ADP-ribosylation of Diadenosine 5',5''-P1,P4'-tetraphosphate by Poly(ADP-ribose) Polymerase in Vitro*

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When the effect of diadenosine 5',5''-P1,P4'-tetraphosphate on a purified poly(ADP-ribose) polymerase reaction was examined, the compound strongly inhibited ADP-ribosylation reaction of histone, while the compound was much less inhibitory of the Mg2+-dependent automodification of this enzyme. In an attempt to study the mechanism of the inhibition, we analyzed the total reaction products, which were synthesized from NAD+ in the presence of diadenosine 5',5''-P1,P4'-tetraphosphate in a reaction mixture for ADP-ribosylation of histone, and found that a new, low molecular product was predominantly synthesized instead of ADP-ribosylated histone in the reaction. Approximately 90% of added NAD+ was converted into this low molecular product under an appropriate reaction condition. Further analysis revealed that the product was mono- and oligo(ADP-ribosyl)ated diadenosine nucleotide and that the bound oligo(ADP-ribose) is elongating at one end of the product during the reaction. Thus, the present study clearly demonstrated that diadenosine 5',5''-P1,P4'-tetraphosphate functions as an acceptor for ADP-ribose in a poly(ADP-ribose) polymerase reaction in vitro. The finding that histone H1 is required in the reaction mixture for the synthesis of this new product suggests that histone H1 and the diadenosine compound interact during this modification reaction.

Poly(ADP-ribose)1 polymerase, which is localized in the eukaryotic cell nuclei, catalyzes the transfer reaction of ADP-ribose from NAD+, resulting in the formation of poly(ADP-ribose) (1-3) and ADP-ribosylated proteins (4-6). Although the exact biological function of this enzyme has not yet been established, its involvement in a wide variety of biological events including DNA synthesis (7-10), DNA repair (11-16), cell differentiation (17-19), and cell transformation (20) has been suggested.

Recent studies on a reaction mechanism with purified enzyme have revealed that the enzyme catalyzes two types of reaction: one is an automodification reaction (enzyme-bound poly(ADP-ribose) synthesis) in the presence of Mg2+; and the other is oligo(ADP-ribose)ylation of histone in the absence of Mg2+ and the presence of histone at an appropriate histone/DNA ratio (21-23).

In an attempt to find a specific inhibitor of these two reactions, we tested diadenosine 5',5''-P1,P4'-tetraphosphate, which is supposed to be a trigger of DNA replication (24, 25), is demonstrated to be a ligand of DNA polymerase a (26), and is reported to be an inhibitor of terminal deoxynucleotidyltransferase (27). The experiment clearly indicated that the compound preferentially inhibits histone modification reaction rather than the Mg2+-dependent automodification reaction of this enzyme (28). In order to investigate a molecular basis for the inhibition we carried out an analysis of the total reaction products, which were synthesized under a histone modification reaction condition in the presence of Ap4A, and found that a new, low molecular ADP-ribosylated compound was predominantly synthesized in place of ADP-ribosylated histone in the reaction. Further analysis revealed that the product was a mixture of mono- and oligo(ADP-ribose)ylated Ap4A, which were not precipitable with trichloroacetic acid.

EXPERIMENTAL PROCEDURES

Materials—[Adenine-2,8-3H]ATP and [adenine-2,8-3H]NAD+ were the products of New England Nuclear. [Adenine-U-14C]NAD+ was obtained from The Radiochemical Center, Amersham, England. NAD+ (grade 5), Ap4A, and ADP-ribose were obtained from Sigma. Snake venom phosphodiesterase (EC 3.1.4.1) was obtained from Worthington, and purified further by the method of Oka et al. (29).

Purified bovine thymus histone H1 was prepared by the method of Bohm et al. (30). [Adenine-2,8-3H]Ap4A was prepared from [H3]ATP and AMP-morpholidate by a modified method of Reiss and Moffatt (31), and Grummt (25). The radiochemical purity of the preparation was 95% as determined by a PEI-cellulose thin layer chromatography according to the method of Randerath et al. (32). [14C]Phosphoribosyl-AMP was prepared by hydrolyzing [adenine-U-14C]poly(ADP-ribose) with snake venom phosphodiesterase and used as an authentic sample.

Preparation of Active DNA—Active DNA, a DNA fraction copurified with and separated from bovine thymus poly(ADP-ribose) polymerase at a relatively later step of the purification of the enzyme, was prepared by the method previously described (33). Although we presumed in early studies (33, 34) that a high enzyme-activating activity of this DNA may be due to the presence of a specific base sequence on the DNA, recent studies (35, 36) have suggested that a high frequency of DNA breakages on the DNA may be the main reason for its high enzyme-activating ability. Active DNA showed a significantly higher activity than native and DNase I-treated calf thymus DNAs, especially when histone modification reaction was carried out using an appropriate concentration of these DNAs as an enzyme activator (36). Thus, we used this DNA as the enzyme activator through this study. A similar DNA in a purified enzyme preparation has been found by Niedergang et al. (37).

Preparation of Enzyme—Bovine thymus poly(ADP-ribose) polymerase was purified according to the method described previously (34). The purity of the enzyme, examined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis followed by Amido black staining.
and densitometric tracing of the stained gel, was approximately 97.
The specific activity was 160 to 180 units/mg.

Assay for Enzyme Activity—The standard assay for poly(ADP-ribose) polymerase and the definition of the unit of the enzyme activity were described in a previous report (34). All enzyme reactions in the present report were carried out under the histone-dependent reaction condition, where this enzyme preferentially ADP-ribosylates histones (22). The standard histone-dependent reaction mixture contained 25 mM Trias-HCl buffer, pH 8.0, 1 mM dithiothreitol, 0.2 μg of active DNA, 20 μg of calf thymus histone H1, 20 μM \([^{3}H]\)NAD\(^+\) (20 cpm/pmol), and 0.1 μg of the purified enzyme in a total volume of 0.2 ml. In some experiments, the concentration and the specific radioactivity of \([^{3}H]\)NAD\(^+\) were changed as indicated or \([^{14}C]\)NAD\(^+\) was used in place of \([^{3}H]\)NAD\(^+\) as indicated. The concentration of active DNA, histone H1, and the enzyme, and the scale of the reaction were also changed in other experiments as indicated.

Determination of Radioactivity—The radioactivity of a sample collected on a glass fiber filter was counted with the use of a toluene-based scintillator as described previously (34). The radioactivity of an aqueous sample was counted with the use of a xylene-based gel scintillator as described previously (22). Double counting of \(H\) and \(C\) was carried out with \(H^{3}/C^{14}\) window and \(H^{14}/C^{14}\) window of a Packard Tri-Carb liquid scintillation spectrometer for the samples in toluene-based and xylene-based scintillators, respectively. The count of \(H\) and \(C\) was calculated from the count of the respective window based on a window ratio of standard \(H\) and \(C\) samples.

Paper Chromatography—Ascending paper chromatography was carried out with a Toyo No. 51 paper, using a solvent system of isoamyl alcohol/acetone/25% ammonium hydroxide/water (66:13, v/v). The paper was cut into strips 0.5-cm wide and the radioactivity on the strips was extracted into 2 ml of 0.1 N HCl by boiling the sample for 30 min. The sample was counted as described above after neutralization.

In order to avoid a degradation of the sample in a preparative experiment, the paper was extracted with 10 mM formic acid at 0 °C overnight. Formic acid in the extract was eliminated by repeating evaporation.

PEI-cellulose Thin Layer Chromatography—PEI-cellulose thin layer chromatography was performed according to the method of Randerath et al. (32), with a solvent system of 1 M LiCl. After the sample was developed, the plate was dried and cut into strips 0.5-cm wide. The sample was extracted from each strip into 2 ml of 0.1 N HCl by boiling at 100 °C for 30 min. The concentration of nucleotides in the extract was determined by measuring the absorbance at 260 nm. The radioactivity of the sample in the extract was counted after neutralization as described above.

DEAE-cellulose Column Chromatography—A DEAE-cellulose column chromatography was performed according to the method of Randerath et al. (32), with a solvent system of 1 M LiCl. After the sample was developed, the plate was dried and cut into strips 0.5-cm wide. The sample was extracted from each strip into 2 ml of 0.1 N HCl by boiling at 100 °C for 30 min. The concentration of nucleotides in the extract was determined by measuring the absorbance at 260 nm. The radioactivity of the sample in the extract was counted after neutralization as described above.

RESULTS

Analysis of Reaction Products by Paper Chromatography—Recently we found that ADP-ribosylation of histone H1 in vitro by purified poly(ADP-ribose) polymerase (the histone-dependent reaction) was strongly inhibited by Ap4A and other diadenosine nucleotides (23). To investigate the molecular basis for this inhibition, the histone-dependent reaction was carried out with and without 0.5 mM Ap4A in the reaction mixture. Without Ap4A, 57% of the radioactivity of added \([^{3}H]NAD^{+}\) (0.1 mM) was incorporated into an acid-insoluble material under the histone-dependent reaction condition, while the incorporation was decreased to 7% by the addition of 0.5 mM Ap4A. In order to examine both the acid-insoluble and acid-soluble products synthesized in the two reactions, the total reaction products obtained from these two experiments were analyzed and compared by paper chromatography (Fig. 1A and B). As shown in Fig. 1A, when the reaction was carried out without Ap4A, the radioactivity of products in the acid-insoluble material was only 25% of the total radioactivity. When Ap4A was added to the reaction mixture, the acid-insoluble material was increased to 57% of the total radioactivity. The acid-insoluble material was only 7% of the total radioactivity as indicated by the addition of 0.5 mM Ap4A.

When the same analysis was carried out for the reaction product synthesized in the presence of 0.5 mM Ap4A in the histone-dependent reaction system, however, the pattern of radioactivity of the products was quite different (Fig. 1B). As expected from a decrease in the amount of acid-insoluble product (the incorporation of \([^{3}H]AP4A\) into an acid-insoluble material decreased from 57% to 7% under this reaction condition as described above), the count of acid-insoluble product located at the origin (shown by a dotted area) on Fig. 1B) was markedly decreased. Under this reaction condition, however, a new major product peak (approximately 30% of added \([^{3}H]NAD^{+}\), indicated by a black area on Fig. 1B) appeared near the position of acid-insoluble marker. This new product showed a slightly lower mobility than ADP-ribose on this paper chromatography and is not ADP-ribose itself, as clearly demonstrated by PEI-cellulose thin layer chromatography of this compound (Fig. 2). This compound is not covalently bound to a macromolecule, such as histone H1 or enzyme, since the incorporation of \([^{3}H]NAD^{+}\) into an acid-insoluble material was only 7% as described above, while the radioactivity recovered in this compound was as large as 30% of added \([^{3}H]NAD^{+}\), indicating that it is a low molecular product.

To determine whether histone H1 is essential for the synthesis of this unidentified compound, the reaction was carried
Filter at 100 °C for 20 min and the radioactivity of [3H]Ap4A, 0.99 pmol, was counted differentially as described under "Experimental Procedures." After incubation at 25 °C for 20 min, the sample was diluted by the addition of 8 ml of ice-cold 5 mM ammonium bicarbonate, pH 8.3, and analyzed by DEAE-cellulose column chromatography as described under "Experimental Procedures." An aliquot of 100 µl of each fraction was dried on a glass fiber paper with 10 mM formic acid at 0 °C overnight. The sample was treated with snake venom phosphodiesterase and then analyzed by paper chromatography as described under "Experimental Procedures." On this analysis more than 90% of the radioactivity recovered at the position of phosphoribosyl-AMP, was shown on Fig. 3. Three peak fractions (A, B, and C) were collected respectively. The hydrolytic product of compound located at the position of phosphoribosyl-AMP on an analysis by paper chromatography as described in Table II. The radioactivity recovered at the position of phosphoribosyl-AMP, was extracted from paper with 10 µl formic acid at 0 °C overnight. The sample was concentrated by evaporation and analyzed by DEAE-cellulose column chromatography with an authentic sample of phosphoribosyl-AMP and cold ADP-ribose as internal standards. Solid line and dotted line indicate the concentration of ammonium bicarbonate buffer, pH 8.3, and absorbance at 260 nm, respectively. Black and white columns indicate [3H] and [14C] radioactivity, respectively.

![Distances from origin (cm)](image)

**Fig. 2.** PEI-cellulose thin layer chromatography of product. The reaction product synthesized in the presence of Ap4A and located at the black area of Fig. 1B on paper chromatogram was extracted from paper with 10 mM formic acid and concentrated by evaporation. The sample was analyzed by PEI-cellulose thin layer chromatography with an authentic sample of ADP-ribose (0.2 pmol) as described under "Experimental Procedures." Black and white columns indicate [3H] and [14C] radioactivity, respectively.

![Radioactivity (cpm x 10^2)](image)

**Fig. 3.** DEAE-cellulose column chromatography of double labeled reaction product. Enzyme reaction was carried out with 10 µg of active DNA, 200 µg of histone H1, 25.8 µM [14C]NAD (10.6 cpm/pmol), 99 µM [3H]Ap4A (9.54 cpm/pmol), and 20 µg of purified enzyme in a total volume of 2 ml of reaction mixture as described under "Experimental Procedures." After incubation at 25 °C for 20 min, the sample was diluted by the addition of 8 ml of ice-cold 5 mM ammonium bicarbonate, pH 8.3, and analyzed by DEAE-cellulose column chromatography as described under "Experimental Procedures." An aliquot of 100 µl of each fraction was dried on a glass fiber filter at 100 °C for 20 min and the radioactivity of [3H] and [14C] was counted differentially as described under "Experimental Procedures." Radioactivity of [3H]Ap4A, ○-○; and [14C]ADP-ribose, ■-■. The elution positions of NAD, ADP-ribose (ADPR), and Ap4A, and starting point of stepwise elution with 0.5 N HCl are indicated by arrows A, B, and C indicate respective fractions recovered in the peak of radioactivity indicated by arrows.

Out as above except that histone H1 was omitted from the reaction mixture, and the product was analyzed. As shown in Fig. 1C, both oligo(ADP-ribose) located at the origin and the unidentified product observed in Fig. 1B disappeared under the reaction condition, and almost all [3H]NAD remained unreacted. These results indicate that both Ap4A and histone H1 are required for the synthesis of this product.

Although only 30% of added [3H]NAD was converted into this unidentified product under the experimental condition of Fig. 1B, its recovery could be increased to 90% by changing the concentration of Ap4A and [3H]NAD to 250 µM and 20 µM, respectively (data not shown). No conversion of NAD to Ap4A was observed.

**Table I**

Molar ratio of ADP-ribose/Ap4A in ADP-riboylated Ap4A compounds

<table>
<thead>
<tr>
<th>Sample</th>
<th>Radioactivity</th>
<th>Ap4A</th>
<th>ADP-ribose</th>
<th>ADP-ribose/Ap4A</th>
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<tr>
<td></td>
<td>[3H]</td>
<td>[14C]</td>
<td>[3H]</td>
<td>[14C]</td>
</tr>
<tr>
<td>A</td>
<td>48,280</td>
<td>50,250</td>
<td>5.06</td>
<td>4.71</td>
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<tr>
<td>B</td>
<td>17,080</td>
<td>37,870</td>
<td>1.79</td>
<td>3.57</td>
</tr>
<tr>
<td>C</td>
<td>8,760</td>
<td>26,580</td>
<td>0.92</td>
<td>2.51</td>
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**Table II**

Analysis of hydrolysates of ADP-riboylated Ap4A compounds

<table>
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<tr>
<th>Sample</th>
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<tr>
<td>A</td>
<td>44</td>
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<td>B</td>
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<td>C</td>
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**Fig. 4.** Identification of a [3H]-labeled phosphoribosyl-AMP. The hydrolytic product of mono[14C]ADP-riboylated-[3H]Ap4A (peak A shown on Fig. 3) contained two compounds, 5'-AMP and a compound located at the position of phosphoribosyl-AMP on analysis by paper chromatography as described in Table II. The radioactivity recovered at the position of phosphoribosyl-AMP, was extracted from paper with 10 µM formic acid at 0 °C overnight. The sample was concentrated by evaporation and analyzed by DEAE-cellulose column chromatography with an authentic sample of [14C]phosphoribosyl-AMP and cold ADP-ribose as internal standards. Solid line and dotted line indicate the concentration of ammonium bicarbonate buffer, pH 8.3, and absorbance at 260 nm, respectively. Black and white columns indicate [3H] and [14C] radioactivity, respectively.

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<td>C</td>
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the product was observed without enzyme in the reaction mixture (data not shown).

**PEI-cellulose Thin Layer Chromatography of Unidentified Product**—To confirm that the unidentified, new reaction product synthesized in the presence of Ap4A in a histone-dependent reaction (shown by black columns on Fig. 1B) is not ADP-ribose itself, the product was extracted from the paper and analyzed by PEI-cellulose thin layer chromatography with an authentic sample of ADP-ribose. As shown on Fig. 2, approximately 95% of applied radioactivity moved slightly slower than Ap4A and was clearly separated from ADP-ribose. The rest of the radioactivity (5%) was located at the position of ADP-ribose.

The findings that this compound is synthesized in the presence of Ap4A, has tritium label derived from ADP-ribose moiety of [3H]NAD+, and shows lower mobility (thus, suggesting its higher negative charge) than Ap4A on PEI-cellulose thin layer chromatography, strongly suggest that Ap4A is ADP-ribosylated by poly(ADP-ribose) polymerase in the reaction.

**DEAE-cellulose Column Chromatography of Product**—In order to confirm ADP-ribosylation of Ap4A by poly(ADP-ribose) polymerase, adenine-2,8-3H]Ap4A (99 μm) and adenine-2,8-14C]NAD+ (25.8 μm) were incubated with purified poly(ADP-ribose) polymerase in the histone-dependent reaction mixture. The total reaction products were analyzed by DEAE-cellulose column chromatography (Fig. 3). On a linear gradient elution of the column with 5 to 40 mM ammonium bicarbonate buffer, pH 8.3, 14C radioactivity was recovered as three main product peaks A(20%), B(18%), and C(11%), and as three main peaks of [3H]Ap4A, as well as 14C counts of approximately 5% were eluted from the column with 0.5 N HCl and approximately 35% remained uneluted on the column. The products A, B, and C were not precipitable and as ADP-ribosylated Ap4A analyzed in this study.

The identification of the [3H]phosphoribosyl-AMP, which was obtained from the hydrolysis of mono-ADP-ribosylated-[3H]Ap4A (A on Fig. 3) by snake venom phosphodiesterase, was performed by DEAE-cellulose column chromatography using an authentic sample of [3H]phosphoribosyl-AMP. As shown on Fig. 4, the peak of 14C radioactivity of the sample completely overlapped with the peak of 3H radioactivity of authentic sample of phosphoribosyl-AMP.

Thus, percentage of recoveries of 3H and 14C radioactivity in phosphoribosyl-AMP and 5'-AMP, respectively, were obtained from the paper chromatography of the hydrolysates of products A, B, and C, as shown in Table II. All of the hydrolysates of these products contained approximately 50% each of [3H]phosphoribosyl-AMP and [3H]5'-AMP. Since the hydrolysis of 1 mol of unmodified Ap4A produces 2 mol of 5'-AMP (29), the result indicates that one of the two adenosine residues of Ap4A was modified by ADP-ribose and the other one remained unmodified in all of these three products analyzed. On the other hand, the ratio of [3H]phosphoribosyl-AMP/[14C]5'-AMP in the hydrolysates of the products A, B, and C was approximately 0:1:2, respectively, indicating that a monomer, a dimer, and a trimer of ADP-ribose are bound to Ap4A for the products A, B, and C, respectively. All of these results indicate that ADP-ribose is bound to Ap4A at one of the two adenosine residues by a ribose-ribose linkage and the bound ADP-ribose is elongating to the direction indicated by a broken line with an arrow, as shown Fig. 5.

**DISCUSSION**

The present study clearly demonstrated that Ap4A is ADP-ribosylated by poly(ADP-ribose) polymerase under the histone-dependent reaction condition. Since a small peak of product was also observed at ADP-ribosylated Ap4A region on a chromatographic analysis of the product from a control experiment, which was carried out without histone H1 and with Ap4A (Fig. 1C), we determined the exact amount of...
ADP-ribosylated Ap4A synthesized in a histone H1-deprived reaction condition of Fig. 3, using DEAE-cellulose column chromatography. Under the reaction condition, the amount of ADP-ribosyl recovered as ADP-ribosylated Ap4A decreased from 50 to 2% of input NAD⁺ and more than 90% of NAD⁺ remained unhydrolyzed (data not shown). Thus, histone H1 appears to be essential for ADP-ribosylation of Ap4A. Since our unpublished results have shown that ADP-ribose covalently bound to histone H1 cannot be transferred to Ap4A, 2 ADP-ribose moiety of NAD⁺ is probably transferred directly to Ap4A. Thus, the effect of histone H1 observed in the ADP-ribosylation reaction of Ap4A suggests the presence of some unknown interaction between histone H1 and Ap4A.

In this regard, our preliminary trial to demonstrate the interaction between histone H1 and Ap4A by an equilibrium dialysis has shown that Ap4A can be bound to histone H1. Although the effect of other proteins on this modification reaction has not been tested, these observations may be considered as suggesting that only protein-bound Ap4A can be ADP-riboseylated.

On an analysis of ADP-ribosylated Ap4A by DEAE-cellulose column chromatography, we used a rather limited elution range (5 to 400 mM of ammonium bicarbonate) to separate mono-, di-, and tri-ADP-ribosylated Ap4A (Fig. 3). When a linear gradient of an extended range from 5 to 500 mM was applied, however, two additional small radioactivity peaks of [3H]ADP-ribose (approximately 2% of added [3H]NAD) emerged at 370 to 500 mM of ammonium bicarbonate buffer, suggesting that a small amount of Ap4A-bound ADP-ribose oligomers with longer chains were also synthesized (data not shown). Thus, even in a restricted manner, ADP-ribose bound to Ap4A appears to be elonged in the reaction.

When ADP-ribosylated [3H]Ap4A was hydrolyzed by snake venom phosphodiesterase and the hydrolysates were analyzed by paper chromatography, an approximately equal amount of [3H]phosphoribosyl-AMP in the hydrolysates strongly suggests that Ap4A functions as a better acceptor for ADP-ribose than ADP-ribose cova-


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REFERENCES


ADP-ribosylation of Diadenosine 5',5''-P1, P4-Tetraphosphate