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Polymerization of ADP-Ribose Pyrophosphatase: Conversion Mechanism of Mg^{2+}-Dependent ADP-Ribose Pyrophosphatase into Mg^{2+}-Independent Form

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ADP-ribose pyrophosphatase (ADPRase) hydrolyzes ADP-ribose (ADPR) into AMP and ribose-5'-phosphate. It is classified into two groups, Mg^{2+}-dependent and Mg^{2+}-independent ADPRase, depending on its Mg^{2+} requirement. Here, we purified Mg^{2+}-dependent ADPRase from rabbit liver and examined what factors affect Mg^{2+} requirement. The purified enzyme showed a single band with the molecular weight of 34 kDa on SDS-PAGE both in the presence and absence of 2-mercaptoethanol. The molecular weight of the native enzyme calculated by gel filtration was 68 kDa, indicating that ADPRase is a dimer made up of two identical subunits. Mg^{2+}-dependent ADPRase with the highest ADPR affinity had a K_m of 160±10 μM and a pH optimum of around pH 9.5. Treatment of the purified ADPRase with heated cytosol fractions at 37°C for 3 h caused some changes in the chemical properties of the enzyme, including an increase in molecular weight, a decrease in solubility, and a loss of Mg^{2+}-dependency. The molecular weight of the cytosol-treated ADPRase measured by gel filtration was over 420 kDa, suggesting, for the first time, that ADPRase could be polymerized by undefined cytoplasmic factors, and that polymerization is accompanied by changes in the solubility and metal ion dependency of the enzyme.

Key words: NAD^+, ADP-sugar, ADP-ribose pyrophosphatase, Polymerization, Mg^{2+}-ion

INTRODUCTION

Adenosine diphosphoribose (ADPR) is a turnover product of NAD^+, including protein-bound ADPR and cyclic ADPR. Free ADPR is involved in various cellular processes such as DNA repair, mRNA processing, cell division, and intracellular Ca^{2+} regulation (Clapper *et al.*, 1987; Jacobson *et al.*, 1994; Ueda and Hayaishi, 1985) and recent evidence shows that free ADPR has a specific cellular role. Free ADPR inhibits the ATP-sensitive K^+ channel in rat ventricular myocytes (Kwak *et al.*, 1996) and, in addition, regulates calcium entry into cells that exhibit calcium-channels containing ADPR pyrophosphatase (ADPRase) (Levitan and Cibulsky, 2001; Perraud *et al.*, 2001). This suggests that free ADPR has ability to gate ion-channels directly, and that free ADPR has a pivotal role in cellular metabolism. However, little is known about how ADPRase controls cellular levels of free ADPR.

ADPRase catalyses the hydrolysis of ADPR into AMP and require Mg^{2+} for its activity. ADPRases have been purified and characterized in various species from *E. coli* to human (Canales *et al.*, 1995; Fernandez *et al.*, 1996; Gabelli *et al.*, 2002; Kim *et al.*, 1998; Miro *et al.*, 1989). Previously, we reported that human ADPRase is a 68 kDa dimer made up of two identical 34 kDa subunits (Kim *et al.*, 1998). Indeed, *E. coli* ADPRase has been shown to be symmetric homodimer with two equivalent catalytic sites, each formed by residues of both monomers, requiring dimerization through domain swapping for substrate recognition and catalytic activity (Gabelli *et al.*, 2001). These results suggest that ADPRase could have a three-dimensional structure which is easily affected by various factors within the cytoplasm. Ribeiro *et al.* (1999) have reported that some changes in the kinetic properties of ADPRase, such as increase of K_m value and decrease of V_{max}, were

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induced by sodium nitroprusside (SNP), indicating that nitric oxide (NO) could negatively regulate ADPRase activity.

In rat liver extract, four distinct ADPRase activities have been described, including three cytosolic activities (ADPRase-I, -II, and Mn) and the mitochondrial ADPRase-m, based on traditional protein purification approach (Canales *et al.*, 1995). The cytosolic ADPRase II has a high K_m and low specificity for ADP-ribose. It may correspond to human erythrocyte ADPRase and human NUDT5. The cytosolic rat liver ADPRase-I and the rat liver mitochondrial ADPRase have very similar characteristics to each other, including a low K_m and high specificity for ADP-ribose and IDP-ribose. ADPRase-Mn is inactive with Mg^{2+} but degrades dinucleotides and CDP-alcohols in the presence of Mn^{2+}. Recently, it has been shown that the *NUDT9* gene encodes a Mg^{2+}-dependent ADPRase that exists as two splice variants, *NUDTα* and *NUDTβ*. It has been suggested that the mitochondrial ADPRase is likely to be the protein product encoded by *NUDTα*. ADPRase I and II are Mg^{2+}-dependent but ADPRase-Mn is Mg^{2+}-independent. The difference in Mg^{2+} dependency of the different ADPRases might occur because of differences in the primary structure, otherwise changes in the three-dimensional structure of the same ADPRase may be responsible.

To clarify these possibilities we purified rabbit liver ADPRase and examined what factors could modulate Mg^{2+} dependency. Consequently, this paper provides the first evidence that ADPRase could be polymerized by undefined cytoplasmic factors, which is accompanied by chemical changes of the enzyme, such as solubility and metal ion dependency.

MATERIALS AND METHODS

Animals and materials

New Zealand white rabbits were used. All experiments were carried out under approval of the Ethics Committee in the Institute for Medical Sciences of Jeonbug National University. Nucleotides, ampholytes, Sephadex G, horse cytochrome C, porcine pepsin, human hemoglobin, and blue dextran were obtained from Sigma Chemical Co. (St Louis, MI, USA). All other reagents were of the highest grade available.

Assay for ADPRase activity

ADPRase was assayed by the determination of ribose-5'-phosphate produced from ADP-ribose using the orcinol reaction (Ashwell, 1957) after the removal of nucleotides by activated charcoal. The reaction mixture containing 1 mM ADP-ribose, 2 mM $MgCl_2$, 50 mM Glycine-NaOH (pH 9.5), and enzyme in a total volume of 0.5 mL was incubated at 37°C for 30 min. After terminating the reaction by the addition of 0.5 mL of 10% trichloroacetic acid, 0.5 mL of 10% activated charcoal was added to remove the nucleotides. The mixture was then shaken for 5 min, and centrifuged to separate the charcoal. A 1.0 mL aliquot of supernatant was mixed with the same volume of orcinol reagent (orcinol 10 mg, 25% $FeCl_2$ 30 μL, and HCl 10 μL). After heating for 20 min in boiling water, the absorbance was read at 660 nm. The absorbances were linear with both reaction time and added enzyme amount under all the conditions used, and showed good correlation with the assay using HPLC described below. One unit of enzyme activity represents the release of 1 μmol of ribose 5'-phosphate per hour.

Phosphodiesterase I activity was assayed by measuring the increase of absorbance at 405 nm in the reaction mixture containing 1 mM of dTMP-*p*-nitrophenyl ester, 50 mM Tris-HCl (pH 8.0) and 5 mM $MgCl_2$, and 0.1 N NaOH as the stopping solution (Bernet *et al.*, 1994).

Chromatography for substrate specificity test

ADPRase was also assayed by analyzing the reaction products produced from various nucleotide substrates on a Vydac 303 NT 405 (0.46×5 cm, Bio-Rad Laboratories) HPLC column (Hellmich and Strumwasser, 1991; Lee *et al.*, 1989). The reaction mixture, containing 50 mM glycine-NaOH (pH 9.5), 2 mM $MgCl_2$ and 1 mM substrates, was incubated at 37°C for 30 min with the enzyme solution in a volume of 100 μL. Ten μL of the reaction mixture was injected onto the Vydac column equilibrated with 0.045 M CH_3COONH_4 (pH 4.6, adjusted with H_3PO_4), and eluted with a linear gradient from 0 to 100% 0.5 M NaH_2PO_4 (pH 2.7, adjusted with CH_3COOH) for 45 min at a flow rate of 1 mL/min. Eluted samples were read at 254 nm. Control samples containing known standards (nicotinamide, NAD^+, AMP and ADP-ribose) were treated under the same conditions.

Electrophoresis for measurement of molecular weight

The samples were dissolved in electrophoresis sample buffer (83 mM Tris, 2% SDS, 10% glycerol, 5% 2-mercaptoethanol, and 0.002% bromophenol blue, at pH 6.8) and boiled for 3 min. SDS-polyacrylamide gel electrophoresis was performed on 10% resolving and 3% stacking gel according to Laemmli (Laemmli, 1970).

Analytical gel filtration

The purified enzyme was applied to a Sephadex G-150 column (1.5×90 cm) equilibrated with 30% glycerol, 10 mM sodium phosphate (pH 7.5), 1 mM $MgCl_2$, 1 mM 2-mercaptoethanol, 0.5 mM EDTA, and 0.15 M KCl. Elution was performed at a flow rate of 5 mL/h. Molecular weight markers for gel filtration were read by the absorbance at 280 nm.

Purification of rabbit liver ADPRase

Ten mM sodium phosphate buffer (pH 7.5) containing 1 mM $MgCl_2$, 1 mM 2-mercaptoethanol, 0.5 mM EDTA, and 30% glycerol was used as standard buffer throughout the purification unless otherwise indicated. After homogenation with 9 volumes of 0.15 M NaCl, cytosol fractions were obtained by centrifugation at 15,000×g for 30 min. The ADPRase cytosol fractions were treated with 1 N acetic acid for 30 min. After centrifugation at 15,000×g for 10 min, 1 N Na_2CO_3 neutralized supernatant. The ADPRase suspensions were mixed with calcium-phosphate gel equilibrated with the standard buffer without 30% glycerol, and stirred for 60 min and centrifuged at 500 g for 5 min. The precipitate was washed with the same buffer, and bound proteins were eluted with the standard buffer containing 60 mM phosphate buffer without 30% glycerol. Solid ammonium sulfate was added again to the enzyme solutions to obtain 55% saturation. The solution was centrifuged after overnight standing. The precipitate was dissolved in 18 mL of standard buffer, and dialyzed against the standard buffer containing 1 M ammonium sulfate for 24 h. The concentrated enzyme was applied to a Sephadex G-150 column (2.6×80 cm) equilibrated with the standard buffer. The flow rate was 2.5 mL/h and fractions of 5 mL were collected. Fractions containing the major portion of the enzyme activity were pooled. The pooled enzyme solution form the previous step was applied to a protein pak glass DEAE-5PW column (0.8×7.5 cm, Waters Co.) equilibrated with the standard buffer. Non-absorbed proteins were washed out, and the enzyme was eluted using a linear NaCl gradient (0-1.0 M) with a flow rate of 1 mL/min and fractions of I mL were collected. The enzyme activity was recovered at 0.7 M NaCl and fractions containing the major portion of the enzyme activity were pooled, concentrated with Centriprep 30 (Amicon), and dialyzed against the standard buffer. Finally, the enzyme solution was applied to preparative isoelectric focusing (IEF, Bio-Rad Laboratories) gel containing ampholyte (pH 2.5/5). ADPRase was eluted at pH 4.2. The enzyme was routinely stored in the standard buffer at -20°C without any loss of enzyme activity for months. The pooled protein from the last step IEF ran as a single band on the SDS-PAGE stained with Coomassie blue.

RESULTS AND DISCUSSION

Animal livers have four distinct ADPRase activities including Mg^{2+}-dependent and Mg^{2+}-independent ADPRase. To analyze factors affecting Mg^{2+} dependency we have purified Mg^{2+}-dependent ADPRase from rabbit liver cytosol by using the modification methods developed by Kim *et al.* (Kim *et al.*, 1998). Purification was achieved to apparent homogeneity by successive anion exchange, hydrophobic interaction, size-exclusion chromatography, and preparative isoelectric focusing. A summary of the typical purification run is shown in Table I. Purification of 7234 fold with a high recovery of 48% was obtained. ADPRase appeared as electrophoretically homogenous, giving a single band with the molecular weight of 34 kDa when stained with Coomassie Brilliant Blue using SDS-PAGE, both in the presence and absence of 2-mercaptoethanol. (Fig. 1 and Table II). The molecular mass of the native ADPRase was determined by gel filtration on a calibrated Sephadex G-150 column chromatography and found to have a molecular weight of 68 kDa (Table II), indicating that the Mg^{2+}-dependent ADPRase of rabbit liver is a dimer made up of two identical subunits. The subunit molecular mass of rabbit liver ADPRase is similar to the molecular masses of subunits of dimeric human erythrocyte ADPRase (Kim *et al.*, 1998).

The rabbit liver ADPRase exhibited affinity only for some ADP-sugars, such as ADP-ribose (ADPR), ADP-glucose, and ADP-mannose, and the enzyme showed the highest affinity with ADPR. The enzyme had no effect on dTMP-*p*-nitrophenyl ester, an artificial substrate for nucleotide pyrophosphatase I. As shown in Table II, the presence of Mg^{2+} was obligatory for the enzyme activity. The maximal activity was found with 2 mM $MgCl_2$. The purified rabbit ADPRase obeyed Michhaelis-Menten kinetics with K_m of 160±10 μM and V_{max} of 7.2±0.5 μmolemin/mg protein for ADP-ribose (Table II). The pH optimum of the rabbit ADPRase was determined in 50 mM Glycine-NaOH buffer (pH range 8.0-10.5). The enzyme showed an optimal activity around pH 9.5 (Table II). The kinetic properties of rabbit liver ADPRase were similar to those of human erythrocyte ADPRase (Kim *et al.*, 1998). Thus, the purified rabbit ADPRase used in this study is sufficiently useful

Table I. Purification of ADP-ribose pyrophosphatase from rabbit liver

Step	Total Protein (mg)	Total Activity (μmol/h)	Specific Activity (μmol/h/mg)	Recovery (%)	Purification (- fold)
1. Cytosol	5,850	3,705	0.63	100	1
2. pH-treatment Fractionation	2,331	3,706	0.59	100	2.5
3. Calcium-phosphate Gel adsorption	250	3,350	14.20	96	22.5
4. Sephadex G-150 Chromatography	55	3,306	60.10	86	95
5. DEAE-5PW Chromatography	1.36	2,408	1,770	65	2810
6. IEF	0.39	1,778	4,558	48	7234

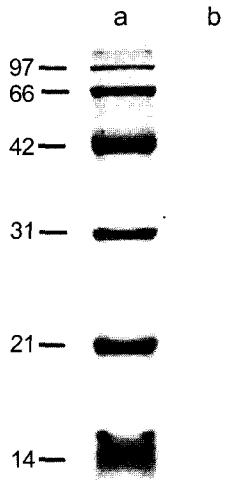

Fig. 1. SDS-polyacrylamide gel electrophoresis of purified ADP-ribose pyrophosphatase. SDS-polyacrylamide gel electrophoresis was performed on 10% resolving and 3% stacking gel and stained with 0.3% (w/v) Coomassie blue. Lane a, molecular weight markers: phosphorylase b (97,400), bovine serum albumin (66,200), ovalbumin (42,699), carbonic anhydrase (31,000), soybean trypsin inhibitor (21,500), and lysozyme (14,400); lane b, purified ADP-ribose pyrophosphatase.

Table I. Properties of purified ADP-ribose pyrophosphatase from rabbit liver

	Properties	
i	Molecular Weight (MW, kDa)*	68
i	Subunit MW (kDa)**	34
iii	Optimum pH***	9.5
iv	Metal ion-dependency	Mg^{2+}, Mn^{2+}
v	K_1 (μM) for ADP-ribose	160 ± 10
vi	V_{max} (μmolemin/mg protein) for ADP-ribose	7.2 ± 0.5

*Determined by size-exclusion chromatography on Sephadex G-150.
**Determined by SDS-PAGE.
***50 mM Glycine-NaOH buffer

to characterize the general properties of Mg^{2+}-dependent ADPRase.

During the process of ADPRase purification we found that partially purified cytosolic ADPRase, showing only Mg^{2+}-dependent activities, was gradually converted into Mg^{2+}-independent form after storage at 4°C for one week. As shown in Fig. 2, the Mg^{2+}-independent activity gradually increased, instead of the reduction of Mg^{2+}-dependent activity as a function of time. Mg^{2+}-dependent activity of cytosolic ADPRase was 85% of total ADPRase on the first day of storage and decreased to 15% at day 20. However, fully purified Mg^{2+}-dependent ADPRase did not show any change in Mg^{2+}-dependency during storage. Furthermore, we found that cytosolic ADPRase also became insoluble after storage at 4°C. This was determined by centrifugation experiments showing an increase of Mg^{2+}-independent ADPRase in precipitates, as opposed to a reduction of Mg^{2+}-dependent form of ADPRase in the supernatant. As shown in Table II, all Mg^{2+}-independent ADPRase was precipitated by centrifugation (20,000×g for 15 min). These results indicate that the storage of cytosolic ADPRase causes a decrease in solubility and the Mg^{2+}-dependency for enzyme activity. Based on the difference of Mg^{2+}-requirement between partially purified and fully purified ADPRase after storage, we speculated that the Mg^{2+}-dependency for ADPRase activity could be controlled by unknown factors in the cytoplasm. Furthermore, purified Mg^{2+}-dependent ADPRase, incubated at 37°C with cyto-

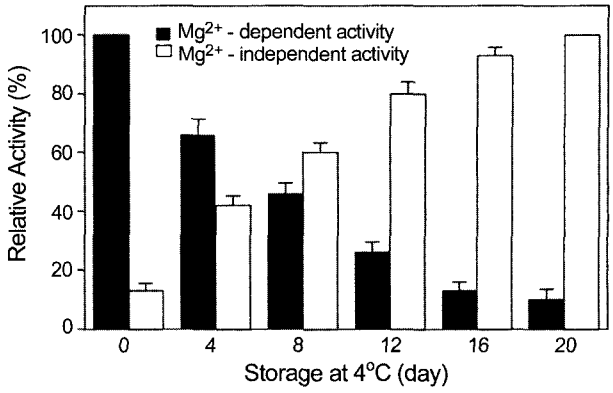

Fig. 2. Conversion of Mg^{2+}-dependent ADPRase into Mg^{2+}-independent form during storage of cytosol fractions at 4°C. Enzymatic activities of both Mg^{2+}-dependent form and Mg^{2+}-independent form of ADPRase were simultaneously determined at the indicated time. The enzyme activity was assayed at 37°C for 30 min in the reaction mixtures (total volume of 0.5 mL) containing 1 mM ADP-ribose, 50 mM glycine-NaOH buffer pH 9.5 in the presence and absence of 2 mM $MgCl_2$ respectively. The relative activities (%) of the Mg^{2+}-dependent form and the Mg^{2+}-independent form of ADPRase were calculated by the absorbance ratio of each sample compared to full absorbance, respectively. Each point represents the mean±S.D. from 3 separate experiments.

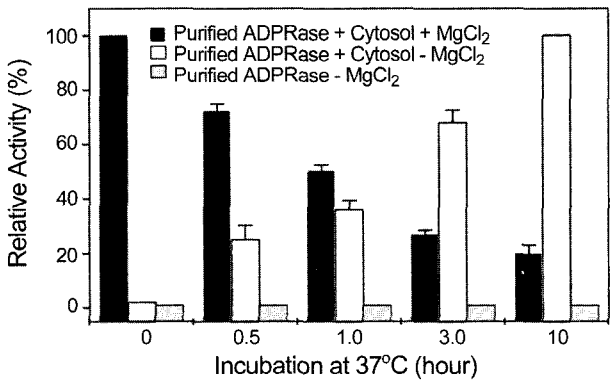

Fig. 3. Conversion of Mg^{2+}-dependent ADPRase into the Mg^{2+}-independent form during the incubation of purified Mg^{2+}-dependent ADPRase with the heated cytosol fraction at 37°C. Enzymatic activities were determined by the same methods as described in Fig. 2.

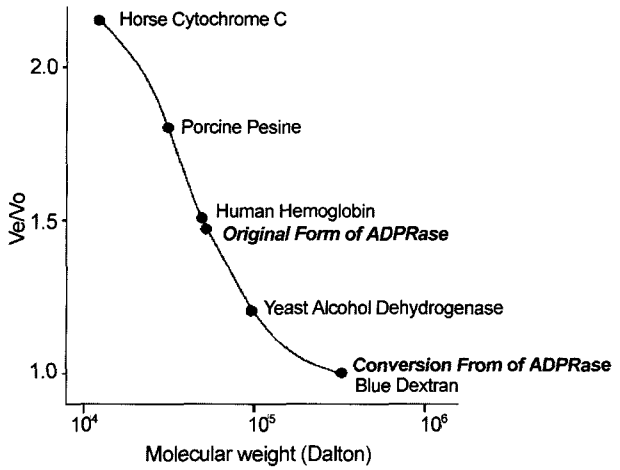

Fig. 4. Determination of molecular weights of ADPRase by Sephadex G-150 gel filtration. The samples were applied to a Sephadex G-150 column (1.5×90 cm) equilibrated with 30% glycerol, 10 mM sodium phosphate (pH 7.5), 1 mM $MgCl_2$, 1 mM 2-mercaptoethanol, 0.5 mM EDTA and 0.15 M KCl. The standard proteins and their molecular weights used were yeast alcohol dehydrogenase (120,000), human hemoglobin (64,000), porcine pepsin (34,700), and horse cytochrome c (12,400). Ve; elution volume of sample, Vo; column void volume.

Table III. Solubility of ADP-ribose pyrophosphatase during storage of liver cytosol. Liver cytosol stored for 6 days at 4°C was centrifuged at 20,000×g for 15 min. ADPRase activities in the supernatant and precipitate were assayed in the presence or absence of 2 mM $MgCl_2$.

Fractions	Activity/0 day		Activity/6 days	
	– $MgCl_2$	+ $MgCl_2$	– $MgCl_2$	+ $MgCl_2$
Supernatant	0.01	0.61	0.01	0.21
Precipitate	0.02	0.04	0.22	0.34

sol fractions stripped of their ADPRase activity (by storing at 56°C overnight), was converted into Mg^{2+}-independent form (Fig. 3). Interestingly, the molecular weight of the cytosol-treated ADPRase, determined by gel filtration, was approximately 420 kDa (Fig. 4), indicating that the polymerization of ADPRase could be induced by undefined factors of the cytosol.

In conclusion, the results suggested that ADPRase could be polymerized by undefined cytoplasmic factors and that this polymerization might be accompanied by chemical changes, such as solubility and metal ion dependency, of the enzyme.

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