PROTEOLYTIC DIGESTION OF HISTONES INDICATES THAT THE NUCLEOSOMES OF NUCLEI AND OF SHEARED CHROMATIN ARE SIMILAR*

Dawn B. Marks and Barbara J. Keller

Department of Biochemistry, Temple University School of Medicine
Philadelphia, Pa. 19140

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SUMMARY: Calf thymus nuclei were treated with trypsin, chymotrypsin or Pronase, and the rate of digestion of the various histone fractions was determined. The results differed from those obtained by digestion of DNA-free histones with the same set of enzymes but were identical to those obtained by digestion of calf thymus chromatin. Because these enzymes have such different specificities, the results of these digestions indicate that the histone fractions have similar locations in the chromosomal substructures of nuclei and chromatin, i.e. that the structure of the nucleosomes which exist within nuclei is not changed markedly when chromatin is isolated from nuclei by a method which involves shearing.

Electron microscopy (1,2) and nuclease digestion (3,4) provided the initial evidence which lead to the current model for chromatin structure. According to this model, chromatin consists of a series of repeating substructures each containing approximately 200 base pairs of DNA wound around a protein core (5,6). This core is composed of two molecules of each of the histones H2A, H2B, H3 and H4.

Efforts to obtain additional evidence in support of this "nucleosome" (7) model have generated concern that the method of isolation of chromatin might disturb these substructures. It has been known for some time that histones dissociate from DNA under certain conditions (8-10) and Doenecke and McCarthy (11) have recently shown that vigorous shearing causes movement of histones along DNA fibers. Noll et al. (12) have found that DNA isolated from intact nuclei which were treated with micrococcal nuclease yielded

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a series of discrete bands on polyacrylamide gels while DNA from sheared chromatin treated with micrococcal nuclease produced only a smear, indicating that the shearing method employed for isolating this chromatin had destroyed the chromosomal substructures. Furthermore, trypsin digestion of chromatin prepared by mild nuclease treatment of nuclei and of chromatin prepared by shearing indicated that the susceptibility of the histone fractions to proteolysis had been altered by the shearing procedure.

On the other hand, Woodhead and Johns (13) obtained the same series of discrete DNA fragments after micrococcal nuclease treatment of nuclei or of chromatin isolated by a method which involved shearing, and Olins et al. (14) have reported that electron microscopy of chromatin sheared by blending or by sonication revealed substructures (ν bodies) throughout the chromatin fibers similar to those observed when intact nuclei were lysed directly onto microscope grids.

We have previously reported the results of digesting calf thymus chromatin with the proteolytic enzymes, trypsin, chymotrypsin, and Pronase (15). Because of the possibility that our method of isolating chromatin had destroyed the chromosomal substructures, we repeated our experiments utilizing intact calf thymus nuclei for the digestion procedure. The results we obtained with nuclei were identical to those we reported for chromatin, supporting the conclusion of Woodhead and Johns that certain conventional preparative methods involving shearing forces yield chromatin which retains the nucleosome structure found within the nucleus.

Materials and Methods: Trypsin and α-chymotrypsin were purchased from Worthington Biochemical Corporation and Pronase from Calbiochem. Nuclei were isolated from frozen calf thymus tissue by the method of Allfrey et al. (16). Nuclei suspended in 0.3 M sucrose, 1 mm $CaCl_2$ (to prevent lysis and aggregation) and 10 mM sodium borate, 2 mM sodium citrate (pH 7.3) at a concentration of 300 µg DNA/ml were treated at room temperature with trypsin (5 µg/ml), chymotrypsin (30 µg/ml), or Pronase (7.5 µg/ml). Two ml aliquots were taken at the times indicated in Fig. 1 and processed as previously described (15).

The acid extracted histones were subjected to electrophoresis according to the procedure of Panyim and Chalkley (17), stained, scanned, and the amounts of the individual histone fractions were determined (15). At each

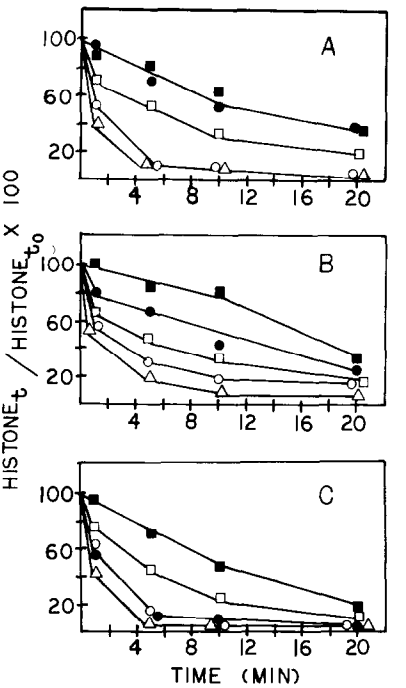

Fig. 1. Proteolytic digestion of nuclei. Calf thymus nuclei (300 µg DNA/ml) were suspended in 0.3 M sucrose, 1 mM $CaCl_2$, 10 mM sodium borate, and 2 mM sodium citrate (pH 7.3) and treated at room temperature with (A) trypsin (5 µg/ml), (B) chymotrypsin (30 µg/ml), and (C) Pronase (7.5 µg/ml). Aliquots were taken at the indicated times and histones were extracted as previously described (15). The acid-soluble proteins were subjected to electrophoresis on polyacrylamide gels in 0.9 N acetic acid. The amount of each histone fraction was determined at each time point and is presented as the amount of protein relative to its amount at zero time. Controls indicated that within this time period there was no change in histone fractions of nuclei in the absence of these enzymes. (Δ), Histone H1; (■), H2A; (□), H2B; (○), H3; (●) H4.

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time point the ratio of the amount of a given histone fraction to the amount present at zero time was calculated. The histone fractions of nuclei incubated in the absence of these enzymes did not change within the time span of these experiments.

Results: The amount of protein remaining in each histone fraction at various times during digestion with each of the proteolytic enzymes is presented in Fig. 1. Although these enzymes have very different specificities, the results in terms of the digestion of the various histone fractions in calf thymus nuclei are similar. Histone H1 is most rapidly digested, and the amount of histone H3 also decreases quite rapidly. H2B is digested more

slowly, and H2A is most resistant. Histone H4 differs for the various enzymes, being almost as resistant to trypsin and chymotrypsin as H2A and as susceptible to Pronase as H3.

These results are virtually identical to those we obtained by trypsin, chymotrypsin and pronase digestion of chromatin (15). This chromatin had been isolated from disrupted calf thymus nuclei by a method which involved shearing in a Waring Blendor at 35 V, the lowest speed at which the blender will operate.

Discussion: Our results indicate that both nuclei and chromatin isolated from calf thymus tissue are similar in terms of the susceptibility of their histone fractions to digestion by proteolytic enzymes. Digestion with these enzymes can be used to determine the location of histone fractions in chromosomal substructures. Trypsin is specific for lysine and arginine residues which tend to be clustered in the NH_2-terminal regions of the histone molecules. Chymotrypsin, on the other hand, has been shown to break bonds in histone molecules which involve phenylalanine, tyrosine and leucine residues (18-22). These hydrophobic amino acids tend to be clustered in the COOH-terminal regions of the histone molecules. Pronase, a mixture of proteolytic enzymes similar to those secreted by the mammalian pancreas, is capable of attacking histones at numerous residues along the chain. Because of the different specificities of these enzymes, we have assumed that histone fractions which are readily attacked by all three enzymes are located in a superficial position of the nucleosome while fractions which are attacked more slowly are in an inaccessible position.

Since histones H1 and H3 are readily digested by all three enzymes, they are probably located on the surface of the nucleosomes. Histone H2A, on the other hand, appears to be buried within the nucleosomes, while H2B may be in a positon intermediate to H3 and H2A. Since H4 is affected differently by each of these enzymes, it is possible that the NH_2-terminal and COOH-terminal portions of the molecule are buried, perhaps in the major groove of the DNA

helix, while portions of the molecule that are susceptible to Pronase are more exposed.

The results presented in this paper indicate that histones are arranged in a configuration in nuclei similar to their configuration in isolated chromatin. Our results support the conclusion of Woodhead and Johns (13) that certain conventional methods of isolating chromatin, even though they involve shearing forces, do not disrupt nucleosome structure. Although Noll et al. (12) observed such disruption, it could be due to the particular method they utilized for isolating chromatin. Perhaps the extent of blending i.e. full speed in a Sorvall Omnimixer (utilized by Noll et al.) versus the lowest speed in a Waring Blendor (our method), is the major factor which determines whether or not the nucleosome structure will be destroyed, although other conditions, such as the ionic environment and pH, might play an equally important role.

It should be noted that we observed a different order of the rate of digestion by trypsin of the histones of calf thymus nuclei (H1 > H3 > H2B > H2A = H4) than that observed by Noll et al. (12) for rat liver (H3 > H2A > H2B = H4).

Both these sets of results differ from those of Weintraub and Van Lente (23) and of Sollner-Webb et al. (24). Although they present no quantitative data, from visual inspection of their gels Weintraub and Van Lente (23) conclude that in trypsin-treated chick erythroblast chromatin histones H1 and H5 are cleaved first, then H3 and H4 are cleaved as a pair followed by H2A and H2B, also as a pair. Sollner-Webb et al. (24) from visual inspection of their gels conclude that in trypsin or chymotrypsin treated duck erythrocyte chromatin histones H1, H5 and H3 are digested at similar rates and H2A, H2B and H4 are also digested at similar rates but more slowly than H1, H5 and H3. They state that H4 is degraded far more slowly in nuclei.

Although our results differ from those mentioned above, they do, however, agree with those of Chatterjee and Walker (25) who also digested calf

thymus chromatin with trypsin and chymotrypsin. A comparison of the rates of digestion of the histone fractions in these five studies suggests that there is a difference in the way in which histones are associated with DNA in different organisms, or perhaps, in different tissues.

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