A nuclease from *Neurospora crassa* specific for d(A-T) rich regions in double stranded DNA*

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Received 22 January 1974

ABSTRACT

A nuclease from *N. crassa* has been prepared to the hydroxylapatite stage of purification described by Rabin and Fraser (1). It degrades single stranded DNA in an essentially exonucleolytic process. It does not give any appreciable acid soluble material with double stranded DNA as substrate. This shows its high degree of specificity towards single stranded DNA.

An extra activity which makes double strand breaks in the AT rich regions of DNA is described. A limited number of breaks are observed with calf or mouse DNAs, as judged by the decrease of sedimentation velocity in alkaline or neutral sucrose gradients. The number of breaks decreases with increasing the ionic strength. λ DNA is broken in half at the AT rich middle region of the molecule. The circular replicative form of fd DNA is converted to linear pieces of a rather homogeneous length. It is concluded from these results that it is an endonuclease activity specific for d(A-T) rich regions in double stranded DNA.

INTRODUCTION

Many nucleases have been isolated from different sources which have a strong preferential activity towards single stranded DNA. Among them are nuclease S_1 extracted from *Aspergillus oryzae* (2,3), Mung bean nuclease I (4) and the nucleases from *N. crassa* (5,6,7,8).

Some of them have been described as having an activity which cleaves double stranded DNA at specific sites.

In the case of Mung bean nuclease I, Johnson and Laskowski (4) have shown that λ DNA was cleaved in the A-T rich region at the center of the molecule.

Beard et al (9) have found that nuclease S_1 converts supercoiled SV 40 DNA to unit length linear molecules-cleaving the DNA molecule at only a limited number of sites, perhaps as few as two.

* Contribution No. 102 from the Center, CNRS.

Wingert and von Hippel (10) - extending previous work by Dirksen and Dekker (11) and von Hippel and Felsenfeld (12) - showed that a non specific DNase, the micrococcal nuclease from Staphylococcus aureus, attacks native DNA initially in dA-dT rich regions.

N. crassa contains two single stranded DNA specific nucleases, one which is an endonuclease (5, 6), and the other, which has been more extensively characterized and is essentially an exonuclease with a slight endonuclease activity (7, 8).

It has been reported as introducing only one nick in the replicative supercoiled form of the circular S 13 and polyoma DNAs (7).

Such an enzyme could be useful for the study of the rapidly reassociating DNA fractions of higher organisms - which contain unmatched sequences (13) covalently linked to the reassociated duplex regions. Its high activity towards single stranded DNA makes it attractive for that purpose. The absence of endonucleolytic breakage of double stranded DNA should also be a great advantage.

The N. crassa exonuclease was therefore prepared. Its specificity towards single stranded DNA was good but it introduced a limited number of breaks in double stranded DNA. This activity has been further characterized.

MATERIAL AND METHODS

Enzyme preparation

The enzyme was prepared according to Rabin and Fraser (1) from N. crassa strain Lindengren (+) F.G.S.C. n° 853 (Fungal Genetics Stock Center), m.t.A., supplied by Dr. R. Peduzzi, University of Geneva. The enzyme was eluted from the hydroxylapatite column by a stepwise process, with phosphate buffers of increasing strength. The 0.2 M phosphate fraction was recovered, rechromatographed on hydroxylapatite and the 0.15 M phosphate fraction was stored, after dialysis, in 0.02 M phosphate pH 6.5 at -20°C or 4°C. No loss of activity was noticed for more than one year.

Substrates

³²P mouse DNA was prepared from L cells. Cold calf DNA was prepared from thymus, λ DNA was a gift of Dr. K. Murray. RF fd DNA was

labelled with ^3H thymidine.

Incubation mixture

Unless otherwise mentioned, the substrates were incubated in the following medium : Tris-HCl 0.1M, pH 7.5 ; MgCl_2 0.01M at 37°C for varying times as specified in the legends of the figures.

Activity measurements

The acid soluble radioactivity of the incubation mixture to which a cold carrier DNA was added, was measured, after precipitation with 1M HClO_4 and centrifugation. The pellet and the supernatant plus pellet washes were counted for ^{32}P by the Cerenkov method. Acid soluble counts were expressed as the percentage of the total substrate radioactivity.

DNA size measurements

The size of the ^{32}P labelled DNA molecules were estimated in either a neutral isocinetuc sucrose gradient (6-20 % ; 0.01M Tris pH 7.4 ; 0.01 M EDTA ; 0.04 % SLS) or in an alkaline sucrose gradient (5-20 % ; 0.9M NaCl, 0.1M NaOH).

In the former case, 28 and 18 S ^3H rRNAs and in the latter a ^3H single strand DNA of 2500 bases were used as markers. Molecular weights were calculated from Studier equations (14).

The gradients were fractionated from the bottom by collecting 0.1 or 0.2 ml aliquots on glass fiber filters, which were dried and counted in a scintillation mixture.

Analytical CsCl centrifugation

Samples were run in neutral CsCl (Merck grade) with *M. lysodeikticus* DNA ($\rho = 1.731 \text{ g/cm}^3$) as marker, at 44 000 rpm, 25°C , in an MSE analytical ultracentrifuge. Photographs were taken at equilibrium, from which microdensitometer tracings were made.

Agarose gel electrophoresis

Electrophoresis was performed according to Aaij and Borst (15) in 0.6 % gels during 3 h at 2.5 mA/gel. The gels were then frozen and sliced in 1 mm segments which were dried and counted in a scintillation mixture.

RESULTS

1) Evidence of an action on high M. W. Double Stranded DNA

In order to characterise the specificity towards SS DNA, the action of the enzyme preparation towards DS DNA was investigated. Compared to SS DNA which was completely acid soluble, the DS DNA showed no detectable acid soluble counts, in the acid precipitation test (fig 1). However, double stranded DNA was broken down to smaller pieces, as shown by the S-measurements of the DNA before and after the action of the enzyme preparation.

Figure 1 : Time course of digestion of denatured and native DNA

^{32}P mouse L-cell DNA was fractionated by stepwise salt elution from hydroxylapatite crystals at 70°C to give native DNA substrate (o—o). For single stranded (●—●) DNA substrate, such preparations were heat denatured, reassociated to a C_0t (product of initial DNA concentration and time) of 1.0 sec. mole. l^{-1} . (0.12 M Na^+ , 60°C) and the reassociated fraction removed by a second hydroxylapatite fractionation. The substrates were then incubated with the enzyme as described in Materials and Methods.

The results also indicated that the DNA fragments reached a minimum size beyond which no further breakdown occurred.

2) Evidence of a size limit : ^{32}P mouse DNA was treated by the enzyme for varying times. The reaction was stopped by EDTA and the products analyzed on alkaline sucrose gradients (fig 2). The results show that a size

Figure 2 : Action of the region specific nuclease on double stranded DNA. After incubation for varying times, the native substrates were layered on alkaline sucrose gradients and spun for 6 h at 48,000 rpm, 8°C in the 3 x 3 ml rotor of an MSE 50 centrifuge. 0.1 ml fractions were collected, ●—● treated native ^{32}P DNA, ○—○ ^3H marker DNA. (a) 20 min, (b) 40 min, (c) 60 min, (d) 2 h, (e) 20 h, (f) 48 h.

limit is reached beyond which no further decrease in molecular weight occurs.

3) Action on sonicated DNA : With sonicated DNA as substrate, no change in size was observed (fig 3) after action of the enzyme. This suggests either a size effect or, more likely, the destruction by sonication of the available sites on the DNA molecules. However, the method is not sensitive enough to detect a small number of breaks due to enzymatic action.

Figure 3 : Action of the region specific nuclease on sonicated DNA. ^{32}P mouse L-cell native DNA was sonicated and fractionated on hydroxylapatite as in fig.1. The substrates, treated for varying times were spun on alkaline sucrose gradients and fractionated as in fig. 2. ●—● sonicated treated native ^{32}P DNA ; ○—○ ^3H marker DNA ; (a) zero time ; (b) 30 min ; (c) 30 h.

4) Effect of the ionic strength : Fig 4 suggests that increasing ionic strength diminishes the number of available sites on the DNA ; while decreasing Mg^{++} content did not affect the availability (experiments not shown here).

In order to check that this change is due to availability of sites and not enzyme inactivation, the experiments shown in fig 5 were done. To a DNA-enzyme mixture which had been incubated for 20 hrs, either more enzyme or more DNA was added. In the first case, no further decrease in DNA size occurred. In the second, the added substrate was broken down to fragments of the same size.

5) Action of the enzyme on λ DNA : The sensitivity of hydrolysis of double stranded DNA to ionic strength strongly suggests that the enzyme recognizes and hydrolyses both the strands in the AT rich regions of DNA.

Figure 4 : Effect of added salt on the size of the product. ^{32}P native L-cell mouse DNA was treated in the standard conditions. The reaction mixtures were made (a) 0.1 M, (b) 0.2 M and (c) 0.3 M in NaCl and incubated for 20 h. Then spun on alkaline sucrose gradients. ●—● treated native ^{32}P DNA ; ○—○ ^3H marker DNA.

Figure 5 : Effect of added salt on the enzyme activity : ^{32}P DNA samples were treated in 0.3 M NaCl for 20 h. (a) without added enzyme, (b) with enzyme. In two samples, after 17 h more enzyme (c) or more substrate (d) were added for 3 h at 37°C . All DNA (●—●) samples were then layered on neutral sucrose gradients and spun for 2 h at 45 000 rpm, 20°C in the SW 50 rotor of a Spinco centrifuge. 0.2 ml fractions were collected. ^3H r-RNAs were added as markers ○—○.

The action of the enzyme preparation on λ DNA (fig 6) which contains an AT rich region in the center of the molecule (16) confirms this suggestion. The two halves obtained are comparable to those of Hershey and Burgi (17) by shearing DNA, and to those of Johnson and Laskowski (4) with Mung bean nuclease I.

Figure 6 : Action of the region specific nuclease on λ DNA . DNA was incubated with the enzyme for varying times. The incubation mixture was then brought to 65°C for 1 min and rapidly cooled. Cold CsCl was added and the sample spun with a DNA marker at 5°C. This was done to prevent possible reannealing, by their cohesive ends, between the two halves obtained after digestion. (a) no enzyme added ; (b) partial digestion ; (c) complete digestion.

6) Action on f_d DNA : The circular replicative form of f_d DNA was treated with the enzyme until a limit was observed by measuring the DNA size in a 0.6 % agarose gel. As it can be seen in fig 7, the size of the final fragments are rather homogeneous. This shows that there are few sites in f_d DNA and they are rather evenly distributed along the 5 500 base pairs of the molecule. These sites have not been characterized further.

DISCUSSION

All the results described here support the idea, already postulated for some other nucleases, that this enzyme preparation is able to "recognize" specifically the AT rich regions present in a double stranded DNA, and to

Figure 7 : Action of the region specific nuclease on the replicative form of f_d DNA. RF II f_d was incubated in standard conditions for varying times and then electrophorised in agarose gels. Two gel patterns are superposed on this figure : o—o untreated DNA, ●—● 20 h treated DNA.

split them with a double break.

Such enzymes cannot be considered as restriction enzymes, although they have some similar features (breakage of double stranded DNA at a limited number of specific sites) because the number of sites is dependent on the physical environment (ionic strength) of the substrates. Another interesting feature is that the sites in the substrate seem to disappear, at least partly, on sonication. These AT rich regions thus appear more fragile - to mechanical shearing - than the rest of the molecule.

Kato et al (18) have reported recently that the N. crassa endonuclease (14, 15) specific for single stranded DNA, was able to convert the super helical RF of ϕ X 174 to the non super helical form. The results were taken as evidence for the presence of at least transient unpaired or weakly hydrogen-bonded region sensitive to the single-strand specific endonuclease. Similar results have been obtained by Godson (19) with nuclease S₁. In both cases, only single nicks (on one strand) are introduced in the DNA. So these enzymes are not comparable with the present enzyme preparation which introduces double breaks in double stranded DNA.

The enzyme preparation used in this work has been described by Rabin et al (7) as having no nucleolytic activity on double stranded DNA. These authors described the introduction of only one nick on the Replicative Supercoiled Form of the circular ϕ 13 and Polyoma DNAs. They characterized this enzyme as an exonuclease specific for single stranded nucleic acids - which always contained a small amount of endonuclease also specific for single stranded DNA (7).

In this study the HAP fraction was found to have the same properties, plus this extra activity on double stranded DNA. This extra activity is very interesting, because of its specificity towards AT rich regions of double stranded DNA.

At the moment, it is not certain whether it is due to the endonuclease specific for single stranded DNA, acting on the weakly hydrogen bonded regions of double stranded DNA which are transiently unpaired - or to a contaminating nuclease which was not present in the preparation of Rabin et al. Experiments designed to clarify this point are being carried out.

Acknowledgements

This work was supported by a grant from the exchange agreement between the Royal Society (Great Britain) and the CNRS (France).

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Non-standard abbreviations used :

HAP, Hydroxylapatite ; SLS, Sodium lauryl sulphate ; RF, Replicative form ; DS, Double stranded ; SS, Single stranded.

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