ADP-ribosyltransferase from Hen Liver Nuclei

PURIFICATION AND CHARACTERIZATION*

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Chromatin-bound ADP-ribosyltransferase from adult hen liver nuclei was purified to a homogeneous state through salt extraction, gel filtration, hydroxyapatite, phenyl-Sepharose, CM-cellulose, and DNA-Sepharose. The ADP-ribosyltransferase has a pH optimum at 9.0 and does not require DNA for reaction. The purified enzyme has a molecular weight of 27,500 ± 500. Aviditmine sulfate, arginine methyl ester, histones, and casein proved to be effective acceptors for the ADP-ribosyl molecule. Among histones, H3 was most active, followed by H2a, H4, and H2b, in that order, the lowest activity seen with H1. With all the acceptors tested, the rate of nicotinamide release was in excess of the ADP-ribosylation. However, changes in the ratio of nicotinamide release to ADP-ribosylation seemed to depend on concentrations of the acceptor used.

ADP-ribosyl-acceptor adducts formed by ADP-ribosyltransferase served as initiators for poly(ADP-ribose) synthesis when these adducts were incubated in the presence of NAD, DNA, Mg²⁺, and the purified poly(ADP-ribose) synthetase, in which poly(ADP-ribose) formation can occur.

ADP-ribosyltransferase catalyzes the transfer of ADP-ribose from NAD to such acceptors as arginine methyl ester and histones, forming the mono(ADP-ribose)-acceptor conjugate (1-12). In eukaryotes, this enzyme activity was found in membranes from turkey and human erythrocytes (10) and thyroid cells (11) and in rat liver 27,000 X g supernatant (12). The purification and characterization of the ADP-ribosyltransferase from turkey erythrocytes membrane and rat liver 27,000 X g supernatant fraction was reported by Moss et al. (12, 13). In contrast to poly(ADP-ribose) synthetase, this enzyme does not require DNA for reaction.

Recently, we observed that the ADP-ribosyltransferase activity in the hen liver nuclei was high compared with findings in chick embryo and rat liver nuclei (14). This enzyme protein associates with chromatin and can be eluted with high concentrations of salt. We now present the first report of the purification and characterization of the nuclear ADP-ribosyltransferase.

EXPERIMENTAL PROCEDURES

Rhode Island Red hens were obtained from Hara Farms, Shimane, Japan. [carbonyl-¹⁴C]NAD (53 mCi/mmol) and [adenine-2,8-³²P]NAD (25 Ci/mmol) were obtained from New England Nuclear. Whole histones, H1, H2a, H2b, H3, protamine, arginine methyl ester, and aggregatine sulfate were obtained from Sigma. H4 histone and calibration proteins for determination of the molecular weight were obtained from Boehringer Mannheim, and precoated thin layer plates, Fixion 50X8, were obtained from Chromatronics Co. Ltd. Individual histones were further purified by the methods of Bohm et al. (15). All other reagents were purchased from Miyata Chemical Co. Ltd., Shimane, and were used without further purification.

Preparation of Nuclei—The preparation of the nuclei from hen liver was carried out as described in a previous report (16).

Enzyme Assays—For standard assay of ADP-ribose-protein formation, the reaction mixture contained 50 mM Tris-Cl buffer (pH 9.0), 1 mM [adenine-2,8-³²P]NAD (0.5 µCi/tube), 100 µg of whole histones, and appropriate amounts of the enzyme in a total volume of 0.2 ml. The reactions were incubated at 25 °C for 30 min, and the reaction was terminated by adding 2.5 ml of cold 25% trichloroacetic acid. Acid-insoluble materials were collected on a glass filter and washed with a total of 15 ml of cold 25% trichloroacetic acid. The radioactivity of the acid-precipitable material was counted using a liquid scintillation spectrometer. For assay of ADP-ribose-small molecule acceptor conjugate formation, the reaction mixture contained 50 mM Tris-Cl buffer (pH 9.0), 1 mM [adenine-2,8-³²P]NAD (0.5 µCi/tube), 1 mM dithiothreitol, indicated amounts of acceptors, and an appropriate amount of enzyme, in a total volume of 0.2 ml. After the incubation for 0 min at 25 °C, a 50-µl sample was applied to TLC thin layer plates, and chromatograms were developed for 4 h in distilled water. Fractions corresponding to the product were scraped and measured by means of liquid scintillation spectrometer. The recovery of the product was 80-82%. In standard assays for nicotinamide release, the reaction mixture contained 50 mM Tris-Cl buffer, pH 9.0, 100 µg of whole histones, 1 mM [carbonyl-¹⁴C]NAD (0.2 µCi/tube), and 1 mM dithiothreitol in a total volume of 0.2 ml; the reaction was initiated with enzyme and incubated for 30 min at 25 °C. The reaction was terminated by adding 0.2 ml of 5 N HCl and 0.4 ml of water-saturated ethyl acetate, and after standing for 15 min, the tubes were centrifuged for 10 min at 5000 X g. The amount of radioactivity remaining in the ethyl acetate fraction after the centrifugation was measured (17).

Gel Electrophoresis—Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed with a 10% gel column (0.6 X 7 cm) containing 0.1% sodium sulfate by the methods of Laemmli and Favre (18), using standard molecular weight markers. The samples were electrophoresed for 9 h at a constant current of 8 mA per gel at room temperature. After electrophoresis the protein band was visualized by staining with 0.1% Coomassie brilliant blue R-250 in 7% acetic acid and destained with 5% methanol in 7.5% acetic acid.

Purification of Poly(ADP-ribose) Synthetase—Poly(ADP-ribose) synthetase from hen liver nuclei was purified to a homogeneous state through salt extraction, first hydroxyapatite column chromatography, ammonium sulfate fractionation, and successive chromatography of gel filtration, second hydroxyapatite, and DNA-cellulose. We have already reported these procedures (19, 20).

Identification of ADP-Ribose-Acceptor Adducts—For identification of ADP-ribose-histone adducts, the reaction mixture containing [adenine-³²P]NAD and whole histones as substrate and acceptor protein, respectively, was incubated, precipitated, and washed with 25% trichloroacetic acid. The radioactive products were dissolved and incubated with snake venom phosphodiesterase and applied on filter paper (Toyo No. 50). Solvent System 1 (isobutyric acid, 1 M NaOH, 0.1 M EDTA) (10:60:1.6) and System 2 (0.1 M potassium phosphate, 2022

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buffer (pH 6.8), (NH₄)₂SO₄, 1-propanol) (100:60:2) were used (21). More than 85% of the radioactivity was identified as 5'-AMP and not isoADP-ribose (data not shown). Thus the product of the nuclear ADP-ribosyltransferase was considered to be ADP-ribosylated histones.

For identification of ADP-ribose small molecule adducts, the thin layer chromatographic analysis was carried out as described under "Enzyme Assays," and the radioactive ninhydrin-positive spot was identified as the adducts.

Protein and DNA Assays—Protein and DNA were determined as described by Lowry et al. (22) and Burton (23), respectively. In some cases, protein determination was carried out by the more sensitive method of Spector (24).

RESULTS

Subcellular Distribution of ADP-ribosyltransferase in the Hen Liver Nuclei

The intracellular localization of ADP-ribosyltransferase in hen liver is shown in Table I. More than half of the ADP-ribosyltransferase was localized in the 600 × g precipitable fraction, when the homogenate was fractionated by centrifugation (25). The nuclei purified from hen liver showed a 3-fold higher specific activity (data not shown) with 38% recovery of the total activity. In addition, the enzyme activity was found in the fraction extracted from the nuclei with 0.6 M KCl (see below). These results indicate that there is a significant amount of ADP-ribosyltransferase in the hen liver nuclei. Purification of the ADP-ribosyltransferase from the hen liver nuclei was then carried out.

Purification of ADP-ribosyltransferase

All procedures were carried out at 4 °C.

Step 1: Preparation of Crude Extract and Gel Filtration—The purified nuclei (600 mg of DNA), prepared as described under "Experimental Procedures" were homogenized in 100 ml of medium containing 0.14 M KCl, 50 mM Tris-Cl buffer (pH 8.0), 2 mM 2-mercaptoethanol, and 1 mM EDTA, and the homogenate was centrifuged at 8,000 × g for 15 min. The pellet was washed twice with 100 ml of the same medium and then resuspended in 100 ml of an extractable medium containing 0.6 M KCl, 2 mM 2-mercaptoethanol, and 1 mM potassium phosphate buffer (pH 6.8), homogenized, and centrifuged at 105,000 × g for 1 h. The supernatant (post-microsomal fraction) obtained from differential centrifugation was concentrated by gel filtration, dialyzed to remove the salt, and assayed for ADP-ribosyltransferase activity as described above, respectively. These enzyme activities represented activities of 600 g sediment, purified nuclei, 8,000 g sediment, 105,000 g sediment, and 105,000 g supernatant, respectively. No significant amount of ADP-ribosyltransferase was detected in the 105,000 g precipitate after repeated washing with the medium containing 0.6 M KCl.

Fractional activity of ADP-ribosyltransferase in hen liver

### Table 1

<table>
<thead>
<tr>
<th>Fractions</th>
<th>Total activity</th>
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<tr>
<td>Homogenate</td>
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<tr>
<td>600 × g sediment</td>
<td>0.67, 55.6</td>
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<tr>
<td>(Purified nuclei)</td>
<td>(0.44, 38.9)</td>
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<tr>
<td>8,000 × g sediment</td>
<td>0.18, 16.7</td>
</tr>
<tr>
<td>105,000 × g sediment</td>
<td>0.12, 11.1</td>
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<tr>
<td>105,000 × g supernatant</td>
<td>0.08, 5.6</td>
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<tr>
<td>Recovery</td>
<td>89.0</td>
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### Table 2

<table>
<thead>
<tr>
<th>Subcellular distribution of ADP-ribosyltransferase in hen liver</th>
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| Thirty five g of the hen liver were homogenized with 4 volumes of medium containing 0.25 M sucrose, 5 mM Tris-Cl buffer (pH 8.0), 3 mM CaCl₂, 1 mM EDTA, 0.5 mM (ethylene glycol bis(β-aminoethyl ether)-N,N',N',N'-tetraacetic acid), and 0.6 M KCl. The homogenate was centrifuged at 105,000 × g for 1 h, and the precipitate was resuspended with same medium (w/v) used for homogenization and centrifuged under the same conditions described above. Most of all of ADP-ribosyltransferase was extracted by this procedure. The extracts were then concentrated to 3 ml by polyethylene glycol (no. 20,000). To separate the ADP-ribosyltransferase from poly(ADP-ribose) synthetase, the concentrated preparation was applied to the Sephadex G-200 column (2.5 × 40 cm) previously equilibrated with 1 mM potassium phosphate buffer (pH 6.8) containing 2 M NaCl and 2 mM 2-mercaptopethanol. The elution was carried out with the same medium at a rate of 10 ml per h. Fractions of 5 ml were dialyzed against 2 liters of 20% propylene glycol, 10 mM Tris-Cl buffer (pH 7.5), and 2 mM 2-mercaptoethanol. The enzyme assay was carried out using [adenine-3H]NAD and whole histones as substrate and acceptor proteins. The total ADP-ribosyltransferase activity in the dialyzed preparation prepared from the liver homogenate represented the homogenous activity. For fractionation of the hen liver, 35 g of the liver were homogenized with 4 volumes of 0.25 M sucrose containing 5 mM Tris-Cl buffer (pH 8.0), 3 mM CaCl₂, 1 mM EDTA, and 0.5 mM EGTA. The homogenate was separated into the principal subcellular fractions by differential centrifugation (25). The purified nuclei were prepared from 35 g of hen liver as described under "Experimental Procedures." To solubilize the ADP-ribosyltransferase from each sediment obtained by centrifugation, each sediment and the purified nuclei were suspended in 1 volume (w/v) of the medium containing 5 mM Tris-Cl buffer (pH 9.0), 0.6 M KCl, and 2 mM 2-mercaptoethanol, respectively, homogenized, and then centrifuged at 105,000 × g for 1 h. This salt extraction was repeated twice, and each of the 105,000 g supernatants combined. Another 105,000 g supernatant (post-microsomal fraction) obtained from differential centrifugation was concentrated, fractionated by gel filtration, dialyzed to remove the salt, and assayed for ADP-ribosyltransferase activity as described above, respectively. These enzyme activities represented activities of 600 × g sediment, purified nuclei, 8,000 × g sediment, 105,000 × g sediment, and 105,000 × g supernatant, respectively. No significant amount of ADP-ribosyltransferase was detected in the 105,000 × g precipitate after repeated washing with the medium containing 0.6 M KCl.
The activity was absent when the column was further washed with the same medium containing 0.05 M NaCl, and 20% propylene glycol.

Step 6: DNA-Sepharose Column Chromatography—The dialyzed enzyme (27 ml) was applied to a DNA-Sepharose 4B column (2 × 8 cm, containing approximately 200 mg of fixed DNA) pre-equilibrated with 50 mM Tris-Cl buffer (pH 7.5) containing 2 mM 2-mercaptoethanol, 0.05 M NaCl, and 20% propylene glycol. When the column was washed with 200 ml of the same medium, the enzyme eluted with a linear gradient of the same medium containing 0.5 M NaCl. The flow rate was 25 ml per h, and each 5-ml fraction was collected. Under these conditions, the enzyme was eluted at approximately 25 ml per h, and each 5-ml fraction was collected. Under these conditions, the enzyme was eluted at approximately between 0.19 and 0.27 M NaCl as a single sharp peak (Fig. 1). The activity was absent when the column was further washed with the same medium containing 2 M NaCl. In the fractions containing the enzyme activity, the protein elution corresponded to the position of the enzyme activity. Furthermore, the specific activity was constant throughout the entire peak indicating the homogeneity of the purified enzyme. The active fractions (tubes 52 to 66) were collected, combined, and concentrated by solid polyethylene glycol (no. 20,000). The concentrated preparations were then dialyzed against 2 liters of 50 mM Tris-Cl buffer (pH 7.5) containing 2 mM 2-mercaptoethanol, 0.05 M NaCl, and 20% propylene glycol. The concentrated enzyme (27 ml) was applied to a DNA-Sepharose 4B column pre-equilibrated with 50 mM Tris-Cl buffer (pH 7.5) containing 2 mM 2-mercaptoethanol, 0.05 M NaCl, and 20% propylene glycol. The enzyme was bound DNA in the nuclei. A summary of a typical purification is shown in Table II. An approximately 620-fold purification was achieved from the gel filtration of the initial extract of the nuclei with a recovery of 36.6%. The specific activity of the purified turkey erythrocyte ADP-ribosyltransferase (13) was estimated by gel filtration and the Molecular Weight—The molecular weight of the purified enzyme was estimated by gel filtration and by gel electrophoresis of the purified enzyme from poly(ADP-ribose) synthetase, as described under "Experimental Procedures."

**General Properties of the Enzyme**

**Estimation of the Molecular Weight**—The molecular weight of the purified enzyme was estimated by gel filtration and electrophoresis of the purified enzyme from poly(ADP-ribose) synthetase, as described under "Experimental Procedures."

**Relative mobility**

**Fig. 2.** Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of ADP-ribosyltransferase fraction after the final purification. The amount of materials applied is: A, 10 μg of each molecular marker protein; a, RNA polymerase α-subunit (Mr = 39,000); b, β-subunit (Mr = 155,000); c, β'-subunit (Mr = 185,000); d, bovine serum albumin (Mr = 68,000); and e, α-lactalbumin (Mr = 14,000). B, 5 μg of the purified ADP-ribosyltransferase. The details are described under "Experimental Procedures." C, semilog plot of Mr (x 10^-4) versus mobility relative to tracking dye in experiments A and B in this figure.
Nuclear ADP-ribosyltransferase

Buffers were adjusted to 50 mM Tris-C1-; and detected by electrophoresis on sodium dodecyl sulfate-polyacrylamide gels.

pH Optimum—Effects of pH on both the ADP-ribosylation and nicotinamide release by the purified ADP-ribosyltransferase were determined over a pH range of 10 units with phosphate (pH 6.0 to 8.0), Tris (pH 8.0 to 9.0), and glycine (pH 8.5 to 10.5), at a concentration of 50 mM. The optimum pH was observed at pH 9.0 for both the ADP-ribosylation and nicotinamide release, when 100 µg of whole histones were used as the acceptor protein (Fig. 3). The reaction rate with Tris-C1- buffer was somewhat higher than the rate with glycine-OH- buffer at pH 9.0 and was twice the value found with sodium phosphate buffer at pH 8.0. The addition of glycine buffer (pH 9.0) at final concentrations of 18 mM as NaOH retained more than 95.6% of the activity found with Tris-C1- buffer alone. Addition of sodium phosphate buffer (pH 8.0) at final concentrations of 50 and 100 mM to the reaction mixture containing 50 mM Tris-C1- buffer (pH 8.0) reduced the activity to 76.3 and 49.8% of the value obtained with 50 mM Tris-C1- buffer (pH 8.0) alone, respectively. Since the reaction rate with Tris-C1- buffer was somewhat higher than with the glycine buffer, the following experiments were carried out at pH 9.0 with Tris-C1- buffer at a concentration of 50 mM. It should be noted that the rate of nicotinamide release exceeded the rate of ADP-ribosylation of whole histones over the pH ranges tested. This is not due to the low recovery rate of ADP-ribosylated histones precipitated by the addition of trichloroacetic acid (see below).

Time Course of ADP-ribosylation Reaction and Effect of Varying Enzyme Concentrations—The amount of ADP-ribosylation and nicotinamide release, as a function of incubation time and enzyme concentration, was tested. The products formed showed a linearity with time over a 2-h period, when 0.05 µg of enzyme and 100 µg of whole histones as acceptors were added to the reaction mixture of 0.2 ml. The amounts of products were in direct proportion to the amount of enzyme added, up to 0.15 µg. Under the conditions used here, the rate of nicotinamide release was in excess of the rate of ADP-ribosylation (see below).

Effect of DNA on the Enzyme Activity—DNA inhibited the ADP-ribosylation reaction, and further addition of histones led to a recovery of the DNA inhibition (data not shown). We, therefore, presumed that the inhibition is due to binding of histones to the DNA. These phenomena were detected in case of rat liver poly(ADP-ribose) synthetase (26) and turkey erythrocyte ADP-ribosyltransferase (10), respectively.

Identification of ADP-Ribose-Agmatine and ADP-Ribose-Arginine Methyl Ester-Adducts Formed by Nuclear ADP-ribosyltransferase—ADP-ribosyltransferase purified from rat liver 26,600 g supernatant or turkey erythrocyte membrane catalyzes the ADP-ribosylation of low molecular weight guanidino compounds (10, 12). We tested this point with hen liver nuclear enzyme. We incubated the mixture of [adenine-3H]NAD with agmatine sulfate or arginine methyl ester, nuclear ADP-ribosyltransferase, and other compounds, as described under "Experimental Procedures," and the products were analyzed by TLC 50x8 thin layer chromatography. As shown in Fig. 4, the radioactive and ninhydrin-positive products were identified as ADP-ribose-agmatine-adducts and ADP-arginine methyl ester-adducts, respectively. In a parallel experiment, [carbonyl-14C]NAD was employed as substrate and the nicotinamide released was also identified by thin layer chromatographic analysis (data not shown). In these experiments, we found that the amount of nicotinamide released was some-
what higher than those of ADP-ribose incorporation, when agmatine sulfate and arginine methyl ester were used as acceptors, at the same concentrations used for the experiment of ADP-ribose incorporation, respectively (see below). These results indicate that the nuclear ADP-ribosyltransferase from hen liver has a substrate specificity similar to that seen with the enzyme from postnuclear fractions of other species (10, 12).

Effects of Acceptors on the Enzyme Activity—The concentrations of each acceptor were adjusted to give the maximum ADP-ribosylation. When each histone was used as an acceptor for the molecule, H3 was most active to be followed by H2a, H4, and H2b, in that order, and the lowest activity was found with H1 (Table III). Besides agmatine sulfate and arginine methyl ester, protamine, casein, and poly-L-arginine can serve as acceptors for the molecule. With all the acceptors tested, the rate of nicotinamide release was in excess of the ADP-ribosylation, and the ratio of nicotinamide release to the ADP-ribosylation varied with the acceptor. These results are similar to findings in the case of ADP-ribosyltransferase from turkey erythrocyte membrane (13). However, changes in the ratio of nicotinamide release to ADP-ribosylation seem to depend on the concentrations of the acceptor used. For example, as shown in Fig. 5, nicotinamide release increased with increasing concentrations of protamine or whole histones. However, in the presence of acceptor proteins at concentrations of over 100 pg of whole histones (Fig. 5A) or 20 pg of protamine (Fig. 5B), the rate of ADP-ribosylation of the acceptor proteins decreased. To demonstrate that the changes in the ratio of nicotinamide released to the ADP-ribosylation are not due to the decrease in the recovery of radioactive product with increasing concentrations of acceptor proteins, the additional protamine was added to the reaction mixture before and after incubation in the presence of 20 pg of protamine as the acceptor. We observed that further additions of protamine after incubation did not change the recovery of the reaction product, [adenine-3H]NAD-ribosylated protamine (data not shown). Similar results were also obtained with histones. Another factor may be the possible contamination of NADase in the histones. We tested this point and observed that high concentrations of histones contained no activity of NADase, under the conditions used for ADP-ribosyltransferase assay. These results indicate that the increase in the rate of nicotinamide release to the ADP-ribosylation was not due to changes in the recovery of ADP-ribose-histone adducts at either the trichloroacetic acid precipitation step and also the radioactivity assay system. Thus, the ratio of nicotinamide release to the ADP-ribose-acceptor protein conjugate formation increased with increasing concentrations of the acceptor proteins.

**TABLE III**

Comparison of acceptor specificities on the release of nicotinamide and formation of ADP-ribose-protein adducts formed by hen liver nuclear ADP-ribosyltransferase

The reaction mixture containing the purified enzyme (0.097 mg), [carbonyl-14C]- or [adenine-3H]-NAD, and the indicated amount of acceptors were incubated for 30 min at 25 °C, in a large volume of 0.2 ml. Other assay conditions are as described under “Experimental Procedures.”

<table>
<thead>
<tr>
<th>Acceptors</th>
<th>ADP-ribose-acceptor adducts formed (a)</th>
<th>Nicotinamide released (b)</th>
<th>b/a</th>
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</thead>
<tbody>
<tr>
<td>Whole histones (100 µg)</td>
<td>3.63</td>
<td>5.12</td>
<td>1.41</td>
</tr>
<tr>
<td>H1 (50 µg)</td>
<td>1.66</td>
<td>2.37</td>
<td>1.43</td>
</tr>
<tr>
<td>H2a (100 µg)</td>
<td>3.53</td>
<td>3.89</td>
<td>1.10</td>
</tr>
<tr>
<td>H2b (100 µg)</td>
<td>2.79</td>
<td>4.16</td>
<td>1.49</td>
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<tr>
<td>H3 (50 µg)</td>
<td>4.85</td>
<td>5.38</td>
<td>1.15</td>
</tr>
<tr>
<td>H4 (50 µg)</td>
<td>2.57</td>
<td>3.12</td>
<td>1.25</td>
</tr>
<tr>
<td>Protamine (20 µg)</td>
<td>2.28</td>
<td>3.76</td>
<td>1.65</td>
</tr>
<tr>
<td>Casein (400 µg)</td>
<td>2.08</td>
<td>3.67</td>
<td>1.76</td>
</tr>
<tr>
<td>Phosvitin (140 µg)</td>
<td>0.39</td>
<td>0.69</td>
<td>1.76</td>
</tr>
<tr>
<td>Poly-L-arginine (50 µg)</td>
<td>2.73</td>
<td>3.49</td>
<td>1.28</td>
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<tr>
<td>Agmatine sulfate (6.25 mM)</td>
<td>1.60</td>
<td>2.94</td>
<td>1.84</td>
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<tr>
<td>Arginine methyl ester (75 mM)</td>
<td>1.15</td>
<td>2.26</td>
<td>1.97</td>
</tr>
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</table>

**FIG. 5.** Effect of increasing concentrations of whole histones and protamine on the release of nicotinamide and formation of ADP-ribose-protein adducts. The reaction mixture containing the purified enzyme (0.1 µg) and increasing amounts of acceptor proteins were incubated for 30 min at 25 °C in a total volume of 0.2 ml. For assays of nicotinamide release and ADP-ribosylation, [carbonyl-14C]-NAD and [adenine-3H]-NAD were used as substrates, respectively. Other assay conditions are described under “Experimental Procedures.” O—O, nicotinamide released; O—O, ADP ribose-protein adducts formed; and X——X, ratio of nicotinamide released to ADP-ribosylation. Whole histones (A) and protamine (B) were used as acceptors, respectively.

**FIG. 6.** Effect of increasing concentrations of various histones on the formation of ADP-ribose-protein adducts. The reaction mixture containing purified enzyme (0.07 µg), 1 mM [adenine-3H]-NAD, and increasing amounts of each histone was incubated for 30 min at 25 °C in a total volume of 0.2 ml. The conditions were as described under “Experimental Procedures.” O, whole histones; O, H1; O, H2a; △, H2b; ▲, H3; and X, H4.
reaction mixture by Lineweaver-Burk procedures. Values for protamine, casein, and poly-L-arginine were 6, 140, and 9 μg, respectively. Furthermore, \( K_m \) values for arginine methyl ester and agmatine sulfate were 24 and 1.9 mM, respectively. The effect of increasing concentrations of NAD under the conditions of fixed concentrations of each acceptor was also investigated in the presence of 100 μg of acceptor proteins. The Michaelis constants for NAD were 0.56 mM with H1 and 0.29 mM with H2a. The values with H2b, H3, and H4 were similar to those with H2a (Fig. 7). These values did not vary with either half or 2-fold concentrations of acceptor proteins (data not shown). The \( K_m \) values with arginine, arginine methyl ester, protamine, casein, and poly-L-arginine at concentrations of 6.25 mM, 75 mM, 20 μg, 400 μg, and 50 μg in a 0.2-ml reaction mixture, were 0.18, 0.07, 0.33, 0.07, and 0.40 mM, respectively.

**Effect of NaCl on ADP-ribosyltransferase Activity**—Recently, Moss et al. (27) reported that the activity of the purified turkey erythrocyte ADP-ribosyltransferase, assayed at low concentrations of acceptors, was increased more than 10-fold by certain inorganic salts. They proposed that the purified erythrocyte ADP-ribosyltransferase exists as a relatively inactive high molecular weight oligomeric form which is converted by salt to a highly active protomeric species (28). We tested this point with a highly purified ADP-ribosyltransferase from hen liver nuclei. As shown in Fig. 8A, increasing the concentrations of NaCl decreased the ADP-ribosyltransferase activity when whole histones at low concentrations were used as acceptor. This reduction was also detected when low concentrations of agmatine sulfate were used as acceptor (Fig. 8B). The inhibition of the ADP-ribose-agmatine adducts formation by NaCl was more predominant than that of nicotinamide release. To determine whether or not the molecular weight of the enzyme would decrease in the presence of NaCl, the enzyme was dialyzed at 4 °C for 24 h against 10 mM Tris-Cl buffer, pH 8.0, in the absence and presence of 200 mM NaCl, respectively. The dialyzed preparations were applied to a Sephadex G-100 column pre-equilibrated with the dialyzed buffer, respectively, and were then eluted with the same buffer in the presence or absence of 200 mM NaCl. The enzyme assay was carried out using agmatine as the acceptor. The enzyme dialyzed and eluted in the absence of NaCl was found in the fraction corresponding to the enzyme dialyzed and eluted in the presence of NaCl. Furthermore, the recovery of the enzyme in the presence or in the absence of salt was the same, when the enzyme fraction was used for assay after dialysis to remove NaCl (data not shown). Thus, we concluded that hen liver nuclear ADP-ribosyltransferase exists in the protomeric and active form, in the absence of salt.

**Effects of Various Compounds on ADP-ribosyltransferase Activity**—We tested the effects of various compounds, known as poly(ADP-ribose) synthetase inhibitors (29), on the purified ADP-ribosyltransferase activity. Five mM each of NADPH, NADP, NADH, benzam ide, and nicotinamide inhibited this enzyme activity in the same order of inhibitory potency found for the synthetase (29), when 1 mM [adenine-\(^{3}H\)]NAD and 100 μg of whole histones were present in 0.2 ml of reaction mixture. Furthermore, the nature of the inhibition by these compounds was competitive for NAD, determined by Lineweaver-Burk plots of the initial velocity of the ADP-ribosyltransferase versus NAD concentration. The assays were carried out as described under "Experimental Procedures" with 0.05 μg of enzyme protein and different concentrations of NAD. ○, 50 μg of H1; ●, 100 μg of H2a; ×, 100 μg of H2b; △, 50 μg of H3; and △, 50 μg of H4 in a 0.2-ml reaction mixture.

![Figure](https://via.placeholder.com/150)

**Fig. 7.** Double reciprocal plots of the initial velocity of the ADP-ribosyltransferase versus NAD concentration. The assays were carried out as described under "Experimental Procedures" with 0.05 μg of enzyme protein and different concentrations of NAD. ○, 50 μg of H1; ●, 100 μg of H2a; ×, 100 μg of H2b; △, 50 μg of H3; and △, 50 μg of H4 in a 0.2-ml reaction mixture.
matography (30). Under these conditions, significant radioactive compounds were detected in the fraction corresponding to the poly(ADP-ribose) molecule. To confirm that the radioactive compounds eluted in the fraction corresponding to poly(ADP-ribose) were actually the poly(ADP-ribose) molecule, the radioactive preparation eluted from the column was further analyzed by paper chromatography, with two solvent systems (21), after degradation with snake venom phosphodiesterase. From the R_v values, the radioactive compound was identified as isoADP-ribose (data not shown). When the total radioactivity applied to the column was considered as 100%, more than 30% of the radioactivity was recovered in the poly(ADP-ribose) fraction, concomitant with the decrease in mono(ADP-ribose) (Table IV). These results indicate that a small but significant amount of mono(ADP-ribose)-histone adducts formed by ADP-ribosyltransferase serves as an initiator for poly(ADP-ribose) synthesis in vitro.

DISCUSSION

We purified to homogeneity the ADP-ribosyltransferase from hen liver nuclei. Evidence for the existence of ADP-ribosyltransferase in the nuclei includes: (a) over half of ADP-ribosyltransferase activity in the whole homogenate was detected in the 600 × g precipitate fraction; (b) the purified nuclei had a higher specific activity than did the crude nuclei; (c) the enzyme could be extracted from nuclei with high, but not physiological, concentrations of NaCl; (d) the enzyme had a high affinity for DNA-Sepharose.

We observed that different properties between hen liver nuclear and turkey erythrocyte membrane enzymes: (a) optimum pH of our enzyme (pH 9.0) is higher than that in turkey enzyme (pH 7.0) (5, 7); (b) histone H1 is a poor acceptor for ADP-ribose with hen liver enzyme while turkey erythrocytes enzyme utilizes this protein as well as H4 as acceptors (10); (c) NaCl inhibits the highly purified ADP-ribosyltransferase activity from the hen liver nuclei, but stimulates the turkey erythrocytes enzyme activity (27). To demonstrate that the different properties between the two enzymes were not due to the different assay conditions, the nuclear enzyme activity was assayed under the conditions of pH 7.0. We observed that H1 is a poor acceptor for ADP-ribose and that NaCl inhibits the enzyme activity, as was seen at pH 9.0 (data not shown).

Recently, Yost and Moss reported two ADP-ribosyltransferases, enzymes A and B, from turkey erythrocyte membrane (31). The former enzyme takes on an oligomeric and inactive form and converts to a monomeric and active form in the presence of chaotropic salts or histones, while the latter enzyme is not activated by these compounds. Whether or not our enzyme corresponds to this ADP-ribosyltransferase B reported by Moss remains to be determined. Finally, K_x values for NAD of hen liver enzyme are much higher than those found in the turkey erythrocyte enzyme (7). These results taken together indicate that the nuclear enzyme shows certain different properties from the erythrocyte enzyme.

We demonstrated that a part of mono(ADP-ribose) molecule formed by ADP-ribosyltransferase serves as initiator for poly(ADP-ribose) synthesis in vitro. The poly(ADP-ribose) bound to the acceptor proteins is attached to the carboxyl group of glutamate/lysine residues (32), and mono(ADP-ribose) is conjugated with the guanidino residues of arginine of acceptor proteins (7). The molecules attached to glutamate and lysine, but not arginine, are sensitive to neutral NH_2OH (33). Thus, if the mono(ADP-ribose) molecule serves as a primer for the poly(ADP-ribose) synthesis, the polymer thus formed should be resistant to neutral NH_2OH. Actually, Brededorst et al. (34) demonstrated two poly(ADP-ribose)-protein conjugates, neutral NH_2OH sensitive and resistant forms. However, proteins modified by mono(ADP-ribosylation) are preferentially present in post-nuclear fractions (35).

Ueda et al. (36) demonstrated that the ADP-ribose-histone H1-adducts, produced nonenzymatically by forming a Schiff base, serve as initiators for poly(ADP-ribose) formation by the purified rat liver poly(ADP-ribose) synthetase. Not only mono(ADP-ribose) formed nonenzymatically (36) but also several diadoseine compounds can serve as initiators for poly(ADP-ribose) synthesis, under certain conditions (37). These results suggest that elongation of mono(ADP-ribose)-chromatin proteins-adducts forming poly(ADP-ribose) molecules in the hen liver nuclei is feasible. Identification of the amino acids modified in the ADP-ribose-protein-adducts formed by ADP-ribosyltransferase in vivo and in vitro is underway.

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REFERENCES


TABLE IV

<table>
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<tr>
<th>Additions</th>
<th>Mono(ADP-ribose)</th>
<th>Poly(ADP-ribose)</th>
</tr>
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<tr>
<td></td>
<td>formed</td>
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<tr>
<td>None</td>
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<tr>
<td>NAD</td>
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<td>0.42 × 10^4 dpm</td>
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Formation of poly(ADP-ribose) from mono(ADP-ribose)-histone adducts by poly(ADP-ribose) synthetase.
Nuclear ADP-ribosyltransferase


