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# IDENTIFICATION OF A 27.5 kDa, THIOL-INDEPENDENT ARGININE-SPECIFIC ADENOSINE DIPHOSPHATE (ADP)-RIBOSYLTRANSFERASE IN CHICKEN BONE MARROW CELLS

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Though two arginine-specific adenosine diphosphate (ADP)-ribosyltransferase cDNAs AT1 and AT2 were cloned from chicken bone marrow cell cDNA library, the transferase corresponding to native AT2 has not been detected. In the present study, we examined whether the native AT2 transferase is present in chicken bone marrow cells. An arginine-specific ADP-ribosyltransferase (AT-H) was separated from the bone marrow cell extract by gel filtration chromatography. AT-H and recombinant AT2, sharing the common chromatographic properties, were thiol-independent and activated by salts while native AT1 required thiol reagents for its activity and was inhibited by salts. These results suggest that the newly detected ADP-ribosyltransferase AT-H is probably the native AT2 transferase.

Key words: ADP-ribosylation, chaotropic salts, ADP-ribosyltransferase, arginine

## INTRODUCTION

ADP-ribosylation is a post-translational modification of proteins in which amino acid-specific ADP-ribosyltransferases transfer ADP-ribose moiety of nicotinamide adenine dinucleotide (NAD) to their target amino acid residues of proteins. An arginine-specific ADP-ribosyltransferase, cholera toxin, ADP-ribosylates an arginine residue of Gs$\alpha$ to inhibit GTPase activity of the G protein, and thus the toxin has been used as a molecular probe to elucidate physiological importance of Gs$\alpha$ function (1). In nitrogen fixing bacteria, *Rhodospirillum rubrum*, dinitrogenase reductase activity has been shown to be regulated by ADP-ribosylation-deADP-ribosylation of specific arginine-residues of the reductase (2). In eucaryotes, arginine specific ADP-ribosyltransferases have been detected together with ADP-ribosylarginine hydrolase (3), which releases ADP-ribose from the arginine-ADP-ribosylated proteins, in animal cells and a reversible regulation of protein functions is thought to be a role of this modification in the cells.

We purified an arginine-specific ADP-ribosyltransferase from chicken polymorphonuclear leukocytes (so-called heterophils) (4). Using the amino acid sequences obtained from the purified heterophil transferase, we cloned two cDNAs encoding arginine-specific ADP-ribosyltransferases, AT1 and AT2, from chicken bone marrow cell cDNA library and expressed them in COS 7 cells (5). The recombinant AT1 transferase required thiol reagents such as dithiothreitol (DTT) for its activity and was inhibited by salts while recombinant AT2 was thiol-independent and rather activated by salts. Based on the identity of the deduced amino acid sequence of AT1 with the partial amino acid sequence of a 27.5 kDa thiol-dependent and salt-inhibited transferase purified from the heterophils (4), we concluded that AT1 cDNA encodes the thiol-dependent and salt-inhibited transferase. However, the transferase activity corresponding to native AT2 enzyme has not been detected in the heterophils.

In the present study, we examined whether native AT2 transferase is present in the chicken bone marrow cells and found thiol-independent transferase (AT-H) that probably corresponds to native AT2 transferase.

## MATERIALS AND METHODS

### *Materials*

Chickens were obtained from a local slaughterhouse.

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NAD was purchased from Boehringer Mannheim (Mannheim, Baden-Württemberg, Ger-many). [adenylate-$^{32}$P] NAD (29.6 TBq/mmol) was from New England Nuclear (Boston, Massachusetts, U.S.A.).

***Extraction of ADP-ribosyltransferase from chicken bone marrow cells***

Bone marrow cells were collected from leg bones of 2-3 month old chickens, washed 3 times with phosphate-buffered saline, layered on 12.5% Ficoll-Metorizoate solution and centrifuged at 800 × g for 30 min. After removal of sedimented erythrocytes, the cells of top fraction were sonicated (10 watts) for 30 sec in 50 mM Tris-HCl (pH 7.5) containing 0.35 M NaCl solution (2 ml/ml packed cell volume) and centrifuged at 105,000 × g for 1 h. The supernatant was used as extract.

***Column chromatography***

Gel filtration chromatography was carried out with a Sephadex G-100 column (2.5 × 60 cm) and running buffer 50 mM Tris-HCl (pH 7.5) containing 0.15 M NaCl. The fraction containing enzymatic activity was dialyzed against 50 mM Tris-HCl (pH 7.5) containing 1 mM ethylenediaminetetraacetic acid (EDTA) and applied to carboxymethyl (CM)-cellulose (1 × 2 cm) equilibrated with 50 mM Tris-HCl (pH 7.5) containing 1 mM EDTA. After washing, the enzyme was eluted with the same buffer containing 0.5 M NaCl.

***Preparation of ADP-ribosyltransferases***

Native AT1 enzyme was purified from chicken peripheral heterophils according to the methods described elsewhere (4). The culture medium of AT2-expressing COS 7 cells was used as recombinant AT2 (5). Bone marrow extract was fractionated on Sephadex G-100 (Fig. 1) and the void fraction was used as AT-H. Specific activities of AT-H, recombinant AT2 and native AT1 were 27.3 nmol/h/mg, 10.6 nmol/h/mg and 400 μmol/h/mg, respectively.

***ADP-ribosyltransferase assay***

For the standard assay of ADP-ribosyltransferase activity, the reaction mixture, containing 400 μg casein, 50 mM Tris-HCl (pH 9.0), 100 μM [$^{32}$P] NAD (120 MBq/mmol), 5 mM DTT, and appropriate amount of the enzyme preparation in a total volume of 0.2 ml was incubated at 25 ℃ for the indicated time (4). The reaction was terminated by adding 10% trichloroacetic acid. Radioactivity of the acid-insoluble fraction collected on a glass filter was counted. In each assay, radioactivity obtained without enzyme was subtracted as a background. Assay of ADP-ribosylarginine formation was done with capillary electrophoresis (6).

***Zymographic in situ gel assay***

The enzyme was separated by 15% SDS-PAGE under reducing conditions. The gel was renatured with 2% Triton X-100 for 30 min twice and then incubated with 10 μM NAD (6.7 kBq/nmol) and 0.2 mg/ml poly-L-arginine for 18 h at room temperature. Following incubation, the gel was fixed with 10% trichloroacetic acid, washed to remove unreacted NAD, dried, and exposed to X ray film.

## RESULTS AND DISCUSSION

When the extract of chicken bone marrow cells was subjected to a Sephadex G-100 column, more than 90% of the activity found in the extract was detected in a fraction of a peak with estimated molecular size of 28 kDa, similar to that of heterophil ADP-ribosyltransferase (4), namely native AT1 transferase (Fig. 1). In addition to this expected peak, a minor peak of activity was detected at the void fraction. When the fraction was incubated with NAD and L-arginine, ADP-ribosylarginine was detected by capillary electrophoresis, confirming the presence of arginine-specific ADP-ribosyltransferase in the fraction. The ADP-ribosyltransferase designated AT-H with such high molecular weight on the gel filtration has not been detected in the chicken. AT-H was not retained to CM-cellulose (data not shown) while native AT1 was adsorbed to the cation exchanger (4). To determine the molecular size of AT-H, we did zymographic *in situ* assay under reducing conditions. As shown in Fig. 1. inset, the molecular size of AT-H was estimated to be 27.5 kDa. Interestingly, though molecular size of recombinant AT2 enzyme was 34 kDa on zymography (5), the recombinant AT2 activity obtained from COS 7

cells transfected with AT2 cDNA was also eluted at the void fraction on the Sephadex G-100 chromatography and passed through the CM-cellulose column (data not shown).

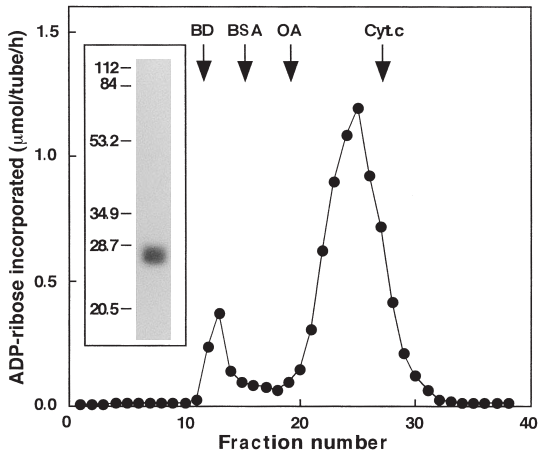


Fig. 1. Sephadex G-100 gel filtration of chicken bone marrow cell extract. Extract from chicken bone marrow cells was fractionated on a Sephadex G-100 column in fractions. ADP-ribosyltransferase activity was assayed at 25 ℃ for 30 min. The arrows indicate the eluted positions of the molecular weight markers: blue dextran (BD), bovine serum albumin (BSA, 67,000), ovalbumin (OA, 43,000) and cytochrome c (Cyt.c, 12,000). Inset showed zymographic *in situ* assay. The void fraction on Sephadex G-100 was subjected to zymographic assay as described in Materials and Methods. Positions of standard proteins (kDa) are indicated on the left.

To investigate the enzymatic properties of AT-H, we examined the effects of NaCl and DTT on the activities of AT-H, recombinant AT2 and native AT1. We first investigated the effect of NaCl concentration on their activities. As shown in Fig. 2, AT-H and recombinant AT2 were enhanced by NaCl while activity of native AT1 decreased with increasing concentrations of NaCl. Maximal activation of AT-H and recombinant AT2 was observed at 200 mM. At this concentration, native AT1 was inhibited by 64%. As reported previously (4, 5, 7), native AT1 activity, hardly detected and not affected by NaCl in the absence of DTT, was greatly enhanced by DTT and the enhanced activity was inhibited by NaCl. Recombinant AT2 was active even in the absence of DTT and was stimulated slightly with NaCl, DTT or both about 1.35, 1.36 and 2.07 times, respectively. Similarly, AT-H showed the transferase activity without DTT and was activated by NaCl, DTT or both about 2.0, 1.76 and 2.5 times,

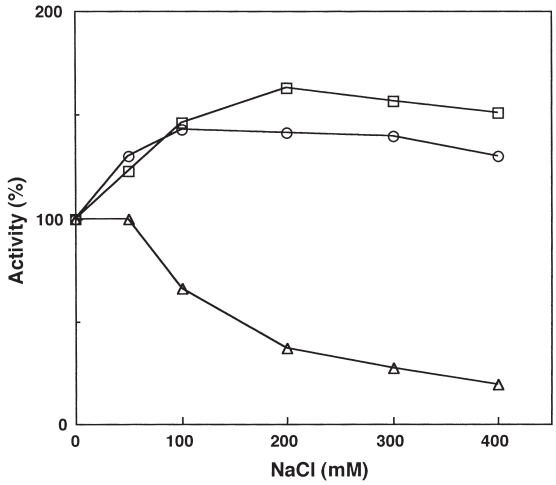


Fig. 2. Effect of NaCl on the activities of AT-H, recombinant AT2 and native AT1. Enzyme activity of AT-H (○), recombinant AT2 (□) and native AT1 (△) was assayed in the presence of 5 mM DTT with the indicated concentrations of NaCl at 25 ℃ for 1 hour. The data are expressed as percentages of the control values in the absence of NaCl, which were 0.67 nmol/h (AT-H), 0.05 nmol/h (recombinant AT2) and 1.18 nmol/h (native AT1).

respectively (Table 1).

Next, we examined the effect of chaotropic salts on their activities and molecular size on the gel filtration. As shown in Table 1, AT-H and recombinant AT2 were activated by chaotropic salts. The order of the stimulatory effect was NaSCN > NaBr > NaCl> NaF. In contrast, native AT1 transferase was inhib-

Table 1. Comparison of some properties of recombinant AT2 (rAT2), AT-H and native AT1 (nAT1)

| | rAT2 | AT-H | nAT1 |
|---|---|---|---|
| Molecular weight (kDa) | | | |
| Zymography | 34 | 27.5 | 27.5 |
| Gel filtration on Sephadex-G100 | >100 | >100 | 28.0 |
| CM-Cellulose | pass | pass | adsorb |
| Effect of NaCl (200 mM) and DTT (5 mM)[a] | | Activation (times) | |
| NaCl | 1.35 | 2.00 | 0.97 |
| DTT | 1.36 | 1.76 | 35.52 |
| NaCl and DTT | 2.07 | 2.50 | 14.23 |
| Effect of chaotropic salts (200 mM)[b] | | Activation (times) | |
| NaSCN | 1.86 | 1.67 | 0.16 |
| NaBr | 1.77 | 1.47 | 0.33 |
| NaCl | 1.61 | 1.36 | 0.39 |
| NaF | 1.35 | 1.26 | 0.65 |

[a] The enzyme activities of rAT2, AT-H and nAT1 in the absence of NaCl and DTT were 0.09 nmol/h, 0.19 nmol/h and 0.01 nmol/h, respectively.

[b] The enzyme activities of rAT2, AT-H and nAT1 in the absence of chaotropic salts were 0.09 nmol/h, 0.67 nmol/h and 1.11 nmol/h, respectively.

ited by them with the same order. Type A transferase from turkey erythrocytes also is activated by chaotropic salts probably via the conversion of the enzyme from inactive oligomer to active protomer (8). However, recombinant AT2 and AT-H enzymes were not converted into small molecular weight form on the gel filtration even in the presence of 1 M NaCl (data not shown). Thus, the activation of AT-H and recombinant AT2 by salts is not due to the formation of the protomeric active form of the enzyme.

In the present study, we demonstrated that another transferase AT-H, detected in high-molecular-weight form on the gel filtration, is present in chicken bone marrow cells and the transferase shares several common characteristics with recombinant AT2 enzyme in terms of chromatographic behavior and enzymatic properties except molecular size as shown in Table 1. The discrepancy in the molecular mass may reflect differences in post-translational modifications such as glycosylation or processing of proteins between chicken bone marrow cells and COS 7 cells originated from African green monkey kidney. Our results suggest that AT-H is probably the native AT2 transferase expressed in chicken bone marrow cells but not similar to turkey erythrocyte type A transferase. To confirm that AT2 cDNA encodes AT-H transferase, determination of primary structure of purified AT-H transferase or production of AT2 transferase antibody is required. Further studies are ongoing.

## ACKNOWLEDGMENTS

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