STUDIES OF SPECIFICITY OF DEOXYRIBONUCLEASE II FROM THYMUS*

BY ULLA-RIITTA LAURILA AND M. LASKOWSKI

(From the Department of Biochemistry, Marquette University School of Medicine, Milwaukee, Wisconsin)

(Received for publication, March 13, 1957)

The early studies on the specificity of different deoxyribonucleases (DNases) have been reviewed by Schmidt (2). Since that time, digests of deoxyribonucleic acid (DNA) by DNase I and DNase II were compared in respect to the size of fragments (3). More recently, a partially purified DNase from Micrococcus pyogenes (4) has been shown to produce fragments terminated in 3'-phosphate (4, 5). Independently, and almost simultaneously, in Sinsheimer’s laboratory, Koerner (6) concluded that DNase II from spleen also produced fragments terminated in 3'-phosphate. Besides the specificity toward the 5'-bond of the diphosphate linkage, no other specificity requirements were observed for the latter enzyme. A study of a similar DNase II from thymus, presented in this paper, also leads to the conclusion that the reaction products are terminated in 3'-phosphate. Furthermore, it will be shown that the sequence 1 Pyp-Pup is absent from the products, thus suggesting that this linkage is preferentially attacked by DNase II.

Methods

DNA was prepared according to Kay, Simmons, and Dounce (9). DNase II was purified through the first three steps of the method of Laskowski et al. (10) and was then chromatographed on substituted celluloses prepared according to Peterson2 and Sober (11). The chromatographic procedure was that of Watson (12). The solution of DNase II, dialyzed against 0.005 M phosphate buffer, pH 6.7, was passed through a column

* Supported by grants from the Atomic Energy Commission and the American Cancer Society. A preliminary report has been published (1).

1 The system of abbreviations according to Smith and Markham (7) is that used previously (8). The capital letter designates a nucleoside; p preceding the capital letter signifies the 5'-phosphoryl group, p between the two capital letters signifies the secondary phosphoryl group linking the 3'-carbon of the preceding nucleoside with 5'-carbon of the following nucleoside, p following the capital letter signifies a 3'-phosphoryl group; (d) signifies that the sugar component is deoxyribose. A = adenosine, G = guanosine, C = cytosine, T = thymine, X, Y, and Z are used for non-specified nucleosides, Pu = purine nucleoside, and Py = pyrimidine nucleoside.

2 We are indebted to Dr. E. A. Peterson for the gift of his substituted celluloses.
(2 × 20 cm.) of diethylaminoethyl-cellulose adjusted to the same buffer. The enzyme emerged in the first peak, whereas a considerable amount of impurities was retained. The enzyme was lyophilized and dialyzed against 0.1 M phosphate buffer, pH 6. The solution of enzyme was placed on the column of carboxymethyl-cellulose (2 × 20 cm.) adjusted to the same buffer. The first peak containing the impurities was discarded. The enzyme was eluted with 0.2 M phosphate buffer, pH 6, and lyophilized. The potency of the fresh preparation was around 4.0 (Δ$E_{260}$ of digest per minute per $E_{260}$ of enzyme solution), expressed as described previously (10). After several months of storage, the potency dropped to 2.5.

Because our present batch of venom (Crotalus adamanteus) differed from that used previously, it was necessary to modify Step 1 of the procedure for preparing phosphodiesterase (13). Venom was dissolved in 0.3 M acetate buffer, pH 6.4, and was passed through a column of Amberlite IRC-50-XE-64. The first peak which contained phosphodiesterase and some 5'-nucleotidase was lyophilized and dialyzed against 0.2 M acetate, pH 6.2. It was rechromatographed on the same resin, adjusted to the above buffer. The first peak was discarded and the enzyme was eluted with 0.6 M acetate buffer, pH 6.4. When assayed for the contaminating 5'-nucleotidase, less than 2.0 per cent thymidine was found after 2 mg. of thymidyl acid were incubated for 3 hours with 0.08 unit of phosphodiesterase. Further procedure of purification of phosphodiesterase was identical with that described (13).

The digested DNA was chromatographed on Dowex 1-X (2 per cent cross-linked) by the slightly modified (4) method of Sinsheimer (14). The fragments thus obtained were identified by using ionophoresis (15), chromatography of mononucleotides (15), nucleosides (16), and bases (17). The bases were obtained after a complete hydrolysis with formic acid (18).

Results

300 mg. of DNA were treated with 12 units (10) of DNase II and incubated at 37° for a total of 18 hours. The rapid phase of reaction evidenced by the loss of viscosity was terminated in 2 hours; no definite statement can be made concerning the termination of the slow phase. The digest was placed on a column of Dowex 1-X2 and was eluted with ammonium formate buffers. Figs. 1 and 2 illustrate the results. The scale on the ordinates is the same in both Figs. 1 and 2, but the horizontal scale in Fig. 1 is 8-fold larger in order to magnify the beginning of the pattern. Com-

3 All samples were purchased from Ross Allen's Reptile Institute, Silver Springs, Florida.

4 Thymidylic acid was found more sensitive to 5'-nucleotidase than the commercial 5'-adenylic acid, which had been used previously.
parison of this pattern with that previously obtained (3) with smaller amounts of enzyme and a shorter incubation period indicates considerable similarity: a very small 0.25 M fraction, a small 0.5 M fraction, a very large 2 M fraction, and a moderate but definite 3 M fraction. The only marked difference is that the amount of mononucleotides is increased in the present experiment. This would appear to be in agreement with the observation of Koerner (6) that mononucleotides appear after the fast phase of the reaction has ended.

Table I shows the relative amounts of different fractions. The results are expressed in per cent of the total material being absorbed at 260 m\textmu.
after the end of the digestion. Recovery higher than 100 per cent is caused by the background of the resin, which, unfortunately, was fairly high when eluting buffers of high molarity were used.

**Table I**

Relative Quantities of DNA Fragments Eluted with Buffers of Increasing Molarity

<table>
<thead>
<tr>
<th>Molarity of eluent</th>
<th>Per cent total optical density</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1*</td>
<td>16.1†</td>
</tr>
<tr>
<td>0.25</td>
<td>1.0</td>
</tr>
<tr>
<td>0.50</td>
<td>8.0</td>
</tr>
<tr>
<td>1.00</td>
<td>24.4</td>
</tr>
<tr>
<td>2.00</td>
<td>46.5</td>
</tr>
<tr>
<td>3.00</td>
<td>11.8</td>
</tr>
<tr>
<td>Total</td>
<td>107.8</td>
</tr>
</tbody>
</table>

* Sum of mono-, di-, and trinucleotides.
† Mononucleotides, 7.3; dinucleotides, 3.3; and trinucleotides, 5.5.

Fig. 3. Chromatography of deoxycytidylic acid: upper, before exposure to crude snake venom as a source of 5'-nucleotidase; lower, after exposure. Incubation 2 hours at 37° with 1 mg. of venom, glycine-NaOH buffer, pH 9.0, in the presence of Mg++. Column Dowex 1-X2, 0.8 X 10 cm. Eluent, 0.01 M ammonium formate, pH 4.5; volumes as indicated.

Peaks 1 and 2 (Fig. 1) were each subjected to a second chromatography to isolate pure mononucleotides. Each mononucleotide was then subjected to the action of crude venom of *C. adamanteus* as a source of 5'-nucleotidase, after which the mixture was rechromatographed under conditions identical with those used before the digestion with venom. Fig. 3 shows an example of chromatography of deoxycytidylic acid before and after digestion with 5'-nucleotidase. No significant quantities of nucleo-
side were observed, whereas the nucleotide emerged at the same place in a good yield. Experiments of this type were performed with each nucleotide and the results are presented in Table II. The average recovery of

<table>
<thead>
<tr>
<th>Deoxynucleoside</th>
<th>Amount before digestion</th>
<th>Recovery after digestion with crude venom</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cytidylic acid</td>
<td>0.79 mg.</td>
<td>0.68 mg.</td>
</tr>
<tr>
<td>Thymidylic acid</td>
<td>0.33 mg.</td>
<td>0.30 mg.</td>
</tr>
<tr>
<td>Adenylic acid</td>
<td>1.18 mg.</td>
<td>1.10 mg.</td>
</tr>
<tr>
<td>Guanylic acid</td>
<td>1.37 mg.</td>
<td>1.19 mg.</td>
</tr>
</tbody>
</table>

Peaks 3 to 8 (Fig. 1), which followed mononucleotides in the original digest, were subjected either to rechromatography or to ionophoresis, which led to a better separation. Fig. 4 is a composite of several standardized ionophoretic runs. Peaks 3, 4, 6, and 8 were successfully separated into several components. Ionophoresis was then repeated on a larger scale with the use of streaks instead of spots. The separated streaks were
eluted, lyophilized, and used for identification by means of degradation with purified phosphodiesterase.

The identification of dinucleotides was accomplished by the same method, which has been already used (4) for identification of (d)ApCp. Since degradation with phosphodiesterase produced from each dinucleotide only a nucleoside and nucleoside diphosphate, the original dinucleotides must have been of a general structure (d)XpYp. The nucleoside was easily identified by paper chromatography (16) or ionophoresis (15). The nucleoside diphosphate was identified by either ionophoresis (15) with a mobility approximately twice that of a corresponding mononucleotide, or by chromatography on Dowex 1, where it emerges with 0.25 m formate.

From the previous considerations, it may be expected that a trinucleotide terminated in 3'-phosphate, (d)XpYpZp, after complete digestion with phosphodiesterase, will yield (d)X, (d)pY, and (d)pZp. The fraction of Peak 8 (Fig. 1), which upon complete hydrolysis with formic acid (18) showed adenine, cytosine, and guanine, was subjected to the action of phosphodiesterase with the results that guanosine was identified by chromatography (16) in Hotchkiss' system and by ionophoresis (15); deoxyadenylic acid was identified by chromatography in Markham and Smith's system and by ionophoresis (15), and 3',5'-deoxycytidine diphosphate was identified by ionophoresis (15). Table III summarizes the results of these experiments. The dinucleotides identified accounted for the three possible combinations. Two of these three were also present in the trinucleotide. The fourth combination, Pyp-Pup, was missing, suggesting that this linkage is preferentially hydrolyzed by DNase II.

**DISCUSSION**

Phosphodiesterase of venom permits the determination of both end groups of fragments of deoxyribonucleic acids terminated in 3'-phosphate.

<table>
<thead>
<tr>
<th>Peak No.</th>
<th>Identified by</th>
<th>Compound</th>
<th>Type of combination</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>Ionophoresis on paper</td>
<td>(d)CpCp</td>
<td>Pyp-Pyp</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(d)CpTp</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>Ionophoresis on paper</td>
<td>(d)ApTp</td>
<td>Pyp-Pyp</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(d)ApAp</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>Chromatography on Dowex 1-X2</td>
<td>(d)GpAp</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>Ionophoresis on paper</td>
<td>(d)GpApCp</td>
<td></td>
</tr>
</tbody>
</table>
The terminal mononucleotide carrying a free phosphoryl group will appear as a nucleoside 3', 5'-diphosphate, whereas the terminal mononucleotide end deprived of the free phosphoryl group will appear as a nucleoside. The method has an obvious and an immediate usefulness for chains of any length, provided that the phosphodiesterase preparation is free from 5'-nucleotidase. In degrading trinucleotides and larger compounds, purity is particularly important since 5'-mononucleotides obtained from the center of the fragment are rapidly dephosphorylated by crude venom. For identifying the 3' end group, the purity of phosphodiesterase is of little significance because mononucleoside diphosphates are resistant to the action of 5'-nucleotidase. Therefore, for identification of dinucleotides, even a comparatively crude preparation is satisfactory.

We have recently postulated (7) that trinucleotides terminated in 5'-phosphate are attacked by phosphodiesterase by a consecutive liberation of mononucleotides from the 5' end. In a trinucleotide terminated in 3'-phosphate the following sequence of events is postulated: first, (d)XpYpZp → (d)X + (d)pYpZp and then (d)pYpZp → (d)pY + (d)pZp. This working hypothesis is based on the observed differences in rates of hydrolysis of (d)pCpA and (d)CpA (8) as well as of (d)ApCp (3).

From the identified products of the reaction, it is tentatively concluded that the linkage Pyp-Pup is preferentially attacked by DNase II. Recently, a similar tentative conclusion was advanced (3) for the explanation of specificity of DNase I, which preferentially splits the sequence pPu-pPy. In both cases, therefore, requirements for specificity involve the side of the internucleotide bond (3'-bond versus 5'-bond) as well as the bases of nucleosides, preceding and following the internucleotide bond. So far, no attempts were made to correlate the proposed preferential hydrolysis of the Pyp-Pup bond with the rapid (or slow) phase of the reaction observed for DNase II (6). In view of persistent efforts in several laboratories to purify DNase II further, it appears wiser to postpone such attempts until a purer enzyme is available.

SUMMARY

Partially purified deoxyribonuclease (DNase II) from calf thymus digested deoxyribonucleic acid into fragments of variable size. The smallest fragments were 3'-mononucleotides. Five dinucleotides and one trinucleotide, all terminated in 3'-phosphate, were isolated and identified. The sequence Pyp-Pup was absent from the identified products. It is suggested that this linkage is preferentially hydrolyzed by DNase II.

BIBLIOGRAPHY

STUDIES OF SPECIFICITY OF DEOXYRIBONUCLEASE II FROM THYMUS
Ulla-Riitta Laurila and M. Laskowski

J. Biol. Chem. 1957, 228:49-56.

Access the most updated version of this article at http://www.jbc.org/content/228/1/49.citation

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 0 references, 0 of which can be accessed free at http://www.jbc.org/content/228/1/49.citation.full.html#ref-list-1