ACTION OF RAT LIVER AP ENDODEOXYRIBONUCLEASE ON DNA TREATED WITH HYDRAZINE OR BLEOMYCIN

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1. Introduction

Endonuclease IV, the minor *Escherichia coli* endonuclease specific for apurinic sites, has been shown [1] to be active on apyrimidinic sites produced by uracil-DNA glycosylase. It was of interest to investigate whether the rat liver enzyme was also active on apyrimidinic sites. However, we have used an AP endodeoxyribonuclease (i.e., an endonuclease specific for apurinic and apyrimidinic sites in DNA) to analyze the products of the reaction of DNA with chemical agents such as hydrazine and bleomycin.

Bleomycin, a glycopeptide with mol. wt of ~ 1500, is an antibiotic produced by *Streptomyces verticillus*, isolated [2,3]. The action of the drug on DNA strongly depends on its concentration. At high concentrations, the four bases are removed from DNA [4] and double-strand as well as single-strand breaks are produced [5]. But, at low concentrations, bleomycin splits off mostly thymine bases [6] and the possibility of intercalation of one of the thiazole rings of the antibiotic in the double-helix at the place of the lost pyrimidine has been suggested [7]. Because bleomycin is currently used in the chemotherapy of neoplastic diseases [8–10], the problem of the repair of DNA lesions produced by this agent is important; we wanted particularly to know whether the base-free sites were accessible to a repair endodeoxyribonuclease.

2. Materials and methods

Labelled *E. coli* DNA is prepared in the presence of [*methyl*-^{3}H]thymidine; it has a specific radioactivity of 60 300 dpm/μg and is kept in 0.15 M NaCl, 0.015 M Na citrate, pH 7.0 (NaCl/cit.) at −30°C.

2.1. *Alkylated-depurinated [^{3}H]DNA*

Alkylation with methyl methanesulfonate followed by heat-depurination has been described [11]. The treated [^{3}H]DNA kept in NaCl/cit., 0.01 M $MgCl_2$, contains 160 apurinic sites and 390 methylated sites/10^6 daltons; less than 1% is soluble in 5% perchloric acid, but NaOH treatment raises the acid-soluble fraction to 35%.

2.2. *Hydrazine-treated [^{3}H]DNA*

To 1 ml NaCl/cit. containing 375 μg [^{3}H]DNA are added 1.7 g hydrazine hydrochloride; it is adjusted to pH 7 with NaOH and brought to 2 ml with NaCl/cit. After 45 h at 37°C, the solution is dialyzed 3 times against NaCl/cit. at 0°C before addition of 0.7 ml 10% sulfonated benzaldehyde in NaCl/cit. The solution (4.2 ml; pH 7) is kept for 4 h at room temperature, then dialyzed 3 times against NaCl/cit., 0.01 M $MgCl_2$. The treatment reduces the specific radioactivity of the DNA to 40 000 dpm/μg; < 1% is soluble in 5% perchloric acid, but NaOH treatment raises the acid-soluble fraction to 5%.

2.3. *Bleomycin-treatment [^{3}H]DNA*

A thiol is necessary for the rapid release of [^{3}H]thymine from the labelled DNA by the antibiotic and the reaction rate at pH 8.2 is far from negligible at 0°C; but the reaction decreases drastically below pH 7.3, the pK_a of the amine group of the amino-alanine (fig.1).

To 0.85 ml NaCl/cit. containing 320 μg [^{3}H]DNA are added 1 ml of 0.1 M Tris. HCl, 0.02 M dithio-

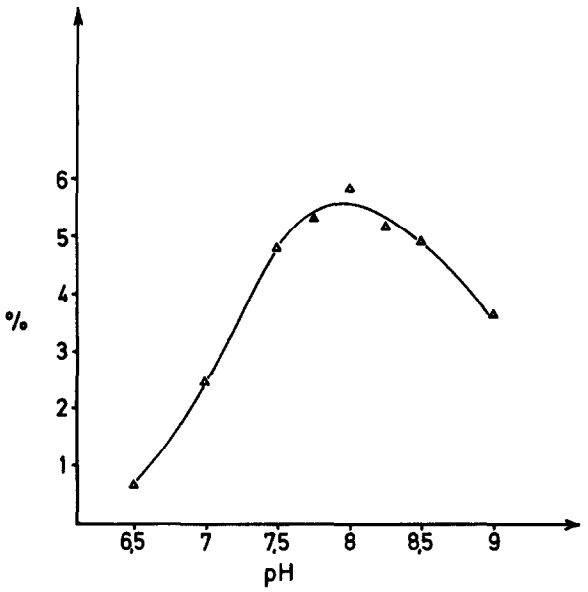

Fig.1. Effect of pH on the release by bleomycin of dialysable radioactivity from DNA labelled in thymine moieties. 400 μl 0.1 M Tris.HCl buffer adjusted at the indicated pH (abscissae) containing 0.020 M dithiothreitol, 4 μg bleomycin and 16 μg [^{3}H]DNA are incubated for 2 h at 37°C; the reaction is stopped by lowering to pH 6.5. The solution is dialyzed at 0°C through a cellulose membrane in a two-compartment lucite apparatus; the radioactivity/ml dialysate is multiplied by the total apparatus solution volume and the result expressed in % substrate DNA radioactivity. Thin-layer chromatography has shown that the radioactivity of the dialysate is only in thymine molecules.

threitol, pH 8.2, containing 200 μg bleomycin (Blenoxane, Bristol Laboratory, Syracuse, New York). After 4 h at 37°C, the reaction is stopped by addition of cold 0.1 M citric acid, 0.2 M Na_2HPO_4, pH 5.9 buffer, which lowers the pH to 6.5. The solution is then dialyzed at 0°C 3 times against NaCl/cit., 0.01 M $MgCl_2$. The treatment reduces the specific radioactivity of the DNA to 43 000 dpm/μg; 5% is soluble in 5% perchloric acid and NaOH treatment raises the acid-soluble fraction to 10%.

2.4. *NaOH treatment*

To 20 μl DNA solution containing ~ 20 μg/ml are added 20 μl 0.4 M NaOH; after 15 min at 37°C, the mixture is neutralized with 20 μl 0.4 M HCl. This treatment hydrolyses a phosphoester bond near each base-free site [11]. The characterization of the alkali-labile sites is however done at a lower pH (pH 12; see section 3).

2.5. *Determination of acid-soluble fraction*

To 60 μl [^{3}H]DNA solution are added 100 μl NaCl/cit. containing 200 μg calf thymus DNA and 900 μl 5.9% cold perchloric acid. After shaking, the tubes are kept 15 min at 0°C, centrifuged at 13 000 × *g* for 15 min, and an aliquot of the supernatant assayed for radioactivity.

2.6. *Rat liver endonuclease specific for apurinic sites*

We followed the purification method in [12] except that the phosphocellulose has been replaced by an affinity chromatography on DNA–Sepharose. The active fractions, dialyzed against 0.05 M Tris.HCl, 0.1 mM EDTA, 0.1 mM dithiothreitol, pH 8, are mixed with an equal volume of glycerol and kept at −50°C. The preparation is without action on normal DNA strands and on alkylated sites; it is devoid of exonuclease activity. The enzyme unit is that used in [13].

3. Results and discussion

3.1. *Hydrolysis rate of the treated [^{3}H]DNAs at pH 12*

We have used the rate of appearance of acid-soluble radioactivity (which is nearly proportional to the rate of formation of strand breaks) at pH 12 and 37°C to characterize the alkali-labile sites. With the alkylated-depurinated DNA, the reaction is first order and the half-life of the alkali-labile sites is 37 min. Reaction of DNA with hydrazine only leaves several kinds of alkali-labile sites with different half-lives, but, after an additional treatment with the sulfonated benzaldehyde, there remains only one kind of alkali-labile sites with the same half-life as the apurinic sites. The alkali-labile sites of the bleomycin-treated [^{3}H]DNA also have the same half-life as the apurinic sites.

3.2. *Action of rat liver endonuclease specific for apurinic sites on the three substrates*

20 μl 0.02 M Tris.HCl, 0.01 M $MgCl_2$, pH 8.0, containing 0.4 μg modified [^{3}H]DNA and 20 μl enzyme (7 units) in Tris.HCl, 0.01 M $MgCl_2$, are incubated at 37°C for various times before measuring the acid-soluble radioactivity; the results are corrected

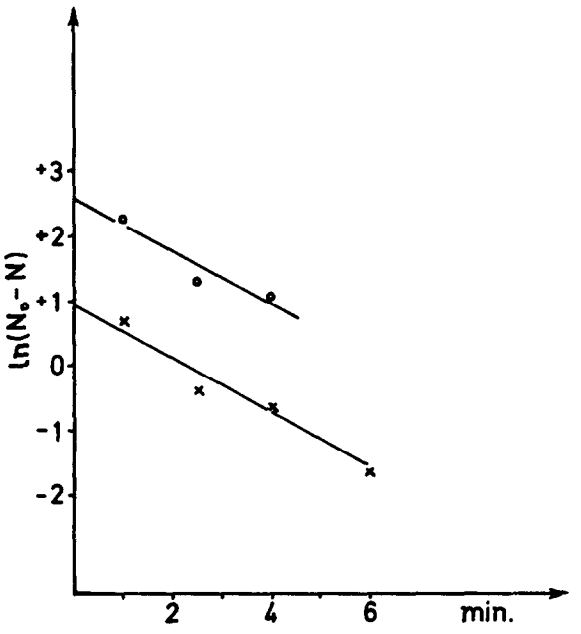

Fig.2. Action of rat liver endonuclease specific for apurinic sites on alkylated-depurinated DNA and bleomycin-treated DNA. The experimental conditions are described in the text. The function $\ln(N_0 - N)$ is given versus time, where N_0 is the maximum acid-soluble radioactivity obtained when the incubation exceeds 10 min and N the acid-soluble radioactivity at the indicated time (min). The best straight lines were determined by the least squares method and their slopes used to calculate the half-lives of the sensitive sites.

for controls without enzyme. The enzyme being in large excess, the reaction follows first order kinetics with a half-life of 103 ± 35 s for the alkylated-depurinated [^{3}H]DNA and 95 ± 14 s for the bleomycin-treated [^{3}H]DNA (fig.2). In both cases, treatment with NaOH after the incubation with the enzyme yields the same acid-soluble radioactivity as treatment with NaOH alone showing that the reactive sites are the same for the alkali and the endonculease specific for apurinic sites. By contrast, the alkali-labile sites in the [^{3}H]DNA treated successively with hydrazine and benzaldehyde are insensitive to the enzyme.

Several conclusions can be drawn from these results:

1. Treatment of DNA with bleomycin leads to a loss of thymine and the appearance of alkali-labile sites; because these alkali-labile sites are sensitive to the rat liver endonuclease specific for apurinic sites, we conclude that this enzyme is also active on apyrimidinic sites. The enzyme is thus an AP endodeoxyribonuclease.
2. Since the enzymic reaction rate on alkali-labile sites appears not to be different for the bleomycin-treated DNA and for the depurinated DNA, it is unlikely that, in our experimental conditions, the antibiotic remained intercalated in the double-helix replacing the departed thymine; such a situation would most probably prevent or at least decrease the activity of the endonuclease. The results also suggest that the base-free sites produced by bleomycin can be repaired.
3. The reaction rates being the same on depurinated and depyrimidinated DNAs, it appears that the nature of the base (purine or pyrimidine) on the complementary DNA strand in front of the base-free site has no influence on the enzyme activity.
4. There exist alkali-labile sites insensitive to the AP endodeoxyribonuclease as those in DNA treated successively with hydrazine and sulfonated benzaldehyde. It is possible that there is no free aldehyde at the deoxyribose C-1′ of these alkali-labile sites; the substituent must however be quickly lost at pH 12 since it does not influence the rate of appearance of breaks near the alkali-labile sites at this pH.

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