thus exposed after the degradation of their associated lysine-rich histones and were then degraded, as was shown by their hyperchromicity (12.6%) and 'melting' curve ($T_m = 72^\circ\text{C}$). After about 48h, only resistant nucleohistone fragments remained, with a molecular weight of 10^5 (as calculated by ultracentrifugation and Sephadex filtration) and DNA saturated (N/P = 3.9) with arginine-rich histones. The DNA composition in these residual fragments of the complex was no longer similar to that of the initial nucleohistone (A+T/G+C 1.45); in particular, there was an increase in the number of guanine-cytosine nucleotide sequences (A+T/G+C 1.21).

Study of fraction III. This fraction, soluble in 0.14M-sodium chloride and at pH 4.6, consisted of low-molecular-weight products, of which 70–75% were diffusible; but, an exhaustive analytic study was necessary to confirm our concept of the autolytic process. Starting from a non-dialysed autolysate, it was necessary to eliminate the 0.14M-sodium chloride-insoluble fraction, together with the fraction precipitated at pH 4.6, and to freeze-dry the entire remaining solution. The subsequent analyses confirmed that the heavily degraded products constituting fraction III have two successive origins, first from fraction I and then from fraction II. At the beginning of autolysis they were mainly of protein nature, and afterwards nucleotide products gradually accumulated. Accordingly, the N/P ratio was maximum (6.3) between 3 and 4h autolysis, and decreased to 4.6 after 24h. In the same way, the lysine/arginine ratio for fraction III, was found to be 3, thus confirming the belief that the lysine-rich histones released from fraction I accumulated in this fraction. Amino acid analysis of this fraction gave higher arginine (9.14%) and aspartic acid (5.25%) values and lower proline (3.10%) and alanine (11.6%) values than expected for a pure F1 histone; this suggests that non-histone proteins present in the dialysis residue were recovered in fraction III.

Table 2. *Composition of histone fractions prepared from control calf thymus and of whole histone from fraction II and from its subfractions obtained by autolysing calf thymus*

The amino acids are expressed as mol/100 mol of total amino acid found, without correction for hydrolytic losses.

Amino acid	Whole histone from autolytic fractions of calf thymus			Histone fractions from control calf thymus*			
	Fraction II	Subfraction IIa	Subfraction IIb	F2a	F2b	F3	F1
Arginine	11.94	12.13	11.60	10.89	7.38	12.04	3.31
Lysine	10.06	8.87	9.26	9.55	12.67	9.29	20.03
Histidine	—	1.56	1.94	2.09	1.82	1.46	0.92
Aspartic acid	6.07	6.40	6.14	5.68	5.86	4.13	3.78
Threonine	5.62	5.53	5.66	5.47	5.78	6.61	5.72
Serine	3.37	4.35	4.56	2.93	7.36	3.78	7.43
Proline	Trace	Trace	—	2.79	4.88	4.86	7.67
Glutamic acid	10.50	11.06	10.40	9.47	10.51	11.95	6.37
Glycine	12.75	11.46	10.81	13.08	7.33	6.40	7.17
Alanine	10.48	10.77	10.70	10.47	12.05	14.46	21.00
Valine	6.75	7.54	8.15	6.66	5.93	4.66	5.93
Methionine	—	0.47	Trace	0.45	0.97	0.81	0.39
Isoleucine	4.96	5.57	5.58	4.95	4.53	5.28	2.34
Leucine	9.36	9.21	9.34	10.34	6.78	9.20	5.09
Tyrosine	3.10	3.12	2.75	3.26	4.13	2.38	1.62
Phenylalanine	2.43	2.68	1.78	1.86	1.95	2.64	1.20
Lysine/arginine ratio	0.84	0.73	0.80	0.87	1.71	0.77	6.05

* From Vendrely & Picaud (1968).

Fig. 3. Starch-gel electrophoresis pattern of fraction II whole histone. (a) Whole histone of calf thymus nucleohistone fraction II after 48h autolysis; (b) control whole histone from calf thymus. Electrophoresis was carried out on starch gel in phosphate buffer, pH 6.86. In HCl, pH 2.2, the electrophoretic pattern of fraction II histone is the same as that of fraction I (see Fig. 2).

Subfractions of fraction II. Characteristics established for fraction II (nucleohistone nature, relative stability after 48h autolysis, low molecular mass, preservation of association between DNA and histone) were such that the products of this nucleohistone fraction were the most favourable material

for our purpose and it was therefore submitted to a more detailed study after subfractionation.

Electrophoresis on starch gel and especially on agarose-Sephadex G-200, proved to be the best method of subfractionation; three bands, a, b and c, were found in a constant and reproducible manner (Fig. 4). Subfraction c, which migrated more slowly and was the most heterogeneous of the three, consisted of products rather similar to those of fraction I which were just at the limit of insolubility in 0.14M-sodium chloride; this subfraction c could be eliminated by a more intensive centrifugation of the total 0.14M-sodium chloride-soluble nucleohistone fraction (Spinco, 40 min, 130 000g, $r_{max.}$). In this way, only the a and b bands remained after electrophoresis; these bands were fast-moving, and fairly homogeneous.

The subfractions a, b and c separated by electrophoresis were true DNA complexes, as shown by four factors. (1) They can be stained both with Amido Schwartz and Toluidine Blue. (2) They migrate in a single mass towards the anode. (3) There is exact coincidence between the Amido Schwartz-stained bands and the radioactive bands detected by labelling the initial nucleohistone DNA with ^{32}P . (4) It is possible to extract DNA and histone from both subfraction a and subfraction b, after having separated their nucleohistone products by electrophoresis and recovered them by elution. These latter nucleohistone products were saturated

Fig. 4. Electrophoresis of calf thymus nucleohistone fraction II. The nucleohistone fraction II applied to the gel was obtained after 48h autolysis of calf thymus chromatin. Electrophoresis was carried out on agarose-Sephadex G-200 gel in phosphate buffer, pH 6.86.

complexes, containing DNA and histone in the same proportion as in the native complex (N/P ratio is equivalent to 3.9 and 4 for IIa and IIb respectively).

The whole histone extracted from subfractions a and b was poor in lysine residues (Table 2); the electrophoretic pattern confirmed the disappearance of the lysine-rich histone fractions, and the remainder of those fractions richest in arginine (F2a and F3). Subfraction IIa was richest in arginine.

There was no difference between the DNA prepared from the two subfractions a and b, neither in mass (DNA sedimentation coefficients are 5S for subfraction a and 5.5S for subfraction b), nor in composition, both subfractions being rich in guanine and cytosine, especially subfraction a.

DISCUSSION

Two conclusions may be drawn from our study. First, at the neutral pH which was used experimentally, and which exists in most living cells, the well known lability of the lysine-rich histone fraction can be specifically induced through a natural physiological mechanism.

Secondly, in native nucleohistone, two different zones seem to be present: short portions of complex (mol.wt. 10^5), whose DNA is richest in adenine-thymine sequences, contained labile lysine-rich

histones, and more stable portions, which appear as resistant 'cores' in our experimental degradation, and which are probably composed of arginine-rich histones, and of DNA slightly richer in guanine-cytosine. This conclusion agrees with the observation of a preferential combination between different histones, or analogous products, and various DNA species (Ohba, 1966; Leng & Felsenfeld, 1966; Hurwitz, Evans, Babinet & Skalka, 1963; Mazen & Champagne, 1968).

Recent results, such as the determination of amino acid sequences in histone fractions F2a1, which appear to be very similar for pea seedlings and calf thymus (Delange, Fambrough, Smith & Bonner, 1968), make a precise specificity between histones and base sequences in DNA appear unlikely; nevertheless, a certain preferential combination may exist in relation to the respective roles of the two main histone fractions, arginine-rich and lysine-rich histones, whose structure appears to be different (Bradbury, 1969; Tuan & Bonner, 1969) and to the different functions of adenine-thymine-rich genes and guanine-cytosine-rich genes suggested by the observations of Martin & Hoyer (1967) and Cummins & Rusch (1968).

Moreover, concerning the structure of nucleohistones and the distribution of the different histone fractions along the DNA chain, our studies of the nucleohistone fractions I and II lead to a conclusion similar to that of Murray (1969). From a different approach, this author concludes that the most probable type of histone distribution would be intermediate between one in which long DNA portions are covered by only one type of histone, and one in which no two like-histone molecules would be adjacent. We are also inclined to adopt the same hypothesis, in view of the existence of some 'cores' in nucleohistone. These resistant, saturated, nucleohistone portions, which we recovered in subfractions IIa and IIb have a rather homogeneous size, since they do not apparently have greatly varying molecular weights. Their consistent molecular weight is in the range of 100 000; since they are saturated complexes, their DNA moiety represents 45% and thus corresponds to a weight of 45 000, which indicates about 70 nucleotide-pairs; the protein part corresponds to a weight of 55 000 and, as it contains essentially F2a and F3, the heaviest histones (average mol.wt. 20 000–30 000), it would consist of two or three histone molecules. Thus, these short portions (representing about 30% of the native nucleohistone) with three analogous histone molecules, would alternate with other complex regions whose histone is of another type. The behaviour of these two main complex zones is different with respect to enzymic degradation (demonstrated above) and also with respect to the support of RNA synthesis *in vitro* (A. Combard

& R. Vendrely, unpublished work). This also supports some of Murray's (1969) results.

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