MAMMALIAN DNA ENDONUCLEASE ACTING AT APURINIC SITES: ABSENCE OF ASSOCIATED EXONUCLEASE ACTIVITY

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1. Introduction

Apurinic (and/or apyrimidinic) sites in DNA occur as a consequence of spontaneous DNA hydrolysis at neutral pH [1], after treatment of DNA with alkylating agents [2] or ionizing radiation [3,4] and by the action of enzymes that cleave *N*-glycosidic bonds in DNA [5]. The apurinic sites are presumably efficiently removed by an excision repair mechanism in vivo and endonucleases that specifically attack DNA at such sites seem to be present in all living cells [6,7]. Yajko and Weiss [8] have recently shown that the *E. coli* enzyme of this type, endonuclease II [6,9] is identical with *E. coli* exonuclease III/DNA 3′-phosphatase [10,11]. It was further found that the endonuclease II and exonuclease III activities of *Hemophilus influenzae* also are associated in the same protein which suggested a more general phenomenon [8,12]. Here it is shown that while the *E. coli* endonuclease II/exonuclease III/DNA 3′-phosphatase activities are all present in a purified preparation of *E. coli* exonuclease III, in confirmation of the work of Yajko and Weiss [8], the otherwise similar mammalian endonuclease [3,7] does not possess associated exonuclease and phosphatase activities. Further, an enzyme activity similar to *E. coli* exonuclease III does not appear to be present in mammalian cells.

2. Materials and methods

The mammalian endonuclease acting at apurinic sites was purified from calf thymus as described [7]. *E. coli* exonuclease III, purified according to Jovin et al. [13], was a gift from Dr. B. Sugden. Crude cell extracts from calf thymus were prepared by disruption of minced thymus in a hand homogenizer with 5 volumes of 0.1 M NaCl, 0.05 M Tris–HCl (pH 7.4), 0.01 M 2-mercaptoethanol, 0.001 M trisodium citrate. After 60 min of gentle stirring at 0°C, the material was centrifuged at 30 000 *g* for 20 min. The supernatant solution was dialyzed against 500 volumes of the extraction buffer for 14 hr, and used directly for enzyme assays. Endonuclease activity at apurinic sites was assayed essentially as described by Verly et al [6], as follows: *E. coli* [^{32}P] DNA (4×10^5 cpm/μg, 50 μg/ml) in 0.05 M potassium phosphate (pH 7.4) was treated with 0.3 M methyl methanesulfonate at 37°C for 45 min. Remaining methyl methanesulfonate was then removed by dialysis against 0.2 M NaCl, 0.01 M trisodium citrate, 0.01 M sodium phosphate (pH 7.0) at 2°C for 16 hr. The alkylated DNA was incubated at 50°C for 6 hr to release methylated purine bases. This heat treatment converts the DNA to substrate for the endonucleases studied [6]. The partly depurinated DNA was dialyzed against 0.1 M NaCl, 0.01 M Na-citrate (pH 6.0) at 2°C for 20 hr, and was stored in several small aliquots at −70°C. In endonuclease assays, the reaction mixtures (0.1 ml) contained 0.1 M Hepes-KOH (pH 8.0), 0.003 M $MgCl_2$ *or* 0.002 M trisodium citrate, 10^{-4} M dithiothreitol, 1 μg depurinated alkylated [^{32}P]DNA, 2 μg yeast tRNA, 50 μg bovine serum albumin, and a limiting amount of endonuclease activity. After 30 min at 37°C, the reaction mixtures were chilled to 0°C, 0.1 ml 0.8 M perchloric acid was added, and after 5 min incubation at 0°C, the tubes were centrifuged at 15 000 *g* for 10 min, and the radioactivity of the supernatants was determined. DNA exonuclease and 3′-phosphatase activities were measured according to Richardson et al. [10,11]. In the exonu-

clease assays, the reaction mixtures (0.1 ml) contained 0.1 M Hepes-KOH (pH 8.0), 0.001 M $MgCl_2$, 10^{-4} M dithiothreitol, 2 μg *E. coli* [^{32}P] DNA, 2 μg yeast tRNA, 50 μg bovine serum albumin, and a limiting amount of enzyme.

3. Results and discussion

A convenient assay method for endonucleases acting at DNA apurinic sites, designed by Verly et al. [6], measures the amount of acid-soluble material liberated from heavily alkylated and depurinated DNA. Due to enzymatic incisions at closely located apurinic sites, a minor part of the total DNA sequences are released in acid-soluble form. In fig.1, *E. coli* endonuclease II and the calf thymus endonuclease acting at apurinic sites have been assayed by this method. The *E. coli* enzyme appears to be a metalloenzyme [9], because it is strongly inhibited by EDTA, but little affected by weaker chelating agents such as citrate. In agreement with Friedberg et al. [9], there was only approximately a 2-fold increase in the rate of attack at the DNA substrate when Mg^{2+} was added. In the presence of citrate and absence of added Mg^{2+}, a maximum of 14% of the substrate was converted to an acid-soluble form (fig.1). These data are similar to those obtained by Verly et al. [6] in a reaction mixture containing an excess of citrate over Mg^{2+}. In the presence of Mg^{2+}, the *E. coli* enzyme degrades the DNA substrate more excessively, apparently due to activation of the intrinsic exonuclease III component [8] of the enzyme. With the mammalian endonuclease, little activity is found in the absence of added Mg^{2+}, and even in the presence of Mg^{2+} there is a maximum release of 14% of the total radioactivity in the DNA substrate. Both enzymes are completely inhibited (< 0.3% residual activity) by addition of 10^{-3} M EDTA (and no Mg^{2+}) to the reaction mixtures.

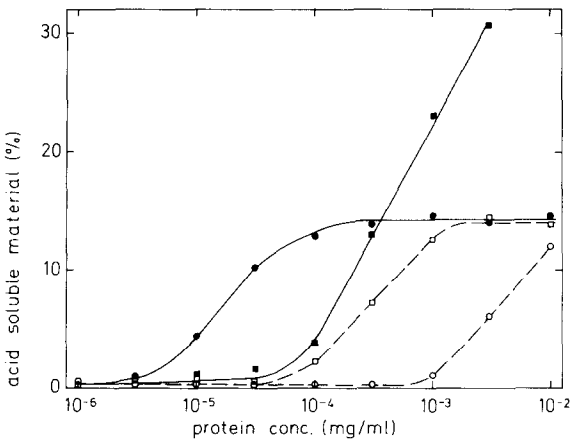

Fig.1. Endonuclease activity against depurinated alkylated DNA by the purified *E. coli* (squares) and mammalian (circles) enzymes. Closed symbols: Mg^{2+}-containing reaction mixtures. Open symbols: Citrate-containing reaction mixtures. For further details, see text.

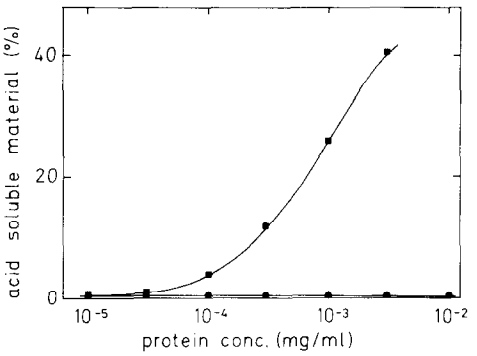

Fig.2. Exonuclease activity against double-stranded DNA by the *E. coli* enzyme (squares) and the mammalian enzyme (circles).

The two enzyme preparations were further tested, in the presence of Mg^{2+}, for exonuclease activity on double-stranded DNA without apurinic sites. As expected, the *E. coli* endonuclease II/exonuclease III degraded this DNA substrate. In contrast, the mammalian endonuclease showed no detectable activity. From figs.1 and 2, it is estimated that when similar amounts of endonuclease activity are compared, the mammalian enzyme is less than 0.1% as active as the *E. coli* enzyme in the exonuclease assay. Addition of Ca^{2+} (10^{-3} M), ATP(10^{-4} M), or boiled crude thymus extract to the reaction mixtures containing the mammalian enzyme did not lead to activation of any detectable exonuclease activity.

Similar results were obtained when the DNA 3′-phosphatase activity of the two enzyme preparations was investigated. The *E. coli* endonuclease II/exonuclease III functioned effectively as a DNA 3′-phosphatase, while the mammalian enzyme showed no detectable phosphatase action up to concentrations of endonuclease activity 1000-fold higher than those used in

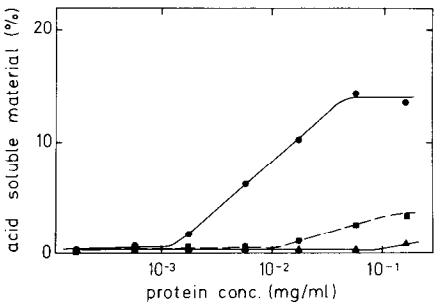

Fig.3. Release of acid-soluble material from [^{32}P]DNA by a dialyzed crude cell extract from calf thymus. Triangles: native DNA. Squares: heat-denatured (100°C, 10 min) DNA. Circles: depurinated alkylated DNA.

linear phosphatase assays with the *E. coli* enzyme.

Because of the relatively high amounts of the endonuclease acting on DNA containing apurinic sites in calf thymus cell extracts, and the low background of unspecific nuclease activity, this endonuclease can be reliably measured in crude cell extracts. Such an experiment is shown in fig.3. The DNA substrate containing apurinic sites is clearly more effectively degraded than either native or heat-denatured DNA, but again a maximum of 14% of the DNA substrate was acid-solubilized. In artificial mixtures of crude cell extract from thymus (0.2 mg/ml) and *E. coli* exonuclease III there was no significant inhibition ($< 10\%$) of the exonuclease. These data indicate that there is no association between the mammalian endonuclease acting at apurinic sites and an '*E. coli* exonuclease III-like' component even in crude cell extracts, and that the latter type of enzyme activity appears to be absent from mammalian cell extracts. The small amount of nuclease activity against heat-denatured and native DNA observed was probably mainly due to mammalian DNase III, a nuclear exonuclease that degrades denatured DNA four times more rapidly than native DNA [14] and is strongly inhibited by the presence of apurinic sites in DNA (S.L., unpublished data).

For the removal of an apurinic site from DNA by excision-repair, the deoxyribose-5′-phosphate residue must be excised after the incision event but prior to repair replication. In preliminary experiments, we have observed excision of deoxyribose-phosphate residues from partly depurinated [^{32}P]DNA by the mammalian endonuclease preparation studied here, but it is not yet clear if this excision activity is due to an intrinsic property of the endonuclease, or to a second enzyme present in the highly purified but non-homogenous endonuclease preparation. It is of interest in the present context that mammalian DNA polymerases also lack associated exonuclease activities [15], in contrast to the *E. coli* DNA polymerases. Further, only very few nucleotides appear to be inserted during repair replication induced by DNA depurination in mammalian cells [16].

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