Volume 213, number 1, 215–220 FEB 04497 March 1987

Separation of damage specific DNA endonuclease activities present in calf thymus

Dag E. Helland, Rune Male and Kjell Kleppe

Laboratory of Biotechnology and Department of Biochemistry, University of Bergen, Bergen, Norway

Received 4 December 1986; revised version received 6 January 1987

A DNA endonuclease activity present in calf thymus specific for incision on DNA damaged by ultraviolet light, osmium tetroxide, potassium permanganate, hydrogen peroxide and acid has been purified from whole cell extracts. The enzymatic activity was heterogeneous both with regard to molecular mass and charge. The molecular mass of the enzyme varied from 25 to 35 kDa, but the different enzymatic species appeared to possess similar activities. The enzymes acted equally well on damage in supercoiled and relaxed forms of DNA. It further had a narrow optimum with regard to salt concentrations, the optimum activity being observed at a concentration of KCl from 40 to 65 mM.

DNA damage Endonuclease; Purification; Enzyme characterization; (Calf thymus)

1. INTRODUCTION

Mammalian DNA endonucleases specific for incision on damaged DNA have been purified and characterized from several sources [1–5]. Waldstein et al. [2] found a high molecular mass activity which they claimed to be specific for pyrimidine dimers. Because of the unstable nature of this enzyme, further characterization proved to be difficult. Other endonucleases studied possess low molecular masses, in the range 25 to 35 kDa, and they act in the absence of Mg^{2+} or other divalent cations on DNAs modified by UV light or other agents like OsO_4. They show no activity toward adducts like psoralen and *N*-acetoxy-*N*-acetylaminofluorene. We have studied in some detail the enzyme from mouse plasmacytoma cells [5–8] and shown that this DNA damage specific endonuclease acts solely on lesions introduced in supercoiled but not in relaxed DNA [6]. This group of enzymes are in the literature frequently referred to as mammalian DNA repair enzymes. Until a definite function of DNA repair in mammalian cells by these enzymes has been shown, we suggest that they should be called damage specific endonucleases.

We have extended the study to similar enzymatic activities from a variety of mammalian cells and tissues and in this report we describe the partial purification and properties of the enzyme present in calf thymus [3]. In contrast to the endonuclease present in mouse plasmacytoma cells, the enzyme found in calf thymus is capable of acting on both supercoiled and relaxed DNA, as well as on short DNA fragments of defined sequences [10]. This damage specific enzyme from calf thymus possesses a substrate specificity nearly identical to that of *E. coli* endonuclease III using DNA damaged by UV-light, OsO_4 and other oxidative agents [9]. Cyclobutyl pyrimidine dimers are not recognized by these endonucleases [10]. Since both the calf thymus endonuclease and the *E. coli* endonuclease III act on DNA modified by oxidation or reduction of the pyrimidine base residues, the name redoxyendonuclease has been suggested for this type of enzyme [9]. Based on analysis of the

Correspondence address: D.E. Helland, Laboratory of Biotechnology, University of Bergen, Box 3152, Årstad, 5001 Bergen, Norway

Published by Elsevier Science Publishers B.V. (Biomedical Division)

3′- and 5′-termini of the enzyme-generated DNA scission products using DNA sequencing methods and HPLC analysis of released material, a novel model for the mechanism of action of redoxyendonucleases was postulated [10]. Some properties of this enzyme from calf thymus have previously been reported by Bacchetti and Benne [3]. The procedure employed for purification in the present report differs markedly from theirs and, moreover, we show that the calf thymus enzyme is separated into subpopulations during the isolation procedure.

2. MATERIALS AND METHODS

2.1. *Materials*

DEAE-cellulose (DE 32 microgranular) and phosphocellulose (P11) were obtained from Whatman. Sephacryl S-200 superfine and Sephadex G-75 superfine were products of Pharmacia A/S. Nitrocellulose membrane filters were purchased from Gelman (Metricell GN-6, diameter 25 mm). [*methyl*-^{3}H]Thymidine (50 Ci/mmol) was obtained from Amersham. Polyethylene glycol (PEG) 35000 was a product of Fluka.

2.2. *Preparation of DNAs*

^{3}H-labelled ϕX174 RFI DNA was prepared essentially as described in [5,6]. After centrifugation in a CsCl gradient, the RFI DNA was subjected to chromatography on a column of Sephacryl S-300 (2.5 × 32 cm) to remove minor impurities of RNA. The DNA was concentrated by ethanol precipitation and resuspended in a buffer (TEN) consisting of 20 mM Tris-HCl, pH 8.0, 1 mM EDTA and 10 mM NaCl. The purified DNA had a specific activity of 2×10^4 cpm·μg^{-1}. Treatments of ϕX174 RFI DNA with UV-light (254 nm), acid and OsO_4 were carried out as described [6].

2.3. *Assay for DNA damage specific endonuclease*

The standard assay mixture of 100 μl consisted of 10 mM Tris-HCl, pH 8.0, 10 mM EDTA, 10 mM β-mercaptoethanol, 40 mM KCl and 0.05–0.1 μg ^{3}H-labelled ϕX174 RFI DNA. The reaction temperature was 37°C and the assay period 30 min. The amount of nicked DNA was determined by the nitrocellulose filter method [5]. When the activity of Mg^{2+} requiring endonucleases was investigated (AP-endonucleases and DNase I), 5 mM $MgCl_2$ was substituted for EDTA in the assay mixture. The average number of breaks introduced per DNA molecule was estimated by assuming a Poisson distribution [5]. One unit of enzyme activity is defined as the amount of enzyme required to give one nick per DNA molecule with 0.1 μg ϕX174 RFI [^{3}H]DNA using the standard assay conditions described above.

2.4. *Determination of protein*

The protein content was estimated using the Biorad protein assay kit with rabbit γ-globulin as a standard.

3. RESULTS

3.1. *Enzyme purification*

3.1.1. Crude extracts

All purification steps were carried out at 0–4°C. Fresh thymus from young calves were cooled on wet ice immediately after they had been removed from the animals. Fat, adipose tissue and blood vessels were removed and the clean tissue (80–250 g) was cut into small pieces by a meat grinder, suspended in 100 ml of the homogenization buffer (0.1 M NaCl, 50 mM Tris-HCl, pH 8.0, 1 mM phenylmethylsulfonyl fluoride (PMSF)), followed by homogenization in a Potter-Elvehjem homogenizer with a teflon piston. The suspension was next filtered through gauze, then homogenized in a Dounce homogenizer, piston B, to break the nuclei. In order to dissociate chromatin-bound enzyme from chromatin, the NaCl concentration was adjusted to 0.7 M. After 10 min the NaCl concentration was reduced to 0.3 M by addition of homogenization buffer minus salt. The suspension was then sonicated for a short period using a Branson sonifier equipped with a microtip. The extract was next subjected to centrifugation for 3 h at 4°C and 20000 rpm using a Beckman 21 rotor. The clear supernatant was used in the further purifications and designated as fraction I. Following this procedure very little if any activity was detected in a resuspended fraction of the pelleted material, indicating efficient extraction of the enzyme.

3.1.2. Ion exchange chromatography

Fraction I was applied onto a column of DEAE cellulose (6 × 17 cm) and eluted with buffer A (10 mM Tris, pH 8.0, 10 mM EDTA, 10 mM β-mercaptoethanol) containing 0.3 mM NaCl. Under these conditions, the DEAE column bound most of the nucleic acids and some of the protein present in fraction I, whereas practically all of the enzyme activity was in the flow through. The enzyme-containing fractions were collected and dialyzed against buffer A containing 50 mM NaCl (fraction II). The dialyzed material was subsequently loaded onto a column of phosphocellulose (6 × 12.5 cm) equilibrated with the same buffer, and washed until the absorbance in the eluate at 280 nm was close to zero. The enzyme activity was eluted with 1.2 l of a gradient of NaCl in buffer A ranging from 50 to 1000 mM NaCl. Fractions of 21 ml were collected and assayed for activity. Two peaks of enzymatic activity were detected (fig.1) eluting at approx. 0.2 and 0.5 M NaCl.

The two peaks were pooled separately, concentrated with PEG 35000 followed by dialyses against storage buffer (50% glycerol, 20 mM Tris-HCl, pH 8.0, 0.1 M KCl and 10 mM β-mercaptoethanol); the final volume was approx. 15 ml. The fractions, designated IIIA and IIIB, were stored at −20°C. Under these conditions, the enzymes were stable for more than a year.

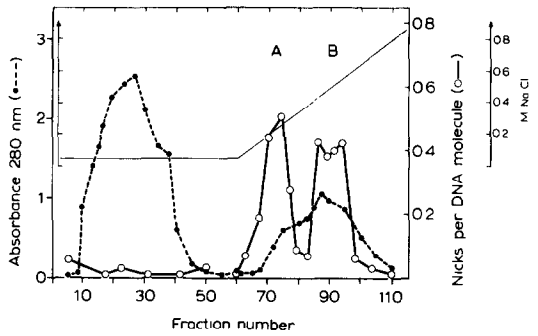

Fig.1. Phosphocellulose ion-exchange chromatography of fraction I. 5 μl extract per assay was used when testing for the DNA-repair endonuclease (o—o) using DNA which had been irradiated with a total dose of UV-light 1.1 kJ·m^{-2} as substrate. No activity was detected in the fractions when nonirradiated DNA was used as substrate. The protein content in the fractions was measured by the absorbance at 280 nm (•---•).

3.1.3. Gel filtration

Fractions IIIA and -B were applied separately to a column of Sephacryl S-200 (2.5 × 100 cm) which had been equilibrated with a buffer consisting of 20 mM Tris-HCl, pH 8.0, 0.2 M NaCl, 1 mM EDTA and 10 mM β-mercaptoethanol. Fractions of 7 ml were collected and assayed for enzymatic activity. The results presented in fig.2 show that the two enzyme populations A and B from the phosphocellulose column were also heterogeneous with regard to molecular mass. Both fractions IIIA and IIIB yielded a major peak of activity with molecular mass in the range 25 to 35 kDa. The enzyme activities in the different fractions were highly active toward damaged DNA since no nicking activity was observed when unirradiated DNA was used as a substrate (not shown). The active fractions were pooled and dialyzed against the storage buffer. These were designated fractions IVA_1, IVA_2, IVA_3, IVB_1, and IVB_2, respectively.

A summary of the purification is presented in table 1. Following the purification of the enzyme a specific protein inhibitor of the DNA repair en-

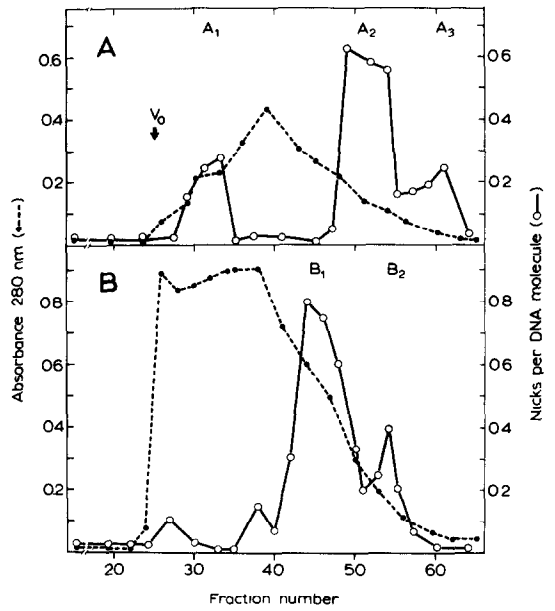

Fig.2. Sephacryl S-200 chromatography of fraction III. The assay for enzyme activity was as described in the legends to fig.1. (A) Fraction IIIA from the phosphocellulose step. (B) Fraction IIIB from the phosphocellulose step.

Table 1

Purification scheme of DNA repair endonucleases from calf thymus

Fraction	Volume (ml)	Protein (mg/ml)	Specific activity (units/mg)	Yield (%)
I. High speed supernatant	180	27.5	9	
II. DEAE-cellulose	483	5.63	18	100
III. Phosphocellulose 0.27 M (A)	225	0.4	250	46
0.50 M (B)	240	0.95	84	39
IV. Sephacryl S-200 IVA_1	48	0.15	933	16
IVB_1	55	0.55	290	18
V. Sephadex G-75 (of IVA_1)	20	0.12	231	1.9

The enzymatic activity was determined using UV-irradiated DNA (1.1 $kJ \cdot m^{-2}$). The Sephadex G-75 step was carried out employing 5 mg of protein from fraction IVA_1 after concentration by dialysis against PEG 35000

donuclease was detected in the crude extract. This inhibitor was not present in the phosphocellulose fractions IIIA and IIIB. If these two pure endonuclease fractions were combined with high speed supernatant or the flow through from the phosphocellulose column, a strong inhibition of the activity was observed. The presence of this inhibitor therefore made the estimation of the total activity in the crude extract somewhat uncertain. This inhibitor appears to be a protein, as indicated by inactivation experiments (not shown).

Judged from SDS-polyacrylamide gel electrophoresis the enzyme containing fractions from gel filtrations of fraction IVA on Sephadex G-75 consist of at least five polypeptides differing in molecular masses from 20 to 40 kDa (not shown). Further purification has proved to be extremely difficult due to loss of activity.

3.2. *Catalytic properties and molecular masses*

When the enzyme fractions from the phosphocellulose and Sephacryl S-200 columns were rechromatographed on the same columns, they eluted in the same fractions as originally detected. This strongly suggests that they are genuine enzyme species and not a result of aggregation or dissociation of subunits.

The phosphocellulose fractions (IIIA and -B) were free of contaminating Mg^{2+}-dependent endonucleases. An AP-endonuclease which also acts in the absence of Mg^{2+} was, however, found to cochromatograph with the DNA repair endonuclease activity. This is similar to that reported for other mammalian DNA damage specific endonucleases and this activity appears to be an inherent property of the enzymatic activity on UV-irradiated DNA [10]. All the five different enzyme fractions from the Sephacryl S-200 purification step responded with a maximum activity on DNA given a total dose of 1 $kJ \cdot m^{-2}$ suggesting that they have similar catalytic properties (not shown).

The DNA damage specific endonuclease isolated here was found to be more sensitive to ionic strength than previously described [3], having a rather narrow optimum of activity between 40 and 65 mM KCl (fig.3). The same ionic strength dependence was found for both of the major fractions from Sephacryl S-200 (fractions IVA_2 and IVB_1).

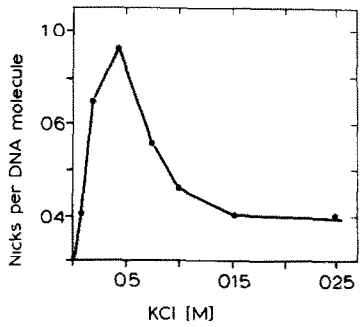

Fig.3. Effect of different concentrations of KCl on the activity of DNA damage specific endonuclease (fraction IVA_2).

The various enzymatic fractions from Sephacryl S-200 were subjected to gel filtration on a column of Sephadex G-75 previously calibrated with marker proteins. Fractions IVA yielded an average molecular mass of 28 kDa, which is similar to that observed by Bacchetti and Benne [3].

3.3. *Substrate specificity*

In addition to UV-irradiated DNA the calf thymus DNA damage specific endonuclease recognizes damage in DNA caused by oxidative agents [9]. Contrary to a previous report [3] we found that all different enzyme fractions obtained following the purification procedure described in this paper also hydrolyzed at AP-sites. In agreement with the observation of Bacchetti and Benne [3], however, the enzymes did not act on DNA crosslinked with 8-methyl psoralen.

In fig.4 the dose dependent nicking activity on osmium tetroxide treated DNA using enzyme fractions IVA_2 is shown. Nearly the same dose dependent nicking activity was observed for all different enzyme fractions giving 0.6 breaks per DNA molecule at the highest osmium tetroxide concentration used to damage the DNA.

We have previously shown that the DNA damage specific endonuclease from mouse plasmacytoma cells only acted on damage introduced in supercoiled and not in relaxed DNA. The calf thymus enzymes were tested in a similar manner using UV-irradiated DNA. In this case, however, there was no difference with regard to activity using relaxed or supercoiled DNA (fig.5).

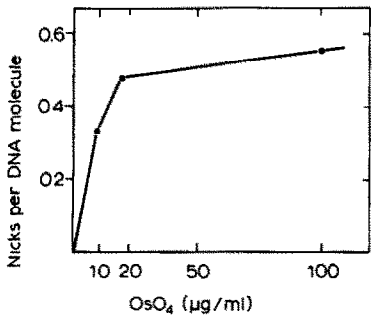

Fig.4. DNA damage specific endonuclease activity on OsO_4 treated DNA. The OsO_4 treatment was carried out using ϕX174 RFI [^{3}H]DNA as described in section 2. 3 units of enzyme from fraction IVA_2 were employed.

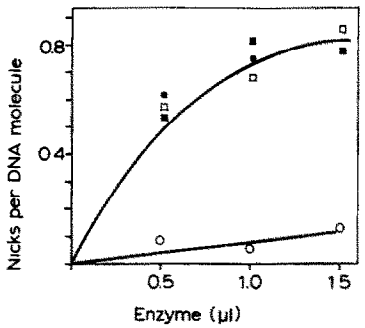

Fig.5. Effect of DNA damage specific endonuclease on UV-irradiated (1.1 $kJ \cdot m^{-2}$) supercoiled (□, ■) and relaxed ϕX174 RF DNA (○, ●). Open symbols are for the mouse plasmacytoma enzyme, and closed symbols are for the calf thymus enzyme (fraction IVA_2). Both enzyme preparations contained approx. 1 unit per μl. The DNA was relaxed by treatment with topoisomerase I as described [6].

This property of the calf thymus enzyme has made it possible to study the mode of action of the enzyme using linear DNA fragments of defined sequences [9,10].

4. DISCUSSION

The present results show that the DNA damage specific endonuclease activity from calf thymus is heterogeneous both with regard to molecular size as well as to ionic properties. A slight heterogeneity in molecular mass was previously observed by Bacchetti and Benne [3], but these authors did not report a marked separation of the enzymatic activities on phosphocellulose, probably due to the fact that they used a single stepwise elution with 1 M NaCl. The elution pattern from the phosphocellulose column resembles that obtained for the purification of the enzyme from mouse plasmacytoma cells. The presence of multiple enzyme species is an indication of the complexity of studying damage specific enzymes from mammalian cells. The reason for this heterogeneity is still unclear.

We have recently described the purification of 3-methyladenine-DNA glycosylase from calf thymus [8]. In this case the chromatin was found to contain one enzyme species with a lower molecular mass than the enzyme present in the cytoplasm. Several explanations can be given for

the presence of the various activities. One possibility is that they are genuine different gene products. Alternatively, the lower molecular mass species could arise from the higher molecular mass forms by proteolytic cleavage, perhaps related to transport to nucleus or binding to chromatin, or by an artefact introduced at the initial purification steps.

ACKNOWLEDGEMENTS

The study was supported by grants from the Norwegian Cancer Society (Landsforeningen mot Kreft). D.E.H. and R.M. are also research fellows of this society.

REFERENCES

[1] Van Lancker, J.L. and Tomura, T. (1974) Biochim. Biophys. Acta 353, 99–114.
[2] Waldstein, E.A., Peller, S. and Setlow, R.B. (1979) Proc. Natl. Acad. Sci. USA 76, 3746–3750.
[3] Bacchetti, S. and Benne, R. (1975) Biochim. Biophys. Acta 390, 285–297.
[4] Brent, T.P. (1983) Biochemistry 22, 4507–4512.
[5] Nes, I.F. (1980) Eur. J. Biochem. 118, 161–168.
[6] Helland, D.E., Nes, I.F. and Kleppe, K. (1982) FEBS Lett. 142, 121–124.
[7] Nes, I.F. and Nissen-Meyer, J. (1978) Biochim. Biophys. Acta 520, 111–121.
[8] Helland, D.E., Raae, A.J., Fadnes, P. and Kleppe, K. (1985) Eur. J. Biochem. 148, 471–477.
[9] Helland, D.E., Doetsch, P.W. and Haseltine, W.A. (1986) Mol. Cell. Biol. 6, 1983–1990.
[10] Doetsch, P.W., Helland, D.E. and Haseltine, W.A. (1986) Biochemistry 25, 2212–2220.
[11] Male, R., Helland, D.E. and Kleppe, K. (1985) J. Biol. Chem. 260, 1623–1629.