A YEAST MITOCHONDRIAL DEOXYRIBONUCLEASE STIMULATED BY ETHIDIUM BROMIDE

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SUMMARY

Several DNase activities, with different substrate and pH requirements, have been identified in yeast mitochondria. One of them is active on double stranded DNA at neutral pH and stimulated by Ethidium Bromide and other DNA intercalating drugs. This activity could be responsible for the yeast mitochondrial DNA degradation induced during mutagenesis by Ethidium Bromide.

Ethidium Bromide (EthBr) intercalates between the base pairs of DNA and its general and physicochemical properties have been reviewed by LE PECQ (1). EthBr displays in yeast several peculiar biological properties : among them, the induction of cytoplasmic respiratory "petite" mutations at a close to 100 % efficiency is remarkable since it occurs in absence of any cell growth (2). This effect, which has not been observed for other intercalating drugs, is concomitant with a shortening of parental mitochondrial DNA (mit-DNA) (3). The extent of the degradation depends both on the concentration of EthBr and the duration of the exposure to the dye. The induced vegetative "petites" contain mit-DNA which displays in most cases a lighter buoyant density (3-4). In some cases, this mit-DNA is also endowed with a reduced size (3-5). After an extensive treatment with EthBr, the "petites" can be completely devoid of extractable mit-DNA (6). Some of these modifications are maintained after many generations of growth in the absence of the dye (5). It should also be stressed that different mit genes can be deleted or kept after mutagenesis in various "petites" in a stable hereditary way (7).

The induction of vegetative "petites" is probably due to a direct action of EthBr on mit-DNA. However, the primary mutagenic event caused by EthBr is unknown since this dye interferes also with yeast mitochondrial protein synthesis (8), with yeast RNA synthesis (9) and binds cooperatively to mitochondrial membranes (10-11). Whatever this primary event is, it triggers directly or indirectly a nucleolytic process which would explain some of the peculiar effects of EthBr on yeast mit-DNA. Therefore, the comparative study of the yeast mitochondrial deoxyribonucleases (DNases) in presence or absence of EthBr could bring informations on the mechanism of the induction

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of mit-DNA degradation by EthBr.

The results described in this paper indicate that a least one yeast mit-DNase is more active in presence than in absence of EthBr.

MATERIALS AND METHODS

Saccharomyces cerevisiae haploid strain IL 8- $C\rho^+$ his trp bearing two mit marker genes C^R_{321} and E^R_{514} and belonging to the ω^+ mitochondrial sexual type (12) is used throughout this work. It was obtained from Dr. P. P. SLONIMSKI (Gif/Yvette).

The cells are aerobically grown at 28°C in yeast extract 1 %, peptone 1 %, Galactose 2 %. The inoculum is a stationary phase preculture grown in the same medium. One generation before the stationary phase, the cells are harvested by centrifugation at 4000 xg. Mitochondria are isolated from protoplasts prepared according to GUERINEAU et al. (13). The mitochondrial pellet is resuspended in 0.6 M sorbitol, 0.001 M EDTA, 0.05 M Tris HCl pH 7.4. This suspension can be further purified by equilibrium sedimentation in a sucrose gradient (30-70 % w/w, 60000 xg for 15 hours at 4°C). The mitochondrial suspension or the gradient fractions are finally lysed by a non ionic detergent : 4 volumes of the mitochondrial material are added to 1 volume of 0.075 M Tris HCl pH 7.4, 0.075 M NH_4Cl, 0.015 Mg Acetate and 10 % w/v of Triton X-100. After one hour at 0°C under periodical shaking, the mixture is centrifuged at 30000 xg for 30 minutes. The pellet is resuspended in the lysis buffer in such a volume as to be 25 fold concentrated. This preparation is designated as the submitochondrial pellet.

^3H or ^{14}C E. Coli DNA are used (14). Their specific activities are respectively 14 x 10^{12} cpm/Mole and 11 x 10^{12} cpm/Mole. Heat denatured E. Coli DNA is obtained by heating DNA in 0.001 M NaCl for 10 minutes at 100°C. After heating, the solution is cooled in an ice bath and immediatly used for the assay. In these conditions, the amount of renatured DNA during the assay is inferior to 1 % of total DNA (15). Proteins concentrations are determined according to the method of LOWRY et al. (16).

DNAses activities are measured by the conversion of labeled DNA to acid-soluble products by 5 µl of the properly diluted submitochondrial pellet at 28°C, either at pH 7.2 (50 mM Tris HCl, 10 mM Mg Cl_2) or at pH 5.2 (50 mM Acetate buffer, 10 mM $MgCl_2$) in presence of bovine serum albumine (BSA) (0.1mg/ml) and EthBr at various concentrations. The final reactional volume is of 215 µl. Samples (40 µl) are removed at different times and mixed with 360 µl of BSA (2.25 mg/ml) at 0°C. Then 400 µl of 1N perchloric acid are added. The mixture is centrifuged for 10 minutes at 8000 xg. It has been verified that the presence of EthBr does not modify the precipitability of DNA and therefore the amount of acid-soluble products. 500 µl of the supernatant fluid are mixed with 10 ml of Bray's scintillation solution and counted in a Packard

Tri-Carb counter. A unit of activity is defined as the amount of enzyme producing 1 nMole of acid-soluble products in 30 minutes.

RESULTS

After Triton X-100 treatment of the mitochondrial suspension, the DNAse activity on native DNA at pH 7.2 is increased by 20-30 fold. After centrifugation of the suspension, part of this activity is recovered in the "submitochondrial pellet" and is apparently bound to membranes. Its proportion depends on several parameters (stage of yeast growth, culture medium, ratio of detergent concentration over mitochondrial protein concentration). It usually lies about 50 %.

The deoxyribonuclease activities have been measured on native and denatured DNA, at pH 5.2 and 7.2 (Table 1).

(a) The activity on native DNA at acid pH is about twice as that at neutral pH.

(b) At neutral pH, the activity on denatured DNA is about 50 fold higher than that on native DNA. This difference might be due to the existence of a yeast DNAse specific for denatured DNA previously described by PINON (17), although this author does not provide any data on its intracellular location.

The effect of EthBr on the rate of DNA degradation has been studied (Fig. 1). It markedly differs, depending on the environmental conditions used for assaying the enzymatic activities. At pH 5.2, the degradation of both native and denatured DNA are inhibited. At pH 7.2, only the activity on denatured DNA is inhibited. In those cases, minimal residual activities which are observed when EthBr concentrations are raised, are respectively 40 %, 0 % and 10 %, of that measured with no EthBr. In contrast, the rate of degradation of native DNA at pH 7.2 is about twice higher in presence of the dye. This activating effect of EthBr, which is the main subject of this report, deeply contrasts with its inhibitory effect on yeast nucleases previously described, on several nucleases from different organisms (18) and on DNA and RNA polymerases (19-20-21-22-23).

Figure 2 shows that the initial rate of liberation of acid-soluble products from native DNA at pH 7.2 is directly proportional to the amount of mitochondrial proteins. This rate is initially constant. However it subsequently increases (presence of the enzyme active on denatured DNA liberated during the reaction). Therefore, we consider only the initial (less than 5 minutes) kinetics, during which the amount of denatured DNA is still very low.

The enzyme activation depends on the concentration of EthBr in the reaction mixture (Fig. 3). It is maximum at a concentration of about 2.3×10^{-6} M. For higher EthBr concentrations, the activation is decreasing but even at very high EthBr concentrations (2.3×10^{-4} M), there is not detectable inhibition. A pretreatment of the submitochondrial pellet with EthBr does not modify its activity on native DNA at pH 7.2.

Table-I

0.4 µg of ^{14}C E. Coli DNA and 5 µl of the submitochondrial pellet (17 mg/ml) kept for 50 days at -20°C are added to the reaction mixture. The experimental conditions are described in material and methods.

Table-1 DNase ACTIVITY IN SUBMITOCHONDRIAL PELLET ON NATIVE AND DENATURED DNA AT pH 7.2 and 5.2

A. Activities (U/Mg of proteins)	
Native DNA	
pH 7.2	100
pH 5.2	170
Denatured DNA	
pH 7.2	5600
pH 5.2	260
B. Ratio of activities	
At pH 5.2 on native DNA / At pH 7.2	1.7
On denatured DNA at pH 7.2 / On native DNA	56

This enzyme is Mg++ dependant and is totally inhibited by EDTA in presence or absence of EthBr.

We have also studied the effect of other drugs displaying DNA binding abilities on this enzymatic activity : spermidine, berenil, and hydroxystilbamidine which do not intercalate between the DNA base pairs and quinacrine, actinomycine and methoxyellipticine which do (24-25-26-27). As shown on Fig. 4, the DNAse activity

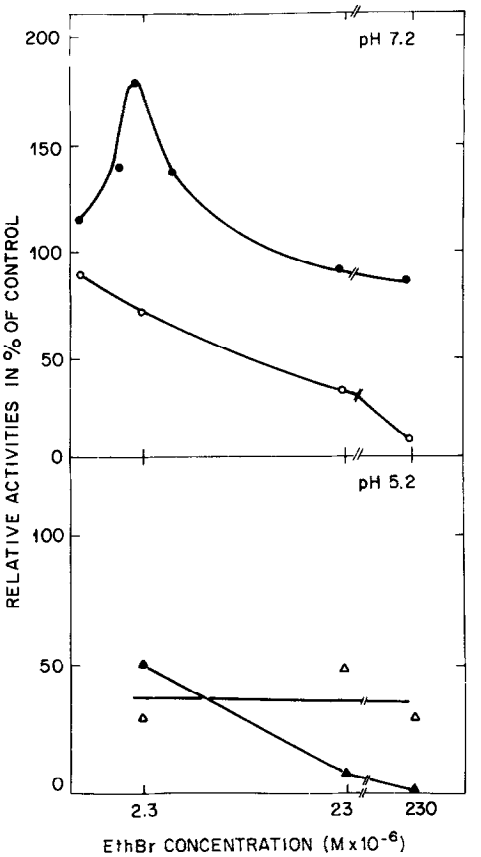

Fig. 1.

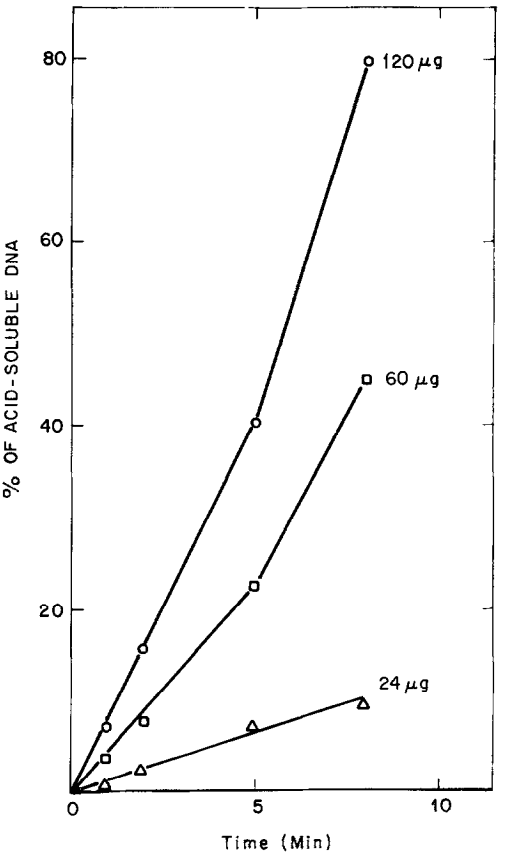

Fig. 2.

Figure-I

Rate of liberation of acid-soluble products from native or denatured DNA by mitochondrial DNase activities in presence of different concentrations of EthBr.

0.4 µg of ^{3}H E. Coli DNA, and 5 µl of the submitochondrial pellet used in Table 1 are added to the reaction mixture ; the submitochondrial pellet is used undiluted in presence of native DNA, and diluted 1/10 in presence of denatured DNA (pH 7.2).

pH 7.2 : ●——● native DNA o——o denatured DNA
pH 5.2 : ▲——▲ native DNA △——△ denatured DNA

Figure-II

Rate of liberation of acid-soluble products from native DNA in presence of various amounts of yeast mitochondrial pellet at pH 7.2

- 0.4 µg of ^{3}H E. Coli DNA, and 2,5 or 10 µl of the submitochondrial pellet (12 mg/ml) are respectively added to the reaction mixture at time 0. Experimental conditions and DNase activities are described in material and methods.

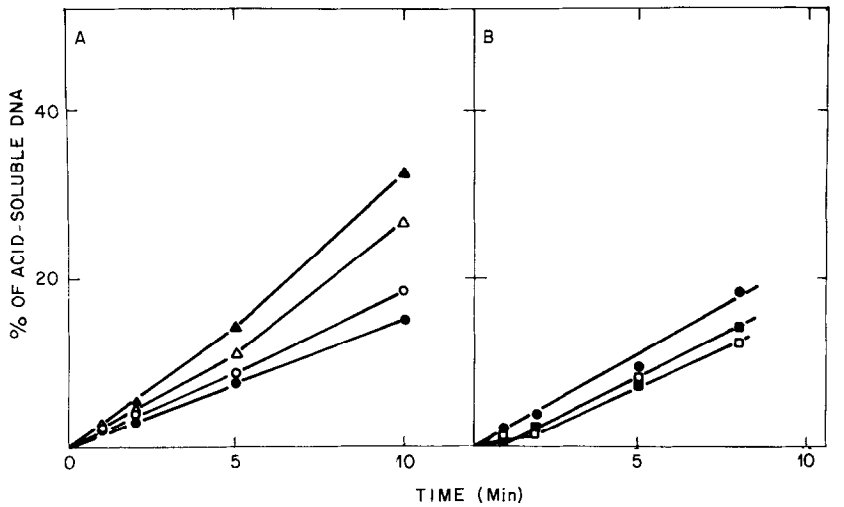

Figure-III

Effect of EthBr at different concentrations on the liberation of acid-soluble products from native DNA by the mit-DNase (pH 7.2)

- 0.4 µg of ^{3}H E. Coli DNA and 5 µl of the submitochondrial pellet (12 mg/ml) are added to the reaction mixture. Experimental conditions are described in material and methods.

(A) ●——● control (0 EthBr), ○——○ EthBr 1.1 x 10^{-6}M,
△——△ EthBr 1.9 x 10^{-6}M, ▲——▲ EthBr 2.3 x 10^{-6}M.

(B) ●——● control (0 EthBr), ■——■ EthBr 23 x 10^{-6}M,
□——□ EthBr 230 x 10^{-6}M.

is not affected or slightly inhibited by the non intercalating drugs, whereas it is activated by the three intercalating products. The activations depends on the nature and concentration of the drugs. In our conditions, the ellipticine derivative is the most efficient (400 % activation) ; however, less EthBr molecules are required to get the maximum activation.

DISCUSSION

Our data indicate that there are several DNases in yeast mitochondria : the major argument is provided by the differential effect of EthBr on the mitochondrial DNase activities. In presence of the dye, the enzyme activity on native DNA is increased at pH 7.2, completely inhibited at pH 5.2. On denatured DNA, the enzymes are differently inhibited depending on the pH. Morever, when the DNase activity is measured in the submitochondrial pellet at different pH, between pH 4 and 8.5, two peaks of activity can be detected (unpublished results).

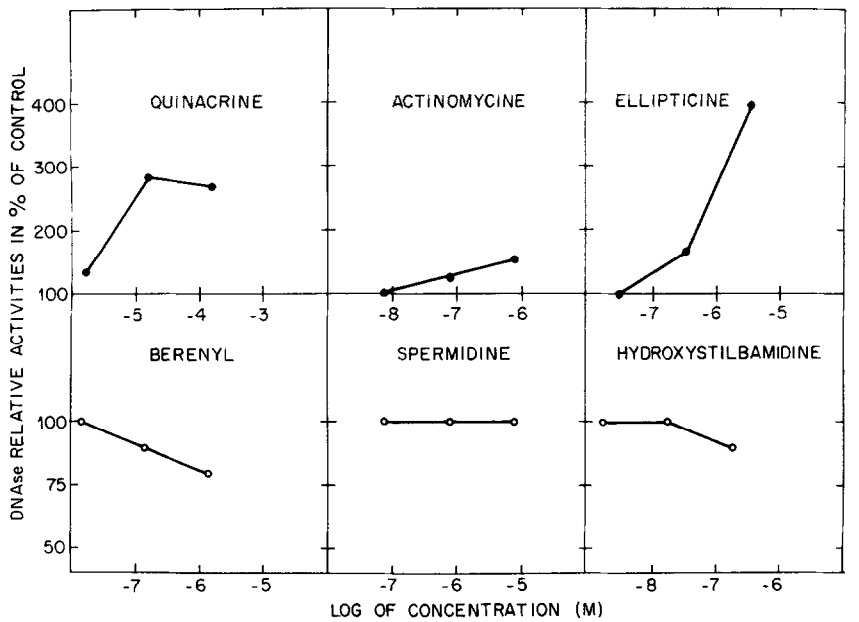

Figure-IV

Effect of non intercalating and intercalating substances on the rate of liberation of acid-soluble products from native DNA by the mit-DNase (pH 7.2).

- 0.4 µg of ^{14}C E. Coli DNA, and 5 µl of the submitochondrial pellet described in Table 1 are added to the reaction mixture. 2 µl of the different drugs are added after proper dilution in distilled water. The experimental conditions are described in material and methods.

The data suggest that among these yeast mitochondrial DNases, at least one of them, working at a neutral pH, is able to recognize sites where intercalating drugs bind to duplex DNA. When no intercalating drug is present, our extracts contain one residual activity which could be related to :

(a) The existence of a single enzyme displaying different affinities for the DNA in presence or in absence of intercalating drugs.

(b) The existence of two distinct enzymes ; one active on native DNA and unable to recognize the intercalated sites, the other one specifically attacking duplex DNA in presence of the intercalating agents.

(c) The creation of denatured zones for the enzyme acting on denatured DNA after the intercalation of the dye along the duplex. This hypothesis is rather unlikely since it does not hold for the Neurospora crassa endonuclease (28), another enzyme specifically active on denatured DNA. We have verified that this endonuclease does not attack duplex DNA in presence of EthBr (unpublished data). Purification of the enzymes will allow us to choose between these hypotheses.

The activation of the DNA degradation by EthBr is very probably due to an action on the substrate rather than on the enzyme.

(a) The relationship between EthBr concentration and the increase of activity is similar to the binding isotherm of EthBr on DNA. The binding coefficient of EthBr on DNA in our experimental conditions (0.05 M Tris HCl, 0.01 M $MgCl_2$ pH 7.2) is 1.5×10^{-6}M (29). The maximum of activation is obtained at about 2×10^{-6}M EthBr. If the intercalating dye was active on enzyme proteins, such close values would be improbable.

(b) Four drugs have been shown to activate the enzymatic degradation of native DNA. They all intercalate between the base pairs of DNA. Therefore, their common intercalating property would likely explain their common effect on the enzymatic system. The relationship between the total concentration of the intercalating drug and the enzymatic activation is not the same for each of them. Such an observation cannot be presently interpreted because the affinity coefficient of the drugs for DNA are not known with precision in our environmental conditions. However, one cannot exclude that the DNAse which recognizes the intercalated sites is also able to discriminate between them according to the rotation angle of the base pairs located on each side of these sites. This rotation angle has been demonstrated to differ for varying intercalating substances (30).

(c) EthBr cannot perform by itself irreversible modifications of the proteins in the submitochondrial pellet which would modify their enzymatic activity (unpublished result).

It would be of interest to know if there is any correlation between the mutagenic effect of EthBr on yeast and its ability to activate the mitochondrial DNase described in this paper. "Petite" yeast strains resistant to the mutagenic action of EthBr have been isolated (P. SLONIMSKI, private communication) ; they are presently under study.

This work has been initiated after discussion with P. SLONIMSKI and J. DEUTSCH.

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