Studies on Poly Adenosine Diphosphate-Ribose

VII. METHODS OF SEPARATION AND IDENTIFICATION OF 2'-(5'-PHOSPHORIBOSYL)-5'-ADENOSINE MONOPHOSPHATE, RIBOSYLADENOSINE MONOPHOSPHATE, AND PHOSPHORIBOSYLADENOSINE*

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SUMMARY

A chromatographic method with a Dowex 1 column is reported for separation of PR-AMP (2'-(5'-phosphoribosyl)-5'-AMP), 2'-(ribosyl)-5'-AMP, 2'-(5'-phosphoribosyl)-adenosine, and 2'-(ribosyl)-adenosine, produced enzymatically from poly ADP-ribose. PR-AMP and related compounds were also separated by paper chromatography.

The formation of polyadenosine diphosphate-ribose from NAD has been well documented by Chambon et al. (1), Nishizuka et al. (2), and us (3, 4). It has been suggested that this polymer occurs naturally (5).

Poly ADP-ribose (I) is hydrolyzed at the pyrophosphate bond by snake venom phosphodiesterase (1), yielding PR-AMP, that is, 2'-(5'-phosphoribosyl)-5'-AMP (II). A small amount of 3'-AMP (III) was also recovered (9) from the hydrolysate. A phosphodiesterase purified from rat liver (10-12) can also separate PR-AMP from its dephosphorylated derivatives, 2'-(5'-phosphoribosyl)-5'-AMP (IV) and 2'-(5'-phosphoribosyl)-adenosine (VI). The nuclear enzyme preparation used for the synthesis of poly ADP-ribose from NAD possessed phosphodiesterase and phosphononesterase activities and it is likely that the terminal structure of polymer with AMP in its intact form is altered by these enzymes, as indicated in Fig. 1. 2'-(Ribosyl)-5'-AMP can be produced by Pathway a, b, or c in Fig. 1. During incubation with the nuclear enzyme preparation and during purification, cleavage may occur at the pyrophosphate bond, as indicated in Fig. 1d. 2'-(5'-Phosphoribosyl)-5'-AMP may be formed through Pathway d, b, or c in Fig. 1.

Phosphodiesterase preparations from snake venom are often contaminated with 3'-nucleotidease (13). 2'-(5'-Phosphoribosyl)-adenosine and 2'-(ribosyl)-5'-AMP may be produced by removal of phosphate from PR-AMP.

For precise determination of the chain length of poly ADP-ribose, and of the terminal structure of this polymer, methods for separation and identification of these compounds are essential. This paper describes the separation of these compounds and related substances, including 2'-(ribosyl)-adenosine, by column and paper chromatography.

MATERIALS AND METHODS

Preparation of Labeled NAD—NAD labeled with 32P in both phosphate groups was obtained from yeast, Saccharomyces cerevisiae, grown in 100 ml of medium containing 2 g of Proteose peptone (Difco No. 3), 2 g of glucose, 0.5 g of yeast extract (Difco), and 10 to 30 mCi of 32P-inorganic phosphate. Cells in the logarithmic phase of growth were harvested and NAD was extracted with 0.5 N perchloric acid after grinding with quartz sand. NAD labeled with 32P purified with an appropriate amount of nonlabeled NAD by Dowex 1 column chromatography, eluting with a gradient of formic acid (0 to 0.8 M). This NAD preparation gave only a single radioactive spot with two different solvent systems on paper chromatography. To obtain NAD labeled only at the phosphate of its AMP moiety, α-32P-labeled ATP and NNN were incubated with NAD pyrophosphorylase and the resulting NAD was purified as described above. NAD labeled at the adenine moiety was obtained by reaction of (S-32P-adenine)-ATP and NNN. NAD labeled with 32P only at the NNN moiety was prepared from ATP and NNN labeled with 32P, obtained from NAD labeled with 32P at both phosphates by hydrolysis with snake venom phosphodiesterase (14).

Preparation of Purified Poly ADP-ribose—A rat liver nuclear enzyme preparation was obtained as described previously (4), with the slight modification that 0.3 mM magnesium ion was added to the 2.4 M sucrose solution used for isolation of cell nuclei. The enzyme preparation was incubated with labeled NAD in 0.1 M Tris-HCl buffer solution (pH 8.0) for 10 min at 37°, as reported before (4). From the acid-insoluble fraction precipitated
by acetate-buffer and ethanol, poly ADP-ribose was purified with Pronase digestion, phenol extraction, RNase and DNase digestion, and gel filtration as reported elsewhere.

Purification of PR-AMP—Purified poly ADP-ribose was hydrolyzed completely with snake venom phosphodiesterase which was free from 5'-nucleotidase. PR-AMP was purified by Dowex 1 formate column chromatography, as described before (7, 8). More than 90% of the total radioactivity in poly ADP-ribose was recovered as PR-AMP.

Preparation of 2'-((Ribosyl)-5'-AMP—2'-((Ribosyl)-5'-AMP was obtained as follows. PR-AMP labeled at the 5'-phosphate of the AMP moiety with 32P was prepared from poly ADP-ribose synthesized from NAD labeled with 32P at the 5'-phosphate of AMP moiety. It was partially digested by alkaline phosphomonoesterase and subjected to column chromatography with Dowex 1, chloride form, as described below. 2'-((Ribosyl)-5'-AMP was recovered as a peak with radioactivity, eluted just before the 5'-AMP used as a marker.

Preparation of 2'-((5'-Phosphoribosyl)-adenosine—2'-((5'-Phosphoribosyl)-adenosine was obtained by partial digestion of PR-AMP labeled with 32P at the 5'-phosphate which was derived from poly ADP-ribose prepared with NAD labeled at the phosphate of the NMN moiety. It was recovered as a peak with radioactivity, between the markers 5'-AMP and 2'-AMP. HCl was removed from the eluate containing 2'-((ribosyl)-5'-AMP or 2'-((5'-phosphoribosyl)-adenosine by evaporation.

Preparation of 2'-((Ribosyl)-adenosine-8-14C—Adenine-labeled PR-AMP, derived from poly ADP-ribose labeled at the adenine, was extensively digested by alkaline phosphomonoesterase and subjected to the same column chromatography as that described in the previous section. 2'-((Ribosyl)-adenosine labeled with 14C-adenine was eluted soon after the start of gradient elution.

Column Chromatography—Column chromatography on Dowex 1-X2 (chloride form) was carried out with convex gradient elution, achieved by placing 420 ml of 0.0035 N HCl in the mixing chamber and 0.25 m NaCl in the reservoir of the column, 0.5 × 20 cm (15).

Paper Chromatography—Descending single dimensional chromatography was carried out with Solvent System 1 or 2 at 25°. System 1 was a mixture of isobutyric acid-concentrated ammonia-water (66:1:33, v/v/v), and System 2 was a mixture of 0.1 M phosphate buffer (pH 6.8)-ammonium sulfate-propanol-1 (100:60:2, v/v/w).

Enzyme and Chemicals—Snake venom phosphodiesterase was purchased from Worthington. Purified snake venom phosphodiesterase was obtained by column chromatography on Dowex 1, chloride form. Alkaline phosphomonoesterase was obtained by column chromatography on Dowex 1, chloride form, as described before (15). Alkaline phosphomonoesterase was purchased from Worthington. Alkaline phosphomonoesterase was partially digested by alkaline phosphomonoesterase and subjected to column chromatography with Dowex 1, chloride form. Pronase digestion, phenol extraction, RNase and DNase digestion was carried out with Solvent System 1, 25°.

RESULTS

Column Chromatography—The typical chromatographic elution patterns of PR-AMP, 2'-((5'-phosphoribosyl)-adenosine, 2'-((ribosyl)-5'-AMP, and 2'-((ribosyl)-adenosine and other related compounds are illustrated in Fig. 2. 2'-((Ribosyl)-adenosine was eluted soon after the start of convex gradient elution. 2'-((Ribosyl)-5'-AMP was eluted just before 5'-AMP and 2'-((5'-phosphoribosyl)-adenosine was eluted between 5'-AMP and 2'-AMP. PR-AMP was eluted immediately after 5'-GMP and overlapped the latter. Inorganic phosphate and ribose 5-phosphate were eluted between 3'-AMP and 5'-UMP.

Paper Chromatography—The running distances of various substances are given in Table I. 2'-((Ribosyl)-adenosine was separated from 2'-AMP with Solvent Systems 1 and 2, and from adenosine with System 2. 2'-((Ribosyl)-5'-AMP and 2'-((5'-phosphoribosyl)-adenosine were well separated from 5'-AMP and 3'-AMP with Solvent System 2, while they move very close to each

other with System 1. PR-AMP can be separated from ATP or 3',5'-ADP with Solvent System 1 and from ADP-ribose with Solvent System 2.

**Table I**

<table>
<thead>
<tr>
<th>Compound</th>
<th>Running distance (cm)</th>
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<tbody>
<tr>
<td></td>
<td>Solvent 1</td>
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<tr>
<td>PR-AMP</td>
<td>16.2</td>
</tr>
<tr>
<td>2'-5'-Phosphoribosyl-adenosine</td>
<td>28.7</td>
</tr>
<tr>
<td>2'-Ribosyl-5'-AMP</td>
<td>26.7</td>
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<tr>
<td>2'-Ribosyl-adenosine</td>
<td>38.9</td>
</tr>
<tr>
<td>ATP</td>
<td>13.2</td>
</tr>
<tr>
<td>5'-AMP</td>
<td>19.2</td>
</tr>
<tr>
<td>2',5'-ADP</td>
<td>16.8</td>
</tr>
<tr>
<td>3',5'-ADP</td>
<td>18.0</td>
</tr>
<tr>
<td>NAD</td>
<td>22.5</td>
</tr>
<tr>
<td>ADP-ribose</td>
<td>15.2</td>
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<tr>
<td>5'-AMP</td>
<td>26.3</td>
</tr>
<tr>
<td>3'-AMP</td>
<td>29.9</td>
</tr>
<tr>
<td>2'-AMP</td>
<td>29.9</td>
</tr>
<tr>
<td>Ribose 5-phosphate</td>
<td>10.0</td>
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<tr>
<td>Adenosine</td>
<td>38.9</td>
</tr>
<tr>
<td>Adenine</td>
<td>42.0</td>
</tr>
<tr>
<td>Inorganic phosphate</td>
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**Table II**

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<tr>
<td>2'-(Ribosyl)-5'-AMP</td>
<td>372</td>
</tr>
<tr>
<td>PR-AMP</td>
<td>9030</td>
</tr>
</tbody>
</table>

It was also observed that phosphate at position 5' was more susceptible to attack by the phosphomonoesterase activity in the rat liver phosphodiesterase preparation than phosphate at position 5' of PR-AMP.

**Table II**

<table>
<thead>
<tr>
<th>Compound</th>
<th>cpm</th>
</tr>
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<tbody>
<tr>
<td>2'-(Ribosyl)-5'-AMP</td>
<td>372</td>
</tr>
<tr>
<td>PR-AMP</td>
<td>9030</td>
</tr>
</tbody>
</table>

It was also observed that phosphate at position 5' was more susceptible to attack by the phosphomonoesterase activity in the rat liver phosphodiesterase preparation than phosphate at position 5' of PR-AMP.

**Formation of Dephosphorylated Derivatives of PR-AMP by Rat Liver Phosphodiesterase**

- PR-AMP is more rapidly as a dephosphorylated derivative than 2'-(ribosyl)-adenosine at position 5' was more susceptible to attack by the phosphomonoesterase activity in the rat liver phosphodiesterase preparation than phosphate at position 5' of PR-AMP.

**Effect of Commercial Preparation of Snake Venom Phosphodiesterase on PR-AMP**

- PR-AMP was incubated with snake venom phosphodiesterase (Worthington, Lot VP 283C) at a concentration of 5000 µg per ml in 50 mM Tris-HCl buffer (pH 8.0) and 5 mM MgCl₂ for 90 min at 37°C. These conditions did not yield any dephosphorylated derivative of PR-AMP. This indicates that the commercial preparation of snake venom phosphodiesterase may be contaminated to a significant extent by enzymes yielding dephosphorylated derivatives of PR-AMP. Thus it is recommended that 5'-nucleotidase activity should be removed by the method of Keller (13).

**Formation of Dephosphorylated Derivatives of PR-AMP by Rat Liver Phosphodiesterase**

- PR-AMP was incubated with rat liver phosphodiesterase under the conditions given in the legend of Fig. 4 and dephosphorylated derivatives of PR-AMP were formed.
ribose. This trimmed poly ADP-ribose was purified by passage through a Sephadex G-50 column and digested by 30 milliunits of alkaline phosphomonoesterase in 0.2 M Tris-HCl buffer (pH 8.0) to remove phosphate in the monooester form at the terminus. After incubation the mixture was extracted with phenol to eliminate the phosphomonoesterase activity. Finally, the poly ADP-ribose preparation was completely hydrolyzed by incubation with 10 units of purified snake venom phosphodiesterase in 50 mM Tris-HCl buffer (pH 8.0) and 5 mM MgCl₂ and subjected to Dowex 1-X2 column chromatography. The results are given in Table II. The ratio of PR-AMP to 2′-(ribosyl)-5′-AMP was about 27, representing the chain length of trimmed poly ADP-ribose.

**DISCUSSION**

The present paper describes the separation of PR-AMP and its dephosphorylated derivatives by paper chromatography and column chromatography.

Snake venom phosphodiesterase is known to hydrolyze poly ADP-ribose to yield PR-AMP (7, 8). The commercial preparation of snake venom phosphodiesterase used was free from activity to dephosphorylate PR-AMP. However, a purified preparation of rat liver phosphodiesterase was also able to dephosphorylate PR-AMP, and snake venom phosphodiesterase may also occasionally contain this activity. When poly ADP-ribose is hydrolyzed by phosphodiesterase contaminated by this activity, the PR-AMP formed will be further converted to 2′-(5′-phosphoribosyl) adenosine or 2′-(ribosyl) 5′-AMP. These two dephosphorylated compounds behave like 5′-AMP under the conditions of paper chromatography or column chromatography normally used for separation of 5′-AMP and PR-AMP. Thus, the chain length of poly ADP-ribose may be underestimated when measured as the ratio of PR-AMP to 5′-AMP plus dephosphorylated derivatives of PR-AMP instead of that of PR-AMP to 5′-AMP. The true chain length can be obtained by separating dephosphorylated derivatives of PR-AMP from 5′-AMP.

Rat liver phosphodiesterase hydrolyzed poly ADP-ribose in an exonucleolytic fashion, while snake venom phosphodiesterase hydrolyzed it in both exonucleolytic and endonucleolytic fashions (16). By examining the occurrence of 2′-(5′-phosphoribosyl)-adenosine and 2′-(ribosyl)-5′-AMP formed by Pathways b and c in Fig. 1, we can determine the terminal structure of poly ADP-ribose in its intact form or after partial digestion with phosphodiesterase. Analysis of the terminal structure of poly ADP-ribose after exonucleolytic digestion by rat liver phosphodiesterase would give information on the direction of hydrolysis of poly ADP-ribose by this enzyme.

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**REFERENCES**

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