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INHIBITION OF ADP-RIBOSYLATION OF HISTONE BY DIADENOSINE 5',5"' -p^1,p^4-TETRAPHOSPHATE

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SUMMARY

Diadenosine 5',5"' -p^1,p^4-tetraphosphate (Ap4A) strongly inhibited ADP-ribosylation reaction of histone by purified bovine thymus poly(ADP-ribose) polymerase. This compound showed a relatively weak inhibitory effect on Mg^{2+}-dependent, enzyme-bound poly(ADP-ribose) synthesis. Among various adenine nucleotides tested, including several diadenosine nucleotides with varying phosphate chain length, Ap4A was the most effective inhibitor of the histone-modification reaction. Ap5A and Ap6A showed slightly lower inhibitory effect than Ap4A. Kinetic analysis of the inhibitor (Ap4A) with varying concentration of substrate (NAD^+) revealed that this compound is a "mixed type inhibitor", with a Ki value of 5.1 μM.

Purified bovine thymus poly(ADP-ribose) polymerase can be activated by binding to a terminal or a nick of double stranded DNA (1) and catalyzes transfer reaction of ADP-ribose from NAD^+ synthesizing protein-bound oligo- or poly(ADP-ribose). Recently we reported two reaction conditions of this enzyme under which ADP-ribosylation of histone and enzyme-bound poly(ADP-ribose) synthesis are preferentially occurring, respectively (2,3). In an attempt to search new inhibitors of this enzyme with these two reaction system, we found that diadenosine 5',5"' -p^1,p^4-tetra-

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Abbreviations:Ap2A; Ap3A, Ap4A, Ap5A and Ap6A; diadenosine 5', 5"' -p1,p2(di-),-p1,p3(tri-),p1,p4(tetra-),-p1,p5(penta-) and -p1, p6(hexa)-phosphate, respectively. pApp, ppApp, pppApp; adenosine 5'-mono-, 5'-di-, and 5'-triphosphate 3'-diphosphate, respectively.

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phosphate (Ap4A), which is known as a ligand of a subunit of DNA polymerase α (4), is a strong inhibitor of poly(ADP-ribose) polymerase and that it specifically inhibits histone modification reaction rather than the auto-ADP-ribosylation of this enzyme.

MATERIALS AND METHODS

Materials [Adenine-2,8-^{3}H]NAD^+ was purchased from New England Nuclear. Diadenosine compounds (Ap2A, Ap3A, Ap4A, Ap5A and Ap6A), 5'ADP, ADP-ribose and NAD^+ were obtained from Sigma. Adenosine, 5'-AMP, and 5'-ATP were obtained from Kojin Co., Ltd., Tokyo. Other adenine nucleotides (pApp, ppApp and pppApp) were products of Sanraku-Ocean Co. Ltd., Tokyo.

Preparation for "active DNA" "Active DNA", a DNA copurified with bovine thymus poly(ADP-ribose) polymerase (5) was prepared accoding to the method described previously (6).

Although we presumed, in early studies (5, 6), that the high enzyme-activating ability may be due to its specific base sequence, recent studies for the enzyme-activation by DNA (1, 7) has suggested that DNA breakages (nicks) concentrated on the DNA may be the main reason for its high activity. The presence of similar small DNA fraction in poly(ADP-ribose) polymerase preparation has been reported also by Nidergang et al. (8).

Enzyme Preparation Bovine Thymus poly(ADP-ribose) polymerase with a purity of appoximately 97% was prepared according to the method described in a previous report (5).

Enzyme Assay The Mg^{2+}-dependent and histone-dependent enzyme reaction was performed as described in a previous report (2). The Mg^{2+}-dependent reaction mixture contained 25 mM Tris-HCl buffer, pH 8.0, 0.5 mM dithiothreitol, 10 mM $MgCl_2$, 0.2 μg of active DNA, 20 μM [^{3}H]NAD^+ (20 cpm/pmol) and 0.1 μg of purified poly(ADP-ribose) polymerase in a total volume of 0.2 ml. The histone-dependent reaction mixture contained 20 μg of purified histone H1 in place of Mg^{2+} in the reaction mixture described above. The reaction was carried out for 10 min and 2.5 min for Mg^{2+}- and histone-dependent reaction, respectively. Other procedures were described in a previous report (2).

In the histone-dependent reaction, at least 70% of the product was oligo(ADP-ribose) bound to histone (2), while almost all reaction product synthesized under the Mg^{2+}-dependent reaction condition was enzyme-bound poly(ADP-ribose) (3).

PEI-cellulose Thin Layer Chromatography. PEI-cellulose TLC of Ap4A (0.25 μmol) was perfomed according to the method of Randerath et al. (9) with a solvent of 1 M LiCl. After desalting with methanol, the plate was dried and cut into pieces with 1.0 cm width. Each pieces were extracted with 5 ml of 3 N NH_4OH. After lyophilization of the extract, each sample was dissolved in 2.6 ml of distilled water.

RESULTS AND DISCUSSION

With the use of histone-dependent and Mg^{2+}-dependent reaction systems we tested the effect of Ap4A. As shown in Fig. 1., Ap4A

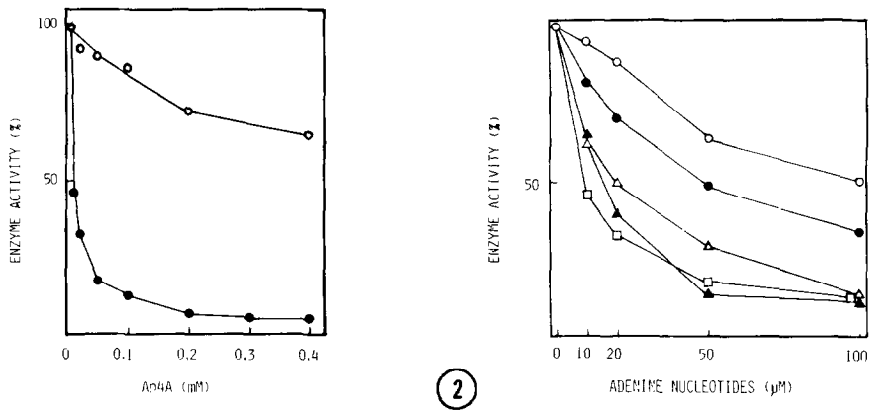


Fig. 1. Effect of Ap4A on Histone-dependent and Mg^{2+}-dependent Reaction.

The enzyme reaction was performed as described under Materials and Methods except that the reaction mixture contained the indicated concentration of Ap4A. The respective enzyme activity without Ap4A (213 pmol/0.1 μg/10 min and 199 pmol/0.1 μg/2.5 min for the Mg^{2+}-dependent and histone-dependent activity, respectively) was set at 100%. open and colsed circles; Mg^{2+}- and histone-dependent enzyme activity, respectively.

Fig. 2. Effect of Various Diadenosine Nucleotides on the Histone-Dependent Enzyme Reaction.

Enzyme assay was performed as described under Materials and Methods with histone-dependent reaction mixture. Varying concentration of diadenosine nucleotides as indicated was added into the reaction mixture. o——o; Ap2A, ●——●; Ap3A, □——□; Ap4A, Δ——Δ; Ap5A, ▲——▲; Ap6A.

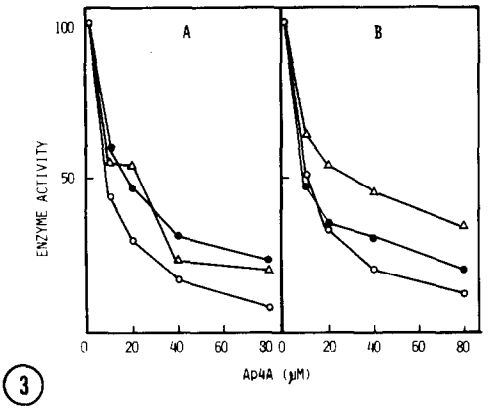
strongly inhibited the histone-dependent reaction, while this compound was much less inhibitory to the Mg^{2+}-dependent, automodification reaction of the enzyme. The concentration of Ap4A required for half inhibition of the histone-dependent and Mg^{2+}-dependent reaction was approximately 10 μM and more than 400 μM, respectively. In order to see whether this inhibition is specific to Ap4A, we tested other related adenine nucleotides including several diadenosine nucleotides with different phosphate chain length (Table 1). Although all of adenine nucleotides were more effective in the histone-dependent reaction than in the Mg^{2+}-dependent reaction, diadenosine nucleotides with 4 to 6 phosphate residues were the most potent inhibitors of the histone-dependent

Table 1. Effect of Adenine Nucleotides on Enzyme Activity

nucleotides (0.1 mM)	Enzyme Activity (%)	
	histone-dependent reaction	Mg^{2+}-dependent reaction
none	100	100
adenosine	90	100
5'-AMP	84	91
5'-ADP	64	93
5'-ATP	55	92
Ap2A	50	92
Ap3A	34	80
Ap4A	12	72
Ap5A	14	84
Ap6A	11	73
pApp	78	103
ppApp	69	106
pppApp	63	100

Enzyme assay was performed as described under Materials and Methods. The indicated concentration of nucleotides was added into the reaction mixture. The enzyme activity obtained with no nucleotide was set at 100%.

reaction, among the compounds tested. Thus further examination for the inhibition of the histone-dependent reaction by these compound was carried out. Although dose responce curves of various di adenosine nucleotides (Fig. 2) revealed a tendency that the compounds with longer phosphate chain are more effective inhibitors, Ap4A was more inhibitory than Ap5A and Ap6A, and was the most effective inhibitor among the tested compounds. These results suggest a specific affinity of poly(ADP-ribose) polymerase for Ap4A. Fig. 3 shows the effect of increasing concentration of DNA (enzyme activator) and histone H1 (acceptor of ADP-ribose) on the inhibition of the reaction by Ap4A. Though a 25-fold increase in DNA and 20-fold decreace in histone H1 concentration apparently



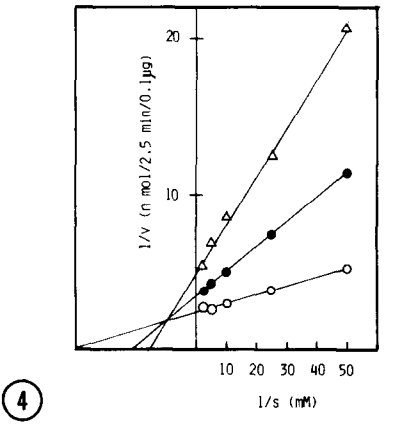


Fig. 3. Effect of Increasing Concentration Active DNA and Histone H1 on the Inhibition by Ap4A.

A: The inhibition by Ap4A was examined in the reaction mixture containing a fixed concentration of histone H1 (20 μg/0.2 ml) and varying concentration of active DNA (o——o; 0.2 μg, ●——●; 0.1 μg, and △——△; 5.0 μg).

B: The inhibition by Ap4A was examined in the reaction mixture containing a fixed concentration of active DNA(0.2 ug) and varying concentration of histone H1 (△——△; 0.1 μg, ●——●; 5 μg, o——o; 20 μg).

In both experiments (A and B), enzyme assay was performed under the histone-dependent reaction condition, as described under Materials and Methods. The indicated concentration of Ap4A was added into the reaction mixture. The respective enzyme activity obtained without inhibitor was set at 100%.

Fig. 4. Double Reciprocal Plot of 1/v versus 1/s(NAD^+) in the Presence of Inhibitor.

Enzyme assay was performed with varying concentration of NAD^+ in the absence (o——o) or presence of 10 μM(●——●) and 30 μM (▲——▲) of Ap4A in the histone-dependent reaction mixture as described under Materials and Methods. Double reciprocal plots of velocity (nmol/2.5 min/0.1 μg) versus substrate concentration (NAD^+, mM) are shown.

did not affect the inhibitory activity of Ap4A so remarkably, both of these changes slightly decreased the inhibition (Fig.3). This observation may be related to our previous one that histone-dependent reaction preferentially occurs at a relatively high histone H1/active DNA ratio (2).

In order to obtain the kinetic parameter of the inhibition, double reciprocal plot of velocity versus substrate (NAD^+) concentration was examined in the absence or the presence of 10 and 30 μM Ap4A (Fig. 4). As shown in this figure, three plots crossed

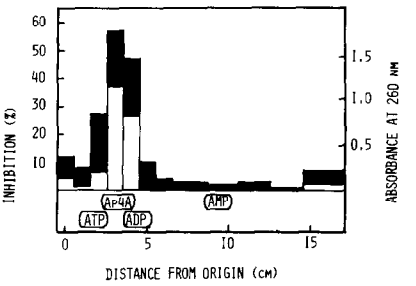


Fig. 5. PEI-cellulose Thin Layer Chromatography of Ap4A.

Ap4A (0.25 μmol) was separated by chromatography on a PEI-cellulose thin layer plate as described under Materials and Methods. After the samples were extracted from PEI-cellulose plate and prepared as described under Materials and Methods, the absorbance at 260 nm (white columns) and the inhibitory activity on the histone-dependent reaction (black columns) were examined. In order to assay the inhibitory actibity, 50 μl of each sample was added into the histone dependent reaction mixture and enzyme activity (0.1 μg) was assayed; the activity without inhibitor was set at 100% and inhibition was expressed by % decrease of the activity. A schematic illustration of the spot of markers (5'AMP, 5'-ADP, 5'ADP and Ap4A on the TLC plate is shown at the bottom of the figure.

at a point and showed different apparent Km values and Vmax, indicating that this compound (Ap4A) is a mixed type inhibitor. Based on these values obtained from these plots, the Ki value of Ap4A was calculated to be 5.1 μM.

In order to confirm that the inhibition of Ap4A is not due to a contaminant in the commercially obtained Ap4A preparation, preparative polyethyleneimine (PEI)-cellulose thin layer chromatography was carried out. After development of the sample, the PEI-cellulose plate was cut into pieces with 1 cm width and each pieces was extracted with 3 N NH_4OH as described under Materials and Methods. When the absorbance at 260 nm and inhibitory activity of each fraction was examined, the main peak of inhibitory activity was recovered at the position of Ap4A, which is shown by the absorbance at 260 nm and a schematic illustration of the spot on TLC plate at the bottom of the figure (Fig. 5). The inhibitory activity of Ap4A was also confirmed by a chromatographic separation

(DEAE-cellulose column chromatography) of this compound according to the procedure described by Rapaport and Zamecnik (10) (data not shown).

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