# DEOXYRIBONUCLEASE II FROM AVIAN LEUKEMIC MYELOBLASTS. II.* SPECIFICITY OF CLEAVAGE, ELECTROPHORESIS OF THE ENZYME AND ITS SUBCELLULAR LOCALIZATION

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DNase II from chick leukemic myeloblasts was characterized from the point of view of specificity of cleavage. The observed resistance of the cleavage products of DNase II toward phosphodiesterase I and their sensitivity to phosphodiesterase II indicate that the cleavage products of myeloblastic DNase II are terminated by a 3′-phosphate. Acrylamide gel electrophoresis confirmed the high degree of purity of the enzyme preparations of myeloblastic DNase II, and two components with different migration rates and different temperature stability with DNase II activity were established. The study of localization of the enzyme in cell fractions showed that the activity of DNase II is associated particularly with the fraction sedimenting at 20 000 g, containing mitochondria and lysosomes.

DNase II isolated and purified from avian myeloblasts was found to possess several properties different from spleen DNase II isolated by Bernardi from hog material[1]. The differences concerned particularly the pH optimum and the effect of univalent and divalent ions.

In the present communication the enzyme is further characterized as to its specificity its electrophoretic behaviour on acrylamide gels and finally as to its localization in isolated cell fractions.

## EXPERIMENTAL

*Specificity of cleavage.* The position of the terminal phosphate in DNA fragments after hydrolysis by DNase II was determined on the basis of chromatography of the products of further cleavage of DNA-oligonucleotides by phosphodiesterase I and II. Cleavage of DNA by myelo-

* Part I: This Journal *36*, 2966 (1971). Abbreviations used: DNA, deoxyribonucleic acid; DNase, deoxyribonuclease; EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)-aminomethane.

blastic DNase II was done under conditions analogous to those of studying the specificity of cleavage by spleen DNase II (ref.[2]) and was modified from the point of view of the enzymological data obtained with the myeloblastic enzyme[3]. The reaction mixture contained: calf thymus DNA (500 μg/ml), potassium acetate of pH 6 (50 μmol/ml), EDTA (10 μmol/ml), DNase II (5 μg/ml). It was incubated in a water bath at 37°C up to an absorbance at 260 nm of 139% ($A_{260}$ was 8·325 but rose to 11·49 within 16 h). After inactivation of the enzyme the product was evaporated. Further cleavage of oligonucleotides with phosphodiesterase I and II was carried out in principle according to Antonoglou and Georgatsos[4]. For cleavage with phosphodiesterase I the following reaction mixture was used: DNase II hydrolysate (equivalent to 750 μg starting DNA); 120 μmol Tris pH 7·8; 7·5 μmol $MgCl_2$; 100 μg snake phsophodiesterase (Worthington Biochemical Corp., USA) in a volume of 250 μl. The enzyme reaction took place in a water bath at 37°C overnight. The reaction mixture for phosphodiesterase II contained in 250 μl: DNase II hydrolyzate (equivalent to 750 μg starting DNA), 37·5 μmol potassium acetate of pH 5, 2·5 μmol EDTA, 100 μg spleen phosphodiesterase (Worthington Biochemical Corp., USA). The enzyme reaction took place in a 37°C water bath overnight.

Chromatographic analysis of fragments after hydrolysis with phosphodiesterases was carried out by two-dimensional paper chromatography[5]. Whole reaction mixtures were analyzed on Whatman No 3 MM at 23°C. The position of the individual spots were established in UV light (Chromatolite).

*Acrylamide gel electrophoresis.* DNase II as a basic protein[6] can be analyzed on acrylamide gels like basic proteins of blood plasma[7]. For electrophoresis of the enzyme isolated from chick leukemic myeloblasts we used 7·5% acrylamide gel. The electrophoresis took place in 0·6% glycine buffer of pH 4·5 in glass tubes 75 mm long and 4 mm in diameter at 4°C for 2 h, at 330 V and 8 mA per 4 tubes. Before polymerization, 48 μg DNase II was added to the sample gel. After electrophoresis the gels were removed from the tubes and either stained with 1% solution of amido-black (Amidoschwarz, Merck) in 7% acetic acid or used for sectioning. The sectioning was done with a blade cutter to 2 mm thickness. The discs were then cut into halves if necessary. One half was stained like the whole gel and bleached in 7% acetic acid overnight, the other half was used for determining the amount of DNase II activity using the isotopic method, at pH 6 (see ref.[3]) when it was found that the results are more reproducible and detection efficiency greater if parts of the gel are left in the incubation mixture and are not removed before precipitation of nonhydrolyzed DNA with perchloric acid. The incubation of the enzyme with substrate took place overnight in a water bath at 50°C.

*Localization of DNase* II *in chick leukemic myeloblasts* was determined by estimating the specific activity of DNase II of isolated cell fractions. The nuclear fraction was isolated according to Busch[8]. Other fractions were isolated from a homogenate of chick leukemic myeloblasts

TABLE I

Specific Activity of DNase II in the Individual Subcellular Fractions of Chick Leukemic Myeloblasts

| Fraction | Nuclear | F 1 | F 2 | F 3 | F 4 |
|---|---|---|---|---|---|
| Specific activity | 0·198 | 25·4 | 34·8 | 10 | 3·04 |
| % | 0·57 | 73 | 100 | 28·8 | 8·7 |

in 3% sucrose in 0·05M Tris-HCl at pH 7. After homogenization, the sucrose concentration was raised to 8·7% (0·25M) and the individual fractions were separated by gradual sedimentation at 1 000 *g* for 20 min, at 5000 *g* for 30 min (Fl), at 20 000 *g* for 30 min (F2), at 105 000 *g* for 30 min (F3), and the final supernatant (F4). The individual cell fractions were stored under dry ice. Nuclei were kept in 0·34M, the other fractions in 0·25M sucrose in 0·05M-Tris-HCl at pH 7. The enzyme activity of DNase II present in the individual fractions was established isotopically after incubating the reaction mixture at pH 6 (see ref.[3]) with aliquots of added cell fractions. The activity observed was expressed as specific activity of DNase II in the given subcellular fraction[3]. Preparation and characterization of calf thymus DNA and the preparatory procedure for myeloblastic DNase II were described before[3].

## RESULTS

It follows from a chromatographic analysis of oligonucleotides formed by hydrolysis with myeloblastic DNase II and exposed to further enzymatic cleavage with phosphodiesterase I and with phosphodiesterase II, myeloblastic DNase II cleavage of the DNA molecules results in an accumulation of cleavage products terminated with 3′-phosphate. This follows from the resistance of the products formed by the action of DNase II toward cleavage with phosphodiesterase I. In this case, there is no difference in the chromatographic pattern of the hydrolytic products of DNA after treatment with DNase II and subsequent action of phosphodiesterase I on the cleavage products (Fig. 1). This finding was confirmed by comparing the effect of phosphodiesterase II, the activity of which is not inhibited by the presence of 3′-phosphate. The contact of phosphodiesterase II with the products of DNA hydrolysis after treatment with DNase II resulted in further profound cleavage of the oligonucleotides present (Fig. 1) which may be seen from the presence of nucleotides and even nucleosides on the chromatograms. According to this characterization, myeloblastic DNase II meets the criteria for DNase II[9] even from the point of view of the effect of exonucleases on the products of DNA cleavage.

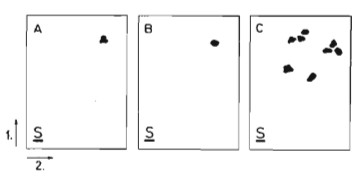


FIG. 1

Hydrolysis of Cleavage Products of DNA by Myeloblastic DNase II Using Phosphodiesterases
S Start. Chromatography of products: A of DNase II, B of phosphodiesterase I, C of phosphodiesterase II. The hydrolytic procedure is described in the text.

Disc electrophoresis was found to be a suitable method for characterizing the degree of purity of our preparation of myeloblastic DNase II on the one hand, while on the other hand it indicated the existence of at least two protein present in the preparations of myeloblastic DNase II which were enzymatically active. A preparation of native DNase II displayed three constant bands, the fastest of these staining richly and displaying marked DNase II activity (Fig. 2*a*), the one migrating more slowly and less richly stained, contained about one-half of the DNase II activity as determined by our procedure. The third band, at the limit of detectability when polymerizing 48 μg DNase II protein with the sample gel, displayed no enzymatic activity. A somewhat different pattern emerged after electrophoresis of the same enzymatic preparation heated to 55°C for 20 min and cooled in ice before polymerization into the gel (Fig. 2*b*). The most rapidly migrating band preserved its activity and stainability, the second band, although clearly present, showed no DNase II activity. The third band could not be distinguished any more and no activity was observed at its assumed location. At the boundary between the starting and the running gel all experiments contained material stainable under the conditions used and containing trace amounts of DNase II activity.

It follows from these findings that the myeloblastic DNase II preparation obtained here is very pure and contains minimum contamination with enzymatically inactive proteins. It followed from these experiments that the enzyme activity of DNase II in chick leukemic myeloblasts is contained in at least two protein species, clearly separated in the system used here, the one migrating more slowly and staining more weakly being sensitive to 55°C.

Determination of specific activity of DNase II in the nuclear fraction of the leukemic myeloblast, in the particulate fractions and in the soluble cell fraction provided information on the intracellular distribution of this enzyme (Table I). The fraction

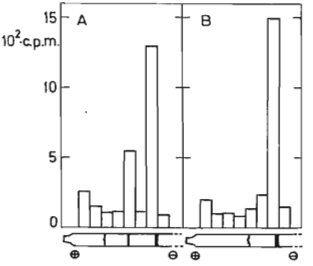


FIG. 2

Disc Electrophoresis of Myeloblastic DNase II and Determination of Enzyme Activity in the Gel

The sample gel was polymerized with 48 μg protein A of native enzyme, B of enzyme heated to 55°C for 20 min. After electrophoresis, 8 discs were sectioned off the column, the first disc being taken from the boundary between the starting and the running gel. The enzyme activity of DNase II was determined by the isotopic method described in the text.

richest in DNase II is the particulate material sedimenting at 20 000 *g* (F 2) and at 5000 *g* (F 1) which consists, according to the data provided by fractionation of chick leukemic myeloblasts, of a mitochondrial and a macrogranular fraction. Another fraction, relatively rich in DNase II, in the fraction sedimenting at 105 000 *g*, containing microsomes (F 3). The specific activity of DNase II is three times lower here than in the F 2 (mitochondrial) fraction. The soluble fraction (F 4) is relatively poor in DNase II and the lowest DNase II activity was found in the nuclear fraction. It follows from these data that DNase II in the chick leukemic myeloblast is a particle-bound enzyme, similar to the DNA-dependent DNA polymerase of mouse fibroblasts during the phase of DNA synthesis[10].

## DISCUSSION

Generally, the specificity of nuclease cleavage is defined by the manner of splitting the phosphodiesterase bond of the corresponding substrate. The product of DNase I action are oligonucleotides terminated with a 5'-phosphate, in the case of DNase II, then oligonucleotides terminated with a 3'-phosphate with a defined sequence of the terminal positions[2,11-13]. The nucleotides thus terminated can serve as substrate either for phosphodiesterase I not inhibited with 5'-P nucleotides, or for phosphodiesterase I, the activity of which is not affected by nucleotides terminated with a 3'-phosphate. The fact that oligonucleotides formed by the action of myeloblastic DNase II on the DNA molecule could be split further by phosphodiesterase II but not by phosphodiesterase I represents another important feature of the enzyme investigated here, placing it among the group of DNases II.

Acrylamide–gel electrophoresis adapted to basic proteins was found to be a suitable method for controlling the purity of the myeloblastic DNase II prepared here. It follows from the present findings that by using a modified isolation procedure one can obtain a protein which was separated electrophoretically into three components of different migration properties, two of which were accompanied by enzymatic activity of DNase II during electrophoresis of the native protein preparation. In the light of these findings, the myeloblastic DNase II purified as shown above[3] may be characterized as a fairly pure preparation with minimum admixtures of protein species without detectable DNase II activity. The presence of two components with enzyme activity of DNase II may be considered from several points of view. It is not likely that they represent an electrophoretic artifact of DNase II splitting during preparation such as might account for the chromatographic findings on columns[14,15] since in the preparatory procedure for pure myeloblastic DNase II the precipitation at pH 2·5 was omitted[3]. On the other hand, it cannot be excluded that DNase II is split artificially during other phases of preparation, bearing in mind the indicated double-peak course of activity of the myeloblastic enzyme released from columns of DEAE-cellulose and hydroxyapatite[3]. It is likely that the two protein

species found in electrophoresis on acrylamide gel and possessing detectable enzyme activity represent rather released subunits of assumed dimer structure of DNase II[15]. Such an explanation is supported by the finding of deviations from the classical course of rate dependence on substrate concentration of this enzyme[3] which constituted the basis for demonstrating oligomeric structure of spleen DNase II[15]. Obviously, one cannot exclude the possibility that DNase II activity in the chick leukemic myeloblast resides in two isoenzymes, separable clearly by acrylamide electrophoresis under the described conditions, the slowly migrating minor species being heat-sensitive. Subcellular fractionation reveals DNase II, together with other ten[16] acid hydrolases, in the lysosomes[17]. This distributions appears to characterize adult tissues as follows from studies on young rats[18]. The myeloblastic enzyme with its pH optimum at 6 was localized in the macrogranular and mitochondrial fractions of the chick leukemic myeloblast. This is in agreement with the lysosomal localization of DNase II since the mitochondrial fraction contains also lysosomes[19]. The exact localization of the enzyme would be established by the use of zonal centrifugation which makes it possible to isolate pure lysosomes[20].

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