Further Studies of a Thymus Nucleohistone-associated Protease*

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SUMMARY

The endogenous proteolytic degradation of the histones of calf thymus nucleohistone has been followed with polyacrylamide disc gel electrophoresis and dansylation of amino-terminal amino acids. The proteolytic enzyme is tightly associated with nucleohistone, is essentially inactive below pH 7.0, is decreased in activity at lower ionic strengths, shows a rather specific bond cleavage for those histone fractions which are susceptible to its attack, and is inactivated by brief heat treatment (2 min at 90°).

The susceptibility of the five histone fractions to proteolysis is critically dependent upon whether the histones form a complex or not with DNA. In the intact nucleohistone three groups of histone are almost or totally resistant to proteolytic attack while the lysine-rich histone and the sulfhydryl-containing histone fractions are rapidly attacked. If histones are freed from DNA, only the lysine-rich fraction is resistant to the protease; all other fractions are rapidly degraded.

In general those tissues having a high cell turnover, e.g., thymus and intestinal mucosa, exhibit a greater rate of proteolysis of nucleohistone than is observed for other tissues.

The proteolytic activity is reversibly inhibited by either the presence of sodium bisulfite or by storing the nucleohistone at low ionic strength (~5 × 10⁻⁴).

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molecular weight 19,500 (9), were dissolved in 0.5 ml of 0.1 M NaHCO₃, pH 8.4, to which 0.5 ml of dansyl chloride in acetone (20 mg per ml, Calbiochem, lot No. 010011) was added. The reaction mixture was agitated for 12 hours, evaporated in a vacuum, and then hydrolyzed (6.4 N HCl) in a vacuum for 16 hours. The hydrochloric acid was removed by flash evaporation over sodium hydroxide pellets. The dansylated amino acids so obtained were redissolved in acetone-0.1 M acetic acid (3:2, v/v) (10).

**Thin Layer Chromatography of Dansylated Amino Acids**—Thin layer chromatography of the dansylated amino acids of histone 1 was done by the method of Morse and Horecker (10) with the exceptions that the thin layer plates were prepared from Silica Gel G-water-methanol (30 g:70 g:1 ml) of thickness 0.25 mm, and they were activated by heating (90° for 1 hour). The developed chromatograph was sprayed with a morpholine-water solution (1:1, v/v) to enhance the fluorescence of the dansylated derivatives (11).

**RESULTS**

**Electrophoretic Assay for Histone Proteolysis**—We had previously assayed for proteolytic activity by observing the decrease in precipitability of the nucleohistone complex in 0.15 M NaCl (5). As this approach is not sensitive to small amounts of proteolysis of histones, we have used a polyacrylamide gel electrophoretic technique which permits us to follow even very low levels of degradation (6). That low levels of degradation can be detected is the result of two factors: (a) the high resolution of the electrophoretic technique and (b) the fact that specific degradation products are reproducibly produced.

The characteristic time-dependent degradation patterns for calf thymus nucleohistone are shown in Fig. 1. The nucleohistone was incubated at 2°. The earliest signs of degradation are the production of several faint bands (arrowed) moving with an electrophoretic mobility slightly greater than the lysine-rich histone (Band 1). Such early degradation is usually detected after 26 hours at 2°. Upon continued incubation (approximately 48 hours), the amount of lysine-rich histone decreases, yielding bands of intermediate mobility and also giving rise to a substantial increase in material in the region of Band 4. Band 2 is also being attacked at this time. The histone pattern obtained after extracting nucleohistone which had been incubated for 6 days at 2° is shown in Fig. 1d. Densitometric analysis shows that although the mobility of some fractions is clearly changed by proteolysis, there has been no net loss of material from the band system. This argues that during an incubation of 144 hours at 2° relatively few bonds have been broken in histone Bands 1, 2, and 3 and that only rather large peptide fragments have been produced.

The effect of heating the nucleohistone at 90° for short time periods was studied. After a brief heat treatment nucleohistone was assayed for proteolytic activity by incubation for 72 hours at 2°. Histone was isolated and assayed as described previously. The results shown in Fig. 2 indicate that exposure to 90° for as little as 2 min serves to inactivate the proteolytic enzyme.

Although proteolysis proceeds more rapidly at 25 or 37° (see below), which are more commonly used incubation temperatures, we have preferred to use 2°, not only because the extent of proteolysis can be controlled with ease at this temperature, but also because it is the temperature at which nucleoproteins (which cannot be frozen) have customarily been stored.

**FIG. 1.** Time dependence of nucleohistone proteolysis. Microdensitometer scans of electrophoretic patterns of histones from calf thymus nucleohistone incubated 26 hours (b), 48 hours (c), 144 hours (d). Scan a is intact histone. The nucleohistone was aged by storing at 2° in 0.01 M Tris-HCl, pH 8.0. Aliquots were withdrawn at the times stated above and immediately extracted with 0.4 N H₂SO₄. Control experiments showed no further degradation after acid treatment.

**The Proteolytic Enzyme Is Bound to Nucleohistone**—The isolation of nucleohistone involves several steps in which the pellet fraction is collected (including the pellet obtained after sedimentation through 2.4 M sucrose). The nucleohistone is then sheared and the supernatant collected. The observation that the proteolytic activity followed the nucleohistone suggested to us that it was firmly bound to it. We have examined this matter further by sedimenting nucleohistone itself into an underlayer of 20% sucrose and testing the resuspended pellet for proteolytic activity. The results shown in Fig. 3 indicate that the proteolytic activity
had stayed with the nucleohistone, the amount of degradation observed in 72 hours being comparable to that observed for a similar time in a sample which had not been sedimented through sucrose.

**Fig. 2.** Heat inactivation of the endogenous proteolytic activity of nucleohistone. Calf thymus nucleohistone was heated at 90° for a, 0 min (control); b, 1 min; c, 2 min; and d, 3 min; and subsequently incubated to test for proteolysis as described under "Materials and Methods." An undegraded control sample, neither heated nor incubated, is shown in Gel e.

**FIG. 3 (left).** Ultracentrifugation does not separate the protease from the nucleohistone. Histones were isolated in the usual manner after treating nucleohistone in the following ways: a, control, not incubated; b, nucleohistone pellet after sedimentation at 100,000 × g for 10 hours and subsequently incubated in 0.01 M Tris, pH 8.0, for 72 hours at 2°; and c, uncentrifuged nucleohistone incubated under identical conditions.

**Fig. 4 (right).** Histone degradation is dependent upon the pH of the nucleohistone solution. Nucleohistone was incubated at 2° for different time periods at the pH described below: a, control, not incubated; b, pH 7.0, 72 hours; c, pH 7.0, 148 hours; d, pH 7.5, 72 hours; e, pH 7.3, 148 hours; and f, pH 8.0, 72 hours.

**pH Dependence of Enzymatic Activity—**The pH dependence of this enzyme is shown in Fig. 4. The enzyme exhibits little activity below pH 7.0. Several solutions of still lower pH were examined, but the proteolytic activity (not shown in Fig. 4) was not detectable in incubations up to 148 hours at 2°.

**FIG. 5.** Degradation patterns of histones 1 and 2. A control, intact nucleohistone was extracted with 5% perchloric acid to give intact lysine-rich histone (b); a sulfuric acid extract of the perchloric acid-insoluble material yielded the intact residual histone fractions (c). Total intact histone is shown in a. A similar John's fractionation upon nucleohistone incubated in 0.01 M Tris-HCl, pH 8.0, for 96 hours yielded degraded lysine-rich histone (e), and degraded residual histone (f) consisting primarily of degraded histone 2 with the other fractions intact. Unfractionated, degraded histone is shown in d.
FIG. 6. Production of new amino-terminal amino acids during degradation. The dansylated amino-terminal amino acids of calf thymus lysine-rich histone were chromatographed on Silica Gel G, benzene-pyridine-acetic acid (80:20:2), following incubation (2⁰) of the nucleohistone for either 5 or 9 days. a, Dansylated derivative of ammonia; b, lysine-rich histone extracted from incubated nucleohistone (5 days); c, undegraded control lysine-rich histone; d, lysine-rich histone extracted from incubated nucleohistone (9 days); e, control undegraded lysine-rich histone; and f, dansylated derivative of ammonia.

Substrate Specificity of Proteolytic Enzyme—In its action upon the intact nucleohistone complex, the enzyme preferentially attacks histone Groups 1 and 2 (F1 and F3 in the nomenclature of Johns (8)) while the other three major groups of histone emerge relatively unscathed, as seen in comparing intact whole histone (Fig. 5a) with degraded histone (Fig. 5d). It is clear from Fig. 5d that some of the degradation products are moving with mobilities such that they overlap pre-existing intact fractions (arrowed, Fig. 5d). Further, from the data of Fig. 5d it can be seen that it is not possible in a single electrophoretic system to differentiate between the degradation products of the two histone groups undergoing proteolysis. It is, however, possible to separate these histone groups because of the preferential solubility of the lysine-rich histone in 5% perchloric acid. Apparently a substantial portion of the degradation products of this histone fraction are coisolated by this procedure and are shown in Fig. 5e. That the additional degraded material is derived from a parent lysine-rich histone is based on observations that the highly characteristic amino acid analysis of the perchloric acid-soluble histone is essentially independent of the extent of degradation.

FIG. 7. The activity of the proteolytic enzyme on isolated histones. Histones were dissociated (2 x NaCl, pH 5.0) from nucleohistone prepared from sucrose-purified calf thymus nuclei. DNA was sedimented (100,000 x g for 18 hours) from the solubilized histones. NaCl was dialyzed from the histones which were then incubated (2⁰) in 0.01 M Tris-HCl, pH 7.0, for 8, 24 hours, and 96 hours. A control sample (a) is also included.

We wanted to test if the new electrophoretic bands we observe following the incubation of calf thymus nucleohistone were a result of true proteolysis, rather than the effect of a change in the charge distribution on the histone molecules as a result, for example, of dephosphorylation or deacetylation. Histone 1 was, therefore, isolated from nucleohistone which had been incubated several days at 2⁰. Both intact and degraded samples of histone 1 were dansylated by the method of Morse and Horecker (10). The interpretation of the results is facilitated by the observation that intact calf lysine-rich histone possesses no free amino-terminal groups. This result is in agreement with that previously
reported (12). On the other hand, degraded lysine-rich histone shows several new amino-terminal groups following a standard incubation of calf thymus nucleohistone (Fig. 6). The new amino-terminal amino acids were identified by eochromatography as alanine, lysine, and either serine or threonine. The time-dependent increase in the intensity, and the number of the dansylated amino acids argues forcibly that the faster moving electrophoretic bands are the result of the formation of new peptides upon incubation of the nucleohistone.

After removal of the lysine-rich histone, it is possible to follow the degradation of the residual histone which is primarily that caused by the proteolysis of the arginine-rich histone Band 2. This is best seen by comparing Fig. 5c showing the intact residual histone with Fig. 5f showing the same fractions after moderate proteolysis. Histones of Band 5 are not degraded by the proteolytic enzyme even when histone Fractions 1 and 2 have been fully attacked. It is not possible to draw conclusions on the susceptibility of Band 4 to proteolysis as this band is masked by degradation products at an early stage.

It is notable that relatively few degradation bands are produced from the destruction of the parent protein and that the mobility of the new bands is highly characteristic and reproducible, arguing strongly for a very specific degradation of those histones which are attacked.

We wondered if the observed substrate specificity lay in the unique chemistry of a given histone molecule or whether it lay in specific conformations imposed upon histones by the interaction with DNA, perhaps exposing some molecules and protecting others.

The histones and the proteolytic enzyme were separated from DNA by sedimenting through 2 M NaCl at pH 5 (at which pH the histones do not aggregate, and the enzyme is inactivated, see above). NaCl was removed by dialysis against water containing enough acid to maintain a pH of 5. The pH was then raised and the system incubated for proteolysis under the standard conditions. The result of such an incubation is shown in Fig. 7 in which it is seen that all the histone molecules are attacked at about the same rate except the lysine-rich histone, arguing strongly that in native nucleohistone, some histone fractions are conformationally protected against proteolytic attack, whereas other fractions are more exposed. The specificity of the enzyme for intact nucleohistone is not affected by exposure to pH 5.

**Inhibition of Proteolytic Activity: Effect of Ionic Strength**—The proteolytic activity of calf thymus nucleohistone can be totally inhibited over a period of several days at 2°C, either in the presence of sodium bisulfite or simply by storing at very low ionic strengths (5 x 10^{-4}). The data of Fig. 8 show the effect of decreasing ionic strength upon the proteolytic activity. The inhibitory effect of low ionic strength is not caused by dissociation of the enzyme from the nucleohistone complex, since sedimentation from 5 x 10^{-4} ionic strength followed by incubation of the pellet in the higher ionic strength (10^{-3}) leads to proteolysis as before.

**Reversibility of Inhibition**—The preparation of calf thymus nucleohistone involves washing frequently with solutions containing 0.05 M sodium bisulfite, an inhibitor of proteolysis in this system. In order to shear the nucleoprotein effectively, the ionic strength is then lowered to 10^{-4} prior to shearing. Thus, the protease has been treated with two of the conditions for full inhibition, and yet upon raising the ionic strength in the absence of bisulfite the proteolytic activity returns as is evidenced by Figs. 1 through 7 in which the nucleohistone used for these experiments was isolated in the presence of bisulfite, and was subsequently sheared at low ionic strength as part of the standard preparative procedure.

**Enzyme Activity Depends upon Origin of Nucleohistone**—Nucleohistone was isolated from calf thymus, intestine, and lung as well as from a mouse ascites tumor line. It is clear from the data of Fig. 9 that the proteolytic activity follows the order: intestine > thymus = tumor > lung.

**Enzyme Activity at Higher Incubation Temperatures**—The bulk
of the experiments described so far were performed at 2° for the reasons given above. However, we have studied the action of the enzyme on nucleohistone at elevated temperatures using otherwise identical conditions. The data of Fig. 10 show that the proteolysis proceeds, as expected, at a much greater rate at 37°, with degradation after 2 hours comparable to that obtained after several days of incubation at 2°. However, the specificity of the enzyme at the two temperatures is strictly comparable as is evidenced by the identity of densitometer patterns following proteolysis at 2° or 37°. In Fig. 10 we see a familiar proteolytic pattern obtained after 18 hours of incubation at 25°.

**DISCUSSION**

We have described the electrophoretic band patterns of peptides obtained from the endogenous proteolysis of incubated whole nucleohistone. One such band has been identified as a very early degradation product, possibly a product of the first scission of a peptide bond in the lysine-rich histone. Measurement of this band by densitometry permits us to detect the initiation of degradation when as little as 1% of the first bond scission has occurred. This is critically dependent upon the assumption that what we have described as the electrophoretic pattern of intact histones is not in fact a product of a limited proteolysis during isolation. We expect the assumption is valid for the following reasons. (a) The histone isolation is performed under conditions which totally inhibit the type of degradation discussed above, (b) very rapid isolations (under 30 min) give identical histone patterns to those described above, and (c) tissues with very low levels of nucleoprotein proteolytic enzyme (e.g. calf lung) closely resemble the histone pattern found for calf thymus.

Initially we found active proteolysis only in nucleohistone isolated from tissues involved in rapid cell division such as calf thymus and intestinal mucosal cells, whereas it was virtually absent in nonreplicating systems such as lung and brain nucleohistone. However, it is clear that a high level of proteolytic activity is not an essential adjunct of rapidly dividing cells, because a rapidly replicating mouse ascites tumor line was found to be devoid of proteolytic activity in its isolated nucleohistone. The most likely explanation for the proteolytic activity of nucleohistone from thymus and intestinal cells lies in the physiology of the cells in question. In both cases there is extensive cell turnover, involving cell destruction and the reutilization of the autolyzed material. An average cell population from these tissues probably always contains a substantial number of cells undergoing autolysis (13), and this may serve to contaminate the nucleohistone following cell rupture during normal isolation. The idea of the role of the proteolytic enzyme associated with nucleohistone as being primarily autolytic and concerned with cell destruction reduces interest in a possible involvement of such a protease in modifying histones during the useful lifetime of the cell.

This is not unexpected since with the possible exception of one histone fraction (the most resistant to proteolysis in intact nucleohistone) there appears to be essentially no biosynthesis of histone in nondividing tissues (14, 15). Furthermore, since the amount of histone relative to DNA in a given nondividing tissue is temporally constant, there would seem little advantage in the possession of a histone protease. However, the great specificity in the way the enzyme degrades histone is remarkable, and it is also of interest that Furlan, Jericijo, and Suhar (16) have shown that a similar enzyme (probably the same enzyme) was much more active against nucleohistone than against albumin or hemoglobin.

The level of proteolytic activity is such that the interpretation of experiments with thymus nucleohistone (such as RNA synthesis in vitro) involving elevated temperatures (37°) for extended time (30 min) must be viewed with suspicion. Furthermore,

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2 Also rat intestinal cells recovering from radiation damage show much reduced nucleohistone proteolysis although the rate of cell division is at a preirradiation level. W. Hansen and J. Cooper, Radiation Research, University of Iowa, personal communication.
unless stored at a very low ionic strength, there is substantial
degradation of nucleohistone even at 37° over an 18-hour period.
The environment of the histone undergoing proteolysis clearly
has a profound effect upon the specificity of the protease. Thus,
the initial effect of the protease upon histone in the intact nucleo-
protein complex is seen primarily in its rapid attack upon the
lysine-rich histone. However, if the entire histone complement
is removed from its association with DNA, the protease attacks
typical of the five histone groups leaving the lysine-rich histone
fully intact even at a stage at which the other histone fractions
are almost totally degraded.

That three of the five histone fractions are completely or
partially resistant to the protease when they are part of the
nucleohistone complex is probably not because of the higher
degree of α helix in these fractions (17). This is deduced from
the observation that the nuclear protease is active against his-
tones even at elevated ionic strengths (3) under which condi-
tions it has been reported that these histones have a high degree
of α helical structure (18). Thus, the enhanced resistance of
these histone fractions to the proteolytic enzyme probably lies
in a protective effect of their interaction with DNA, indicating
that these fractions are most likely in a much more intimate
association with DNA than are the lysine-rich histone or the
sulfhydryl-containing histone. This is in agreement with many
previous suggestions that the lysine-rich histone is relatively
exposed and serves to cross-link nucleohistone molecules (19, 20).
However, it is curious that the lysine-rich histone when dissoci-
ated from DNA and primarily in the random coil conformation
(21), is exceedingly resistant to attack by the protease. A
small fraction of the free lysine-rich histone in solution has,
however, been reported to exist in the β conformation (18), and
this may exert a protective effect on enzyme-susceptible regions
of the molecule.

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