INHIBITION OF RAT LIVER Ca^{2+}, Mg^{2+}-DEPENDENT ENDONUCLEASE ACTIVITY BY NICOTINAMIDE ADENINE DINUCLEOTIDE AND POLY(ADENOSINE DIPHOSPHATE RIBOSE) SYNTHETASE.

Koichiro Yoshihara, Yoshinori Tanigawa and S.S. Koide

Biomedical Division, The Population Council
The Rockefeller University, New York, N.Y. 10021

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SUMMARY: Incubation of rat liver chromatin with NAD^+ resulted in an inhibition of the Ca^{2+}, Mg^{2+}-dependent endonuclease while the Mg^{2+}-dependent endonuclease was not affected. To establish that the endonuclease was blocked directly by adenosine diphosphate ribosylation purified enzymes were used in the reaction mixture. The following ingredients were required in order to demonstrate the inhibitory effect: partially purified Ca^{2+}, Mg^{2+}-dependent endonuclease, purified poly(adenosine diphosphate ribose) synthetase, NAD^+ and DNA.

INTRODUCTION

Rat liver nuclear chromatin possessed poly(ADP-Rib)* synthetase which transfers the ADP-Rib moiety of NAD to nucleoproteins to form a polymer (1-3). However, the biological significance of ADP-ribosylation of nuclear proteins and poly(ADP-Rib) formation is not yet fully understood. It was suggested that the formation of poly(ADP-Rib) might play a role in the regulation of DNA synthesis (4,5). The inhibition of DNA synthesis affected by the formation of poly(ADP-Rib) *in vitro* was correlated with a suppression of the template (priming) activity (4,5) and DNA polymerase activity (6). The apparent inhibitory effect on DNA polymerase activity of rat liver chromatin was due to a release of the enzyme into the reaction medium during incubation with NAD^+ *in vitro* (7). In a previous communication evidence was presented which suggested that incubation of rat liver nuclei with NAD^+ affected a block of endonuclease activity which prevented activation of the template for DNA synthesis (8).

In the present study evidence will be presented to show that chromatin bound Ca^{2+}, Mg^{2+}-dependent alkaline endonuclease is inhibited by ADP-ribosylation and that the inhibition of the endonuclease is manifested as a suppression of the template for DNA synthesis.

MATERIALS AND METHODS

Materials. [2-^{3}H-adenine]NAD^+ was a gift of Dr. Shall, Sussex, England. Rat liver DNA was prepared by the method of Kay, Simmons and Dounce (9). Highly polymerized [^{3}H]DNA was prepared from *E. coli* K 38, cultured by incubating with [^{3}H-methyl]thymidine according to the method of Smith

* Abbreviation used: ADP-Rib, adenosine diphosphate ribose; Nam, nicotinamide.

(10). Several cultures of E. coli were obtained through the generosity of Dr. P. Model, Rockefeller Univ., USA. Histones were prepared from isolated rat liver nuclei by extraction with 0.25 N HCl. DNA-cellulose was prepared from calf thymus DNA (Miles Lab) by the method of Alberts and Herrick (11). It contained 570 μg DNA/ml of packed volume.

Assay for protein and DNA. Protein and DNA were determined as described by Lowry et al. (12) and Burton (13), respectively.

Preparation of chromatins. Liver nuclei were prepared from Holtzman adult male rats according to Chauveau et al. (14) with slight modification (7). Preparation of chromatin was described in a previous report (7) . The chromatin obtained after step 2 washing procedure was used in the present study unless otherwise indicated.

Assay for poly(ADP-Rib) synthetase activity. The standard assay mixture for poly(ADP-Rib)synthetase activity contained 10 μM [^{3}H]NAD (75,000 cts/min/nmole, 10 mM $MgCl_2$, 50 mM Tris-HCl buffer (pH 8.0), 1 mM EDTA, 2 μg each of rat liver DNA and histones in a total vol of 0.2 ml. The mixture was incubated for 10 min at 25°C and the reaction stopped by the addition of 2.5 ml of 10% ice cold trichloroacetic acid. The acid-insoluble material was collected on a glass-fiber filter and washed twice with 2 ml of 10% trichloroacetic acid and with 2 ml of 95% ethanol. The filter was dried and the radioactivity determined in a toluene-base scintillation medium. One unit of enzymic activity was arbitrarily defined as equivalent to the incorporation of 10 pmoles of [^{3}H]ADP-Rib into the acid-insoluble material.

Assay for endonuclease activity. [^{3}H]DNA-gel was prepared according to Melgar and Goldthwait (15). The final suspension of [^{3}H]DNA-gel contained 20,000 cts/min/0.5 μg DNA/100 μl.

Two assay systems were used to measure endonuclease activities. The Ca^{2+}, Mg^{2+}-dependent endonuclease assay system contained 100 μl of [^{3}H]DNA-gel, 50 mM Tris-HCl (pH 8.0), 10 mM $MgCl_2$, 2 mM $CaCl_2$ 10 mM 2-mercaptoethanol and appropriate amounts of enzyme preparations in a total vol of 0.2 ml. The Mg^{2+}-dependent endonuclease assay system contained 100 μl of [^{3}H]DNA-gel, 50 mM potassium phosphate buffer (pH 6.15), 5 mM $MgCl_2$, 10 mM 2-mercaptoethanol and appropriate amount of enzyme preparation in a total vol of 0.2 ml. The mixture was incubated for 15 min at 37°C. The reaction was stopped by placing the tube in ice and by adding 0.2 ml of 20 mM EDTA (pH 8.0). The tube was centrifuged for two min at 1000g. An aliquot of 0.2 ml of the supernatant was mixed with 10 ml of Aquasol and the radioactivity measured. A unit of endonuclease activity is equivalent to 4000 cts/min of radioactivity solubilized from [^{3}H]DNA-gel under the prescribed condition.

Extraction and partial purification of endonuclease. Mg^{2+}-dependent acid endonuclease was extracted from chromatin with a buffer solution containing 5% glycerol; 50 mM Tris-HCl (pH 7.4); 2 mM EDTA; 0.1 M NaCl. The sediment was subsequently extracted with the same buffer containing 0.35 M NaCl to obtain the Ca^{2+}, Mg^{2+}-dependent endonuclease. For each mg of DNA in chromatin 0.5 to 1.0 ml of the extraction buffers was used.

The endonucleases were purified by ammonium sulfate fractionation (50-85% saturation) and/or carboxymethyl-cellulose column chromatography. The poly(ADP-Rib) synthetase activity present in the partially purified preparation of rat liver Ca^{2+}, Mg^{2+}-dependent endonuclease fraction was destroyed by incubation in a buffer (pH 5.1) at 37°C for 10 min. This heat-treated endonuclease preparation was used in subsequent experiments. The rat liver Mg^{2+}-dependent endonuclease and bull semen Ca^{2+}, Mg^{2+}-dependent endonuclease preparations contained minimal poly(ADP-Rib) synthetase activity.

Preincubation with NAD. Chromatins containing 0.5 to 3.0 mg DNA were incubated in a total vol of 2.0 ml of a medium containing 50 mM Tris-HCl (pH 8.0), 10 mM $MgCl_2$, 2 mM 2-mercaptoethanol, 5 mM NAD^+. The system was incubated at 25°C for 15 min.

Purification of rat liver poly(ADP-Rib) synthetase. Rat liver chromatin containing 350 mg of DNA was extracted with 100 ml of a medium composed of 5% glycerol, 0.35 M NaCl, 50 mM Tris-HCl (pH 7.4), 2 mM EDTA. After the extraction it was found that about 70% of poly(ADP-Rib) synthetase activity remained with the residual chromatin. The enzyme was dissociated from chromatin by centrifugation in a CsCl density gradient as described in a previous report (16). For large scale preparation the centrifugation was performed in a Type 60 Ti rotor for 40 hr at 50,000 rev/min.

The enzyme fraction (70 ml) was collected and the pH adjusted to 7.2 by the addition of 0.2 vol of 2 M Tris-HCl buffer (pH 6.8). Hydroxylapatite was washed and suspended just before use in a medium which contained 30% glycerol, 50 mM Tris-HCl (pH 7.2), 0.5 M NaCl. Equal vol of the hydroxylapatite slurry was added to the enzyme solution and the mixture stirred for 30 min at 0°-4°C and centrifuged at 2000g for 10 min. The sediment was washed with 20 ml of a solution which was composed of 50 mM Tris-HCl (pH 7.2), 30% glycerol, 0.5 M NaCl, and extracted twice with 20 ml of a buffer which contained 0.2 M potassium phosphate (pH 7.2), 30% glycerol 1 mM EDTA. The extracts were combined and dialyzed against 1 liter of Medium A which contained 30% glycerol, 50 mM Tris-HCl (pH 8.0), 2 mM EDTA, for 20 hr at 0°-4°C. The buffer was changed 3 times. The retentate was centrifuged and the precipitate discarded. The supernatant containing the enzyme (25,000 units, 12.2 mg protein, equivalent to 0.5 vol of the preparation) was placed on a carboxymethyl-cellulose column (1 x 5 cm) which was equilibrated with Medium A. The column was washed with 20 ml of Medium A containing 0.1 M NaCl. The enzyme was eluted off with Medium A containing 0.2 M NaCl. Fractions of two ml were collected. The fractions containing the enzyme were pooled and dialyzed against one liter of Medium A overnight at 0°-4°C and stored at -70°C. The purified poly(ADP-Rib) synthetase fraction possessed minimal Ca^{2+}, Mg^{2+}-dependent endonuclease activity [ca. 3 units/208 units of poly(ADP-Rib synthetase activity/10 µg protein]. Following the described isolation procedure a 90 fold purification of the enzyme from chromatin was achieved. Poly(ADP-Rib) synthetase showed an absolute requirement for DNA and was stimulated 2 to 3 times by the addition of histones to the assay system (16).

TABLE I

Extraction of Endonuclease Activity from Rat Liver Chromatin

Fractions	Endonuclease Activity (units/mg DNA)	
	Mg^{2+}-dependent	Ca^{2+}, Mg^{2+}-dependent
0.1 M NaCl extract	74	19
0.35 M NaCl extract	6	106
Residual	2	13

RESULTS AND DISCUSSION

Our previous findings (8) suggested that preincubation of nuclei with NAD^+ affected an inhibition of Ca^{2+}, Mg^{2+}-dependent endonuclease present in rat liver nuclei. This enzyme was described by Hewish and Burgoyne (17) and Ishida et al. (18). To show a direct effect of poly

TABLE II

Effect of Preincubation of Rat Liver Chromatin with NAD on Endonuclease Activity

Treatment	Endonuclease activity (units/mg DNA)					
	Supernatant		0.1 M NaCl extract		0.35 M NaCl extract	
	A	B	A	B	A	B
Control	6 (>4)	50	>4	33	108 (105)	>4
+ 5 mM NAD^+	1 (>4)	69	>4	16	7 (6)	>4
+ 5 mM NAD^+ 20 mM Nam	3 (>4)	64	>4	16	100 (78)	>4

Rat liver chromatin was preincubated with or without NAD and nicotinamide as described under Methods. The mixture was cooled in ice for 5 min and centrifuged for 10 min at 2000g. The supernatant was assayed. Chromatin (sediment) was extracted in sequence with buffers containing 0.1 M NaCl and 0.35 M NaCl. After centrifugation 5 µl of the supernatants were assayed for Ca^{2+}, Mg^{2+}-dependent endonuclease (A) and the Mg^{2+}-dependent endonuclease activities (B). The activities were expressed as units per mg of DNA content of chromatin. The enzyme activities determined after dialysis of the fraction against a medium containing 20% glycerol, 50 mM Tris-HCl (pH 7.4), 2 mM EDTA, 2 mM 2-mercaptoethanol are shown in parenthesis.

(ADP-Rib) formation on the endonuclease activity, the endonucleases were extracted with 0.1 and 0.35 M NaCl from rat liver crude chromatin (Table I). Two types of endonuclease activities were detected in crude chromatin fraction which could be assayed at different pHs. The acid and alkaline endonucleases were differentially extracted with 0.1 to 0.35 M NaCl, respectively. The endonuclease in the 0.1 M NaCl extract showed maximum activity at pH 6.15 and the activity was increased by divalent cations in the system. Mg^{2+} or Ca^{2+} stimulated the activity but the addition of Ca^{2+} to the assay medium containing Mg^{2+} did not enhance further the activity. The endonuclease in the 0.35 M NaCl extract showed maximum activity at pH 8.0-8.2 and required both Mg^{2+} and Ca^{2+} for activity, as was described by other investigators (17,18). The activities of the acid endonuclease in the 0.35 M NaCl extract and of the alkaline endonuclease in the 0.1 M NaCl extract were estimated to be less than 10%.

To determine whether ADP-ribosylation affected the endonuclease or altered the structure of chromatin so that it became inaccessible to endonucleolytic attack, i.e., preventing endonuclease from acting on DNA, rat liver chromatin was incubated with or without NAD^+, extracted with 0.1 and 0.35 M NaCl as described and the endonuclease activities measured (Table II). When chromatin was incubated with NAD^+, the Ca^{2+}, Mg^{2+}-dependent endonuclease activity was inhibited (Table II). The endonuclease activity was not detected in any of the fractions (Table II, column A). On the other hand, a

significant amount of Mg^{2+}-dependent acid endonuclease was released from chromatin into the reaction medium during the preincubation step. However, the total activity of the acid endonuclease (supernatant plus 0.1 M NaCl extract) was not suppressed on preincubation with NAD^+ (Table II, column B). Nicotinamide added to the incubation mixture prevented the inhibitory effect of NAD^+ by blocking poly(ADP-Rib) synthetase activity (Table II, 2-4). The present data showed clearly that Ca^{2+}, Mg^{2+}-dependent alkaline endonuclease in rat liver chromatin is specifically inhibited on preincubation with NAD^+ and that the Mg^{2+}-dependent acid endonuclease was not affected. To establish that this contention is correct, Ca^{2+}, Mg^{2+}-dependent endonuclease of rat liver and bull semen, and rat liver nuclear Mg^{2+}-dependent endonuclease were partially purified. About 370, 17 and 190 folds purification were achieved from chromatin or bull seminal fluid, respectively. The purification procedure will be described in greater detail in a subsequent report. The bull semen endonuclease was included in this study since its properties were similar to that of rat liver (unpuslished data). The endonucleases were preincubated with NAD^+ and purified poly(ADP-Rib) synthetase as described in the legend of Table III and an aliquot was directly assayed for endonuclease activity. The Ca^{2+}, Mg^{2+}-dependent endonuclease obtained from rat liver and bull semen were inhibited on incubation with NAD^+ while Mg^{2+}-dependent acid endonuclease was not affected at all (Table III).

To ascertain that the inhibition of the endonuclease was due to ADP-ribosylation the condition and ingredients of the assay system were varied. The complete system contained appropriate amounts of rat liver Ca^{2+}, Mg^{2+}-dependent endonuclease, poly(ADP-Rib) synthetase, NAD^+ and DNA-cellulose as described in the legend to Table IV. Various ingredients were omitted from the reaction mixture to establish whether or not they participated in the inhibition. After 30 min of preincubation at 25°C, DNA-cellulose was added to the mixture. The total endonuclease activity was found to be bound to DNA-cellulose and was dissociated by suspending the DNA-cellulose in an extraction buffer containing 0.5 M NaCl. The result clearly demonstrated that poly(ADP-Rib) synthetase was required in the reaction medium in order to demonstrate an inhibition of the endonuclease by NAD^+. NAD^+ alone was incapable of inhibiting the endonuclease in the absence of poly(ADP-Rib) synthetase. Furthermore, DNA was required for maximum inhibition which is in agreement with the finding that it is necessary for maximal poly(ADP-Rib) synthetase activity (16). When DNA-cellulose was omitted from the reaction mixture, the inhibition of the endonuclease was minimal in the presence of both NAD^+ and poly(ADP-Rib)

TABLE III

Endonuclease Activities after Treatment with Poly(ADP-Rib) Synthetase and NAD^+

Treatment	Endonuclease activity (cts/min)		
	Rat liver Ca^{2+} Mg^{2+}-dependent	Rat liver Mg^{2+}-dependent	Bull semen Ca^{2+} Mg^{2+}-dependent
No incubation	1,296	2,598	2,056
Control	1,288	2,627	2,218
" + NAD^+	493	2,634	826

Partially purified rat liver Ca^{2+}, Mg^{2+}-dependent endonuclease (15 units), rat liver Mg^{2+}-dependent endonuclease (15 units) and bull semen Ca^{2+}, Mg^{2+}-dependent endonuclease (20 units) were incubated individuallv in a mixture containing 50 mM Tris-HCl (pH 8.0), 10 mM $MgCl_2$, 2 mM 2-mercaptoethanol, 10 µg rat liver DNA, 1 mM NAD, 15 units of poly(ADP-Rib) synthetase in a total vol of 0.2 ml at 25° for 15 min. NAD^+ was omitted from the non-incubated and control samples. The "no incubation" sample was kept at 0°. After incubation the tubes were placed in ice bath and aliquots of 10 µl were assayed for endonuclease activity.

TABLE IV

Ingredients Required in the Reaction Mixture to Demonstrate Inhibition of Endonuclease Activity.

Systems	NAD 5 mM	Nam 20 mM	Poly(ADP-Rib) synthetase (25 units)	DNA-cellulose	Endonuclease Activity (%)
Control	---	---	---	---	100
"	---	---	+	---	100
"	+	---	---	---	100
"	+	---	+	---	78
Complete	+	---	+	+	24
"	+	+	+	+	90

The complete system contained 50 mM Tris-HCl (pH 8.0), 10 mM $MgCl_2$, 5 mM NAD^+, 35 units of rat liver Ca^{2+}, Mg^{2+}-dependent endonuclease (1.6 µg protein), 25 units of poly(ADP-Rib) synthetase (1.2 µg protein) and 100 µl of DNA-cellulose slurry (27 µg DNA) in a total vol of 0.5 ml. In the control systems some of the ingredients were omitted. The mixture was incubated for 30 min at 25°C. The reaction was terminated by placing the tube in ice. To the control tubes in which DNA-cellulose was omitted, 100 µl of DNA-cellulose slurry (27 µg DNA) was added after the incubation (tube 1 to 4) and the tubes placed in an ice bath for 10 min and centrifuged at 2000g for 5 min. The pellet of DNA-cellulose was washed with 1 ml of a solution composed of 20% glycerol, 50 mM Tris-HCl (pH 7.4), 2 mM EDTA and subsequently with 0.2 ml of the same buffer containing 0.5 M NaCl to extract the endonuclease from the DNA-cellulose. An aliquot of 10 µl of the extract was assayed for endonuclease activity.

synthetase in the reaction mixture (Table IV). Hence, it can be concluded that the inhibition is due to ADP-ribosylation of nuclear proteins. This contention was further substantiated by the finding that deamido-NAD, 3-acetylpyridine NAD, ADP-Rib or nicotinamide used in place of NAD^+ were

incapable of affecting an inhibition. These compounds are known to be poor substrate for poly(ADP-Rib) synthetase (19).

Yamada et al. (20) reported that a deoxyribonuclease obtained from rat liver nuclei hydrolyzed synthetic poly(dA-dT) exonucleolytically and was inhibited by purified polymers of ADP-Rib. In our hands, when the free polymers were added to the Ca^{2+}, Mg^{2+}-dependent endonuclease system, the inhibition was minimal. When a large amount of poly(ADP-Rib) (1.28 μg) was added to the endonuclease assay system the enzymic activity was inhibited by about 28%. The amount of polymer added was about 20 times the estimated amount of the poly(ADP-Rib) synthesized during the preincubation of endonuclease and poly(ADP-Rib) synthetase with NAD^+. In other words, when only 5% of poly(ADP-Rib) equivalent to 0.05 μg was synthesized during the preincubation of endonuclease with NAD^+ and poly(ADP-Rib) synthetase the endonuclease activity was inhibited by 70-85%. Furthermore, all of the synthetic deoxyribo-and ribonucleotide homopolymers such as poly(dA), poly(dG), poly (dC), poly(rA), poly(rU), poly (rG) were more potent inhibitors than poly(ADP-Rib); i.e., the Ca^{2+}, Mg^{2+}-dependent endonuclease activity was inhibited by 70-95% when 1 μg of the above mentioned polynucleotides were added to the reaction mixture.

It can be concluded from our present study that the basis for the inhibition of the Ca^{2+}, Mg^{2+}-dependent endonuclease mediated by poly(ADP-Rib) synthetase and NAD^+ is due to a direct effect of ADP-ribosylation on the enzyme rather than on association of the polymers of ADP-Rib with the endonuclease. Our recent results with radioactive NAD^+, purified endonuclease, and poly(ADP-Rib) synthetase showed that ADP-ribosylation of Ca^{2+}, Mg^{2+}-dependent endonuclease does take place (unpublished data). This finding is in agreement with the report that various nuclear proteins including histones and acidic proteins are ADP-ribosylated (21). The fact that poly(ADP-Rib) formation caused a block of a specific endonuclease suggests that the poly(ADP-Rib) synthetase might participate in the regulation of metabolic processes of mammalian cell nuclei by modifying enzymic activities by ADP-ribosylation.

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