The Separation of the Phosphodiesterase and Deoxyribonuclease II Activities of Bovine Spleen

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SUMMARY

Deoxyribonuclease II (Deoxyribonucleate 3'-nucleotidohydrolase, EC 3.1.4.6) was purified 1250-fold from bovine spleen. The specific activity of the final preparation was 408. It was free of phosphatase and alkaline ribonuclease, and almost free of nonspecific phosphodiesterase, acid ribonuclease, and adenosine triphosphatase. Chromatography on carboxymethyl cellulose prior to heating caused the bulk of the deoxyribonuclease to be eluted early, in a symmetrical peak containing phosphodiesterase (orthophosphoric diester phosphohydrolase, EC 3.1.4.1). After the crude preparation was heated at 60°, the symmetrical peak disappeared and was replaced by separate phosphodiesterase and deoxyribonuclease peaks. Repeated chromatography of the unheated preparation or mild heating (37°, with or without 2-mercaptoethanol) of the symmetrical peak caused changes similar to those seen after heat treatment of the crude preparation. The chromatographic data, heat inactivation, pH optimum, and activity on a number of phosphodiesters support the contention that the phosphodiesterase and deoxyribonuclease activities of the unheated preparation are on separate proteins.

Recently, the suggestion was made that the ability to hydrolyze this substrate is an intrinsic, albeit weak, property of pure DNase II (1). This claim was based largely on the tendency of the two activities to be eluted in constant proportion from chromatographic columns, as well as on certain other properties shared by the activities. In this paper, some conditions under which DNase II and the nonspecific phosphodiesterase chromatograph together are shown, and the conditions for the separation of the enzymes are described. Furthermore, it is shown that the trace of phosphodiesterase remaining in the DNase II peak bears no constant relationship to the latter activity, is almost devoid of activity on dinucleotide analogues, and has no activity against the oligonucleotides of ribonuclease-resistant "core." The phosphodiesterase is therefore a contaminant, and probably has little or no significant bearing on the biological actions of DNase II.

EXPERIMENTAL PROCEDURE

Materials

Cm-cellulose and sodium bis(p-nitrophenyl) phosphate were purchased from Sigma, DEAE-cellulose for thin layer chromatography from Bio-Rad Laboratories, Richmond, California, and p-nitrophenyl thymidine 5'-phosphate and ATP from Calbiochem. Thymidine 3'-p-nitrophenyl phosphate was a gift from Dr. Koert Gerzon of Eli Lilly and Company, who prepared it according to the procedure of Turner and Khorana (2). Bovine spleen and thymus were obtained from a local slaughterhouse and kept frozen until used. Calf thymus DNA was prepared by salt extraction and deproteinization with sodium dodecyl sulfate (3).

Methods

Column Chromatography—Carboxymethyl cellulose was cycled according to the procedure of Stehelin (4). Chromatography was performed at 4°, under a hydrostatic head of 30 cm.

Enzyme Assays—The assays for DNase, RNase, phosphodiesterase, and phosphatase have been described before (5). The results for phosphodiesterase are reported in the units described.
by a modification of the method of Laskowski and Filipowicz assayed by the method of Hilmoe (7). ATPase was determined by thin layer chromatography (9). Activity against RNase-resistant core was assayed by Laskowski (6). Activity against RNase-resistant core was assayed by the method of Hilmo (7). ATPase was determined by a modification of the method of Laskowski and Filipowicz (8) permitting separation of the nucleotide products of digestion by thin layer chromatography (9).

RESULTS

The experiments were performed on DNase II prepared by a slight modification of the method of Hodes and Swenson (10). A typical preparation is summarized in Table I.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Volume</th>
<th>Total A280</th>
<th>Total DNase II</th>
<th>Specific activity</th>
<th>Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH 4.5</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Halted</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dialyzed</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cm-cellulose</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>274</td>
<td>82.2</td>
<td>3,365</td>
<td>40.9</td>
<td>46</td>
</tr>
<tr>
<td>II</td>
<td>15</td>
<td>21.3</td>
<td>3,000</td>
<td>141</td>
<td>41</td>
</tr>
<tr>
<td>III</td>
<td>9</td>
<td>6.2</td>
<td>2,040</td>
<td>40.7</td>
<td>39</td>
</tr>
</tbody>
</table>

by Laskowski (6). Activity against RNase-resistant core was assayed by the method of Hilmo (7). ATPase was determined by a modification of the method of Laskowski and Filipowicz (8) permitting separation of the nucleotide products of digestion by thin layer chromatography (9).

In tube 100 the phosphodiesterase to DNase ratio was 1:20,000. By the third rechromatography, the preparation was almost entirely devoid of phosphodiesterase (Table I), and the enzymes had no constant relation to each other.

A similar change in elution position of DNase II after heating at 37° for 2 hours in 0.05 M 2-mercaptoethanol was noted by Kates and McAuslan (11). This was attributed to a molecular modification induced by the mercaptoethanol. However, they did not study phosphodiesterase activity. The experiments of Kates and McAuslan was repeated with measurement of both DNase and phosphodiesterase activities. An unheated preparation was divided in half after the dialysis step. One portion was chromatographed on Cm-cellulose, whereas the other half was heated before chromatography at 37° for 2 hours in the presence of 0.05 M 2-mercaptoethanol in sodium acetate buffer, pH 5.5. The results are shown in Fig. 3. The DNase and phosphodiesterase activities of the unheated portion were eluted together. However, in the portion heated with 2-mercaptoethanol, the DNase and phosphodiesterase were separated by 40 tubes (80 ml).

In order to determine the role of the 2-mercaptoethanol the experiment was repeated, but this time the unheated portion was warmed at 37° for 2 hours in the buffer alone. The shift in elution position was identical with that obtained in the presence of 2-mercaptoethanol. Thus, the shift in position was due to the reducing atmosphere of the 2-mercaptoethanol but not to the heating.

These results could be interpreted as a tendency for DNase II and phosphodiesterase to aggregate at low (cold room) temperatures. Chromatography at higher temperatures might then allow for separation of these enzymes.

![Fig. 1. Initial chromatographic purification of deoxyribonuclease II on Cm-cellulose. The unheated extract obtained from the dialysis step (see Table I) was applied to the column. The DNase II was eluted with 0.5 M ammonium acetate buffer, pH 5.5. Column dimensions were 3.5 X 9.0 cm; fraction size, 2 ml; flow rate, 1 ml per min; total volume, 200 ml.](http://www.jbc.org/cover.html)
promote separation. However, all attempts to perform the separation at 25°C resulted in large losses of DNase activity.

Specificity of Phosphodiesterase—The small amounts of p-nitrophenyl phosphodiester available made it necessary to determine the activities of the preparations under a single set of conditions. Those were optimal for activity against the bis(p-nitrophenyl) phosphate, and may or may not have been best for the other substrates. Activity was always greater with bis(p-nitrophenyl) phosphate than with the thymidine 3'- or 5'-diesters. The bulk of the activities against all three substrates was eluted before the DNase, and the phosphodiesterase trailing into the DNase peak exhibited almost no activity against the thymidine 5'-diester. The exonuclease activity was eluted with the bulk of the other phosphodiesterases and, in consonance with the findings of other authors (12, 13), the DNase peak was devoid of action against small oligonucleotides. The chromatographic behavior of the phosphodiesterases is portrayed in Fig. 4. In this preparation, previously chromatographed only once, more than the usual amount of the activity against the 3'-diester was carried into the DNase peak.

None of the peaks exhibited much activity against ATP, and indeed the ATPase activity was eluted as a separate peak after the bulk of the DNase II (data not shown).

Heat Inactivation of Purified Enzyme—A preparation that had been subjected to a single chromatography on Cm-cellulose but had not been heated was used for this experiment. The eluate was adjusted to pH 3.5 and then was held at 50°C or 60°C for 5 to 15 min. Both DNase and phosphodiesterase were equally sensitive to heating at 60°C, but at 50°C the latter was inactivated more rapidly (Fig. 5).

The DNase II of crude extracts was more stable at elevated temperatures and most preparations were processed at 60°C (Table I).

![Fig. 2. The second fractionation of deoxyribonuclease II on Cm-cellulose. Both unheated and heated preparations are shown. For details, see the text. The DNase II was eluted with a linear gradient of 0.05 M to 0.5 M ammonium acetate buffer, pH 6.0. Column dimensions were 18 × 2.4 cm; flow rate, 1 ml per min; fraction size, 2 ml; total volume, 250 ml.](image_url)
**FIG. 4.** Separation of DNase II from accompanying phosphodiesterase activities. The eluate from the first Cm-cellulose column of a heated preparation was dialyzed and applied to a column, 11 × 1.8 cm. Elution was as in Fig. 2, except that total volume was 400 ml. Core refers to the nondialyzable oligonucleotides remaining after exhaustive digestion of RNA with pancreatic RNase (10), bis to the action against sodium bis-(p-nitrophenyl) phosphate, 3' to that against thymidine 3'-p-nitrophenyl phosphate, and 5' to that against p-nitrophenyl thymidine 5'-phosphate.

**FIG. 5.** Stability of deoxyribonuclease and phosphodiesterase activities of the partially purified enzyme as a function of time of heating at 50° and pH 3.5.

**DISCUSSION**

One important aspect of the study of the phosphodiesterase activity accompanying or contaminating the DNase II is the relevance of that activity to the action of DNase itself. Unfortunately, no really satisfactory method is available for determination of DNase II, and determination of phosphodiesterase activity is complicated by the presence of a number of enzymes of sometimes overlapping activity able to hydrolyze doubly esterified orthophosphoric acid. Nevertheless, it seems clear, from studies on both DNase II (12, 13) and "nonspecific" phosphodiesterase, that the activity of the former is endonucleolytic and is limited to larger polynucleotides. The phosphodiesterase that remains associated with the DNase after extensive purification has, as shown here and by others (1), an activity that is almost limited to bis(p-nitrophenyl) phosphate. This enzyme is nearly inert against the p-nitrophenyl analogues of the dinucleotides and the ribonuclease-resistant oligonucleotides of core, whereas the spleen exonuclease (EC 3.1.4.1) readily hydrolyzes thymidine 3'-p-nitrophenyl phosphate and core, and snake venom diesterase (EC 3.1.4.1) hydrolyzes the 5'-analogues. This contaminating bis(p-nitrophenyl) phosphodiesterase is also not active against polyphosphates, such as ATP.

If the bis(p-nitrophenyl) phosphate diesterase activity associated with the DNase is not likely to interfere with or complicate the action of DNase on DNA, does it represent an incidental action of that enzyme, a "mistake," or is it due to a persistent contaminant? The experiments reported here support the latter contention. The heat stabilities (Fig. 5), as well as the pH
optimum and stability to sodium dodecyl sulfate (data not presented), differ significantly for the two enzymes.

A number of the enzymes hydrolyzing phosphate mono- and diesters have similar chromatographic properties (14, 15) and various ancillary techniques must be used to separate them. The nature of the association between bis(p-nitrophenyl) phosphodiesterase and DNase is not clear, but physical treatments, such as mild heating, freezing and thawing and, exposure to salt, make it relatively easy to separate the activities. Separation is accompanied by a shift in the positions of both the diesterase (to the low salt side) and the DNase (to the high salt side). In order to show this effect, all columns used in refractionation (Figs. 2 and 3) were of the same dimensions and were prepared in the same manner. The changes in elution position are very obvious. These shifts could be due to changes in charge (increasingly negative for phosphodiesterase and positive for DNase) or conformation or both. Neither electrophoresis in polyacrylamide gel nor chromatography on Sephadex has proved useful in resolving this problem.

Whatever the nature of the bonding forces, these changes, once initiated, are permanent. They are not necessarily, as thought by Kates and McAuslan (11), associated with changes in sulfhydryl linkages. It is of course obvious that the two activities are present on separate proteins and are not, as previously thought (1), due to a single active site. Furthermore, the separation of DNase II from the activity hydrolyzing the “specific” phosphodiester, thymidine 3’-p-nitrophenyl phosphate, makes

REFERENCES

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