Proc. Natl. Acad. Sci. USA
Vol. 82, pp. 5603–5607, September 1985
Biochemistry

Reversibility of arginine-specific mono(ADP-ribosyl)ation: Identification in erythrocytes of an ADP-ribose-L-arginine cleavage enzyme

(ADP-ribosylation/cholera toxin/adenylate cyclase)

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Communicated by E. R. Stadtman, April 22, 1985

ABSTRACT **Enzymes have been identified in animal tissues that catalyze the mono(ADP-ribosyl)ation of arginine and proteins. Since these NAD:arginine ADP-ribosyltransferases under physiological conditions do not appear to catalyze the degradation of the product ADP-ribose-arginine, the possibility was investigated that a different family of enzymes exists that cleaves the ADP-ribose-arginine linkage. An enzyme was identified in and partially purified from turkey erythrocytes that catalyzed the degradation of ADP-ribose-[^{14}C]arginine synthesized by a salt-activated NAD:arginine ADP-ribosyltransferase, resulting in the release of a radiolabeled compound that was characterized chromatographically and by amino acid analysis as arginine. This putative arginine product was converted in a reaction dependent on NAD and the NAD:arginine ADP-ribosyltransferase to a compound exhibiting properties characteristic of ADP-ribose-arginine. Action of cleavage enzyme on [*adenine*-U-^{14}C]ADP-ribose-arginine resulted in the release of a radiolabeled compound that behaved chromatographically like [*adenine*-U-^{14}C]ADP-ribose. Since degradation of ADP-ribose-arginine appears to generate an arginine moiety that is a substrate for the NAD:arginine ADP-ribosyltransferase, it appears that ADP-ribosylation may be a reversible modification of proteins.**

ADP-ribosylation is a covalent modification catalyzed by viral, bacterial, and animal enzymes (1, 2); the ADP-ribose moiety of NAD is transferred to either an acceptor protein or amino acid or to another ADP-ribose moiety to yield an acceptor-linked monomer or polymer (1, 2). These synthetic reactions are catalyzed by two classes of enzymes: (*i*) mono(ADP-ribose)transferases, which catalyze the mono(ADP-ribosyl)ation of proteins, and (*ii*) poly(ADP-ribose) synthetases, which catalyze the initial ADP-ribosylation of protein and subsequent chain elongation (3–6). The importance of the mono(ADP-ribosyl)ation reaction has been best documented for bacterial toxins that exert their effects on cells by modifying critical enzymes (7–13). Choleragen (cholera toxin) and *Escherichia coli* heat-labile enterotoxin, agents involved in the pathogenesis of cholera and traveler's diarrhea, respectively, modify a stimulatory GTP-binding protein of the adenylate cyclase system, leading to an increase in cellular cyclic AMP (9, 12, 13). Pertussis toxin (islet-activating protein) ADP-ribosylates and inactivates an inhibitory component of adenylate cyclase (10–13). Diphtheria toxin and *Pseudomonas* exotoxin A modify elongation factor II, thereby inhibiting protein synthesis and causing cell death (7, 8); an ADP-ribosyltransferase endogenous to animal cells is believed to catalyze a reaction similar to diphtheria toxin (14). Although the metabolic role of poly(ADP-ribose) synthetase has not been defined, it is closely linked to chromatin-associated events (1, 6).

Among the ADP-ribosyltransferases endogenous to animal cells are those that catalyze the formation of ADP-ribose-arginine (15–19). These enzymes, which also modify proteins, presumably using a guanidino moiety as an ADP-ribose acceptor, have been identified and purified from turkey erythrocytes, chicken liver, and rabbit muscle (15–19). A family of NAD:arginine ADP-ribosyltransferases has been identified in turkey erythrocytes, where different transferases are localized in the nucleus, cytosol, and membrane fractions (16, 17, 20). These transferases differ in physical, kinetic, and regulatory properties (20). The bacterial toxins, choleragen and *E. coli* heat-labile enterotoxin, also possess NAD:arginine ADP-ribosyltransferase activity (21, 22); toxin-catalyzed activation of the adenylate cyclase system is believed to result from modification of an arginine residue on a cyclase regulatory protein (23).

The mechanism for the reversal of the mono(ADP-ribosyl)ation reaction has not been elucidated fully. The pyrophosphate moiety of protein-bound ADP-ribose is cleaved by a phosphodiesterase activity that is fairly common in animal tissues (24, 25). In extracts from Simian virus 40-transformed mouse fibroblasts (SVT2), ADP-ribose-protein was degraded by endogenous enzymes to release compounds tentatively identified as ADP-ribose and phosphoribose, although other products were formed consistent with proteolytic digestion to yield an ADP-ribose-(amino acid) peptide conjugate(s) (26). To study the reversal of ADP-ribosylation, it was decided to examine the degradation of a model compound, ADP-ribose-arginine, and thus to circumvent the problems inherent in characterization of the products of a more complex ADP-ribose-protein degradation. Since turkey erythrocytes were the source of a family of NAD:arginine ADP-ribosyltransferases responsible for the synthetic reaction, this tissue was examined for the presence of ADP-ribose-arginine cleavage enzymes.

MATERIALS AND METHODS

Materials. Turkey blood was purchased from Pel-Freez; $MgCl_2$, potassium phosphate, and NaCl, from Fisher; Affigels 601 and 501, from Bio-Rad; agmatine, arginine, ovalbumin, phosphodiesterase I, alkaline phosphatase, and NAD, from Sigma; [*carbonyl*-^{14}C-]NAD (52 mCi/mmol; 1 Ci = 37 GBq), [^{14}C]arginine (336 mCi/mmol), [^{3}H]arginine (51 Ci/mmol), and [*adenine*-U-^{14}C]NAD (280 mCi/mmol), from Amersham; dithiothreitol, from Bethesda Research Laboratories; thin-layer chromatograms, from Sybron/Brinkmann; DE-52 cellulose, from Whatman; phenyl-Sepharose, from Pharmacia; and cholera toxin, from List.

Assays. NAD:arginine ADP-ribosyltransferase activity was determined in a total volume of 0.3 ml containing 50 mM

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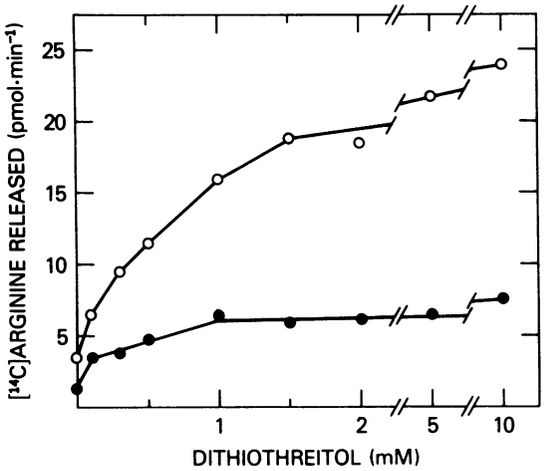

FIG. 1. Effect of dithiothreitol and Mg^{2+} on the activity of the ADP-ribose-arginine cleavage enzyme. Phenyl-Sepharose-purified ADP-ribose-arginine cleavage enzyme (32.6 μg) was assayed without (●) or with (○) 10 mM $MgCl_2$ and the indicated concentration of dithiothreitol, with other conditions as described for the standard assay. Means of values from quadruplicate assays are shown.

potassium phosphate (pH 7.0), ovalbumin (1 mg/ml), 32.4 μM [*carbonyl*-^{14}C]NAD (40,000 cpm), 200 mM NaCl, and 10 mM agmatine. The reaction was initiated with transferase (0.02 milliunits). After incubation at 30°C for 30 min, two 0.1-ml samples were transferred to columns (0.2 × 4 cm) of AG1-X2 (Bio-Rad) equilibrated with water to isolate [*carbonyl*-^{14}C]nicotinamide (21, 27). One unit of enzyme catalyzes the transfer of 1 μmol of ADP-ribose to agmatine per min. All assays were run in duplicate.

ADP-Ribose-Arginine Cleavage. ADP-ribose-arginine cleavage activity was assayed in a total volume of 0.1 ml containing 50 mM potassium phosphate (pH 7.5), 10 mM $MgCl_2$, 50 μM ADP-ribose-[^{14}C]arginine (≈5000 cpm), 5 mM dithiothreitol, and other additions as indicated. The reaction was initiated with cleavage enzyme. After incubation for 30 min at 30°C, samples (80 μl) were transferred to phenylboronate affinity columns (0.2 × 4 cm) (Affi-Gel 601) to separate the substrate from [^{14}C]arginine. All assays were run either in triplicate or quadruplicate.

Protein was determined by a modification of the method of Lowry *et al.* (28) with bovine serum albumin as a standard or by a dye-binding assay (Bio-Rad).

Protein Purification. NAD:arginine ADP-ribosyltransferase A purified from turkey erythrocytes as described (16) exhibited one major protein band on $NaDodSO_4$/polyacrylamide slab gels.

The ADP-ribose-arginine cleavage enzyme was partially purified from the supernatant fraction (27,000 × *g*, 20 min) of a turkey erythrocyte homogenate by successive chromatography on DE-52 cellulose, phenyl-Sepharose, and organomercurial agarose. This procedure, although it produced only ≈200-fold purification, did remove a number of contaminating activities, such as pyrophosphatases, that act on the ADP-ribose moiety (25).

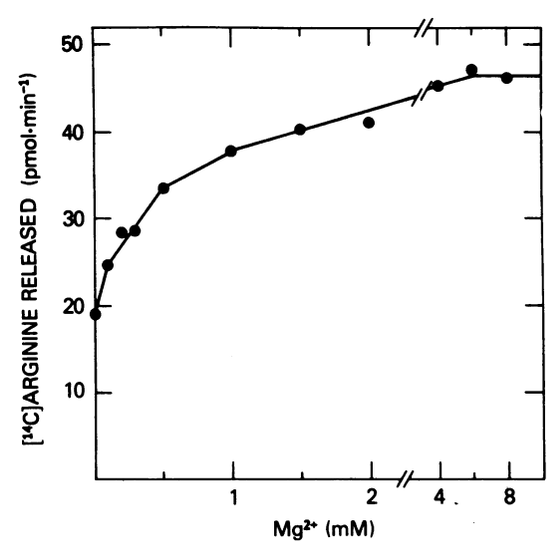

FIG. 2. Effect of $MgCl_2$ on the activity of the ADP-ribose-arginine cleavage enzyme. Organomercurial agarose-purified cleavage enzyme (55.2 μg) was assayed under standard conditions except that the concentration of $MgCl_2$ was varied as indicated. Means of values from quadruplicate assays are reported.

RESULTS

Degradation of ADP-ribose-L-[^{14}C]arginine by the partially purified cleavage enzyme was enhanced by dithiothreitol (Fig. 1) and $MgCl_2$ (Figs. 1 and 2). Activation was maximal with 5–10 mM dithiothreitol and 5–10 mM $MgCl_2$ (Fig. 2).

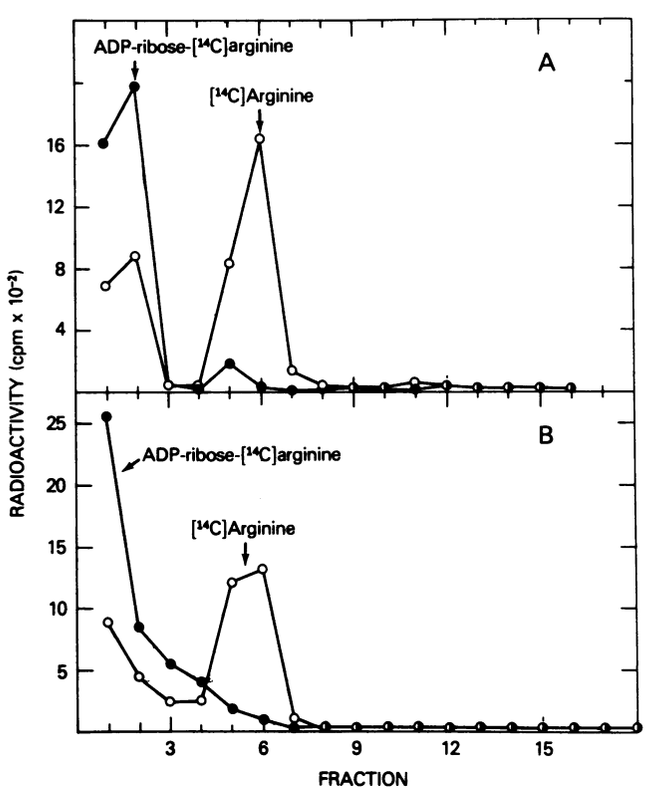

FIG. 3. Identification of the product of enzymatic cleavage of ADP-ribose-[^{14}C]arginine. ADP-ribose-[^{14}C]arginine (11.5 μM; 21,600 cpm) was incubated for 20 min at 30°C without (●) or with (○) organomercurial agarose-purified ADP-ribose-arginine cleavage enzyme (9.65 μg) as described for assay of its activity. A sample (0.02 ml) was applied to a sheet (0.1 mm) of polyethyleneimine-cellulose MN 300 impregnated with fluorescent indicator. After development with 1-butanol/acetic acid/H_2O, 12:3:5 (vol/vol) (*A*) or ethanol/ammonia/H_2O, 80:4:16 (vol/vol) (*B*), chromatograms were cut into 1.0-cm segments, which were transferred to vials containing 2.5 ml of water, and radioactivity was determined after the addition of Beckman Ready-Solv MP. Recovery of applied radioactivity was 97.6%. The migration of arginine is shown; it was separated from other standards that included 5'-AMP, ADP, and NAD.

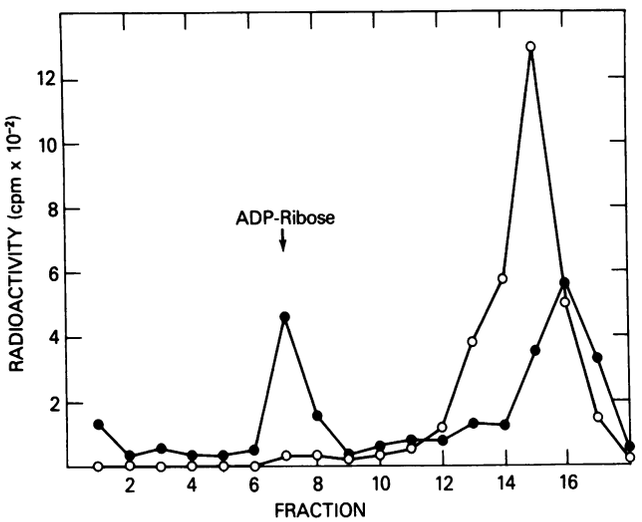

FIG. 4. Identification of the product of the enzymatic cleavage of [*adenine*-U-^{14}C]ADP-ribose-arginine as [*adenine*-U-^{14}C]ADP-ribose. [*adenine*-U-^{14}C]ADP-ribose-arginine was generated in the presence of the purified NAD:arginine ADP-ribosyltransferase (100 milliunits) in a mix containing 50 mM Hepes at pH 8.0, 20 mM arginine, 10 mM [*adenine*-U-^{14}C]NAD (9.9×10^6 cpm), 1 mg of ovalbumin per ml, and 100 mM NaCl in a total vol of 1.0 ml. After 90 min at 30°C, the mix was applied to a boronate affinity column (1 × 2 cm); the column was washed with 0.1 M glycine, pH 9.0/10 mM $MgCl_2$/0.1 M NaCl, and [*adenine*-U-^{14}C]ADP-ribose-arginine was eluted with 50 mM sodium acetate (pH 5.0) (eight 2-ml fractions). Fractions were neutralized with 0.1 ml of 1 M dibasic potassium phosphate. To test for the cleavage products of [*adenine*-U-^{14}C]ADP-ribose-arginine, the substrate (0.177 mM; 17,400 cpm) was incubated in a mix containing 50 mM potassium phosphate at pH 7.0, 10 mM $MgCl_2$, and 5 mM dithiothreitol in a total vol of 0.1 ml. The reaction was initiated with (●) or without (○) organomercurial affinity column-purified cleavage enzyme (16.1 μg) and was allowed to run for 1 hr at 30°C; 0.02 ml was then applied to 0.1-mm polyethyleneimine-cellulose MN 300 TLC plate impregnated with fluorescent indicator (Brinkmann). The chromatogram was run in 1 M acetic acid until the solvent front reached the origin. The plate was then transferred to 0.9 M acetic acid/0.3 M LiCl. The migration of ADP-ribose is shown; it was separated from other standards that included 5′-AMP, ADP, and NAD; the major radioactive peak corresponds to [*adenine*-U-^{14}C]ADP-ribose-arginine. Fractions were cut and counted after addition of 2 ml of water and 9 ml of Beckman Ready-Solv MP. Of the applied radioactivity, 66.1% was recovered on the chromatogram; 23% of the recovered radioactivity corresponds to [*adenine*-U-^{14}C]ADP-ribose.

In the standard assay procedure, a phenylboronate resin, which binds compounds containing vicinal hydroxyl groups, was added to separate the substrate, ADP-ribose-L-[^{14}C]arginine, from [^{14}C]arginine, the presumed product of the cleavage enzyme-catalyzed reaction. The product was characterized further as [^{14}C]arginine by thin-layer chromatography in two solvent systems (Fig. 3 *A* and *B*). A product identical to arginine was formed as well with ADP-ribose-L-[^{3}H]arginine as substrate (data not shown). Other possible degradation products of ADP-ribose-L-[^{14}C]arginine, phosphoribosyl-[^{14}C]arginine and ribosyl-[^{14}C]arginine, were generated by incubation with a snake venom phosphodiesterase or with both phosphodiesterase and alkaline phosphatase, respectively. Both compounds, which contain the vicinal hydroxyls of ribose, bind to phenylboronate resins and can be distinguished readily from [^{14}C]arginine.

To determine whether cleavage of ADP-ribose-L-arginine resulted in the formation of ADP-ribose as well as arginine, [*adenine*-U-^{14}C]ADP-ribose-L-arginine, synthesized from [*adenine*-U-^{14}C]NAD and excess L-arginine by the erythrocyte ADP-ribosyltransferase, was incubated with Mg^{2+}, dithiothreitol, and cleavage enzyme. A radiolabeled product exhibited a mobility similar to that of ADP-ribose by thin-layer chromatography (Fig. 4).

Table 1. Identification by amino acid analysis of a product of the enzymatic cleavage of ADP-ribose-L-[^{14}C]arginine as [^{14}C]arginine

Product assay	Arginine in column fraction, nmol	
	No enzyme	With enzyme
Amino acid analysis	0.4	11.8
Radioassay of ^{14}C	2.9	10.0

ADP-ribose-[^{14}C]arginine was synthesized in a reaction mix containing 50 mM Hepes at pH 8.0, 40 mM [^{14}C]arginine (3.74×10^7 cpm), 20 mM NAD, 100 mM NaCl, and 1 mg of ovalbumin per ml (total vol, 2 ml). Reaction was initiated with NAD:arginine ADP-ribosyltransferase (200 milliunits) and run for 90 min at 30°C. The substrate, [^{14}C]arginine, was separated from the product, ADP-ribose-[^{14}C]arginine, by chromatography on a column (1.6-ml bed vol) of phenylboronate. ADP-ribose-[^{14}C]arginine was eluted with 50 mM sodium acetate (pH 5.0). The solution was neutralized with 1 M dibasic potassium phosphate. Conversion of [^{14}C]arginine to ADP-ribose-[^{14}C]arginine was 27.0%; to determine the products of ADP-ribose-[^{14}C]arginine cleavage, 0.596 mM ADP-ribose-[^{14}C]arginine (2.79×10^5 cpm) was incubated in 50 mM potassium phosphate, pH 7.0/10 mM $MgCl_2$/5 mM dithiothreitol in the presence or absence of organomercurial agarose-purified cleavage enzyme (0.161 mg) in a total vol of 1 ml for 3 hr. The solution was then applied to a phenylboronate column (1.5-ml bed vol). The sample volume (1 ml) and 4 ml of a 5 mM potassium phosphate, pH 8.0/10 mM $MgCl_2$ wash were collected (total vol, 5 ml). Two 0.1-ml aliquots were applied to an amino acid analyzer (Beckman 6300) to determine the effect of cleavage enzyme on generation of [^{14}C]arginine. The recovery of [^{14}C]arginine in the 5-ml eluate was 39.3%.

These studies tentatively identified the products of ADP-ribose-L-[^{14}C]arginine degradation by the cleavage enzyme as ADP-ribose and L-[^{14}C]arginine. To confirm further the identity of the cleavage enzyme product as arginine and to demonstrate that the guanidino moiety was preserved during the cleavage reaction (i.e., excluding ornithine and citrulline as possible products), the radiolabeled product formed during degradation of ADP-ribose-L-[^{14}C]arginine by the cleavage enzyme was resolved by chromatography on a phenylboronate resin and identified as arginine by amino acid analysis (Table 1). This radiolabeled compound was incubated with excess NAD and the purified erythrocyte NAD:arginine ADP-ribosyltransferase; the radiolabeled product of the transferase-catalyzed reaction adhered to the phenylboronate resin and had a mobility identical to that of ADP-ribose-L-[^{14}C]arginine by thin-layer chromatography (Table 2 and data not shown). Formation of the presumed ADP-ribose-L-[^{14}C]arginine conjugate was dependent on NAD and ADP-ribosyltransferase (Table 2). Addition of unlabeled arginine in excess of the NAD inhibited the incorporation of radiolabel into ADP-ribose-L-[^{14}C]arginine (Table 2).

DISCUSSION

The present report documents the presence of an ADP-ribose-L-arginine cleavage enzyme in turkey erythrocytes. The enzyme appears to catalyze the degradation of ADP-ribose-L-arginine to ADP-ribose and arginine in a reaction that is stimulated by Mg^{2+} and dithiothreitol. The purification procedure separated this activity from other enzymes that act on the substrate, such as phosphodiesterases (25).

In animal tissues, enzymes have been identified that transfer ADP-ribose to at least two different amino acids in acceptor proteins, glutamate and arginine (15–19, 29–32). The glutamate residues of histones are modified by poly(ADP-ribose) synthetase, an enzyme that catalyzes both the initial ADP-ribosylation of protein as well as the subsequent chain elongation and branching to yield a poly(ADP-ribosyl)ated protein (1, 3–6). Degradation of the polymer can

Table 2. Formation of ADP-ribose-L-[^{14}C]arginine from the product of the cleavage reaction presumed to be [^{14}C]arginine

Additions	cpm		Unadsorbed [^{14}C]arginine, cpm*	Adsorbed ADP-ribose-[^{14}C]arginine, cpm*
	Applied	%recovered		
NAD (1 mM)	871	97.1%	815 (93.4%)	32 (3.7%)
Transferase (1 milliunit)	860	101.9%	870 (98.9%)	26 (3.0%)
Transferase/NAD	1005	90.2%	114 (11.3%)	793 (78.9%)
Transferase/NAD/arginine (10 mM)	867	107.8%	837 (96.5%)	98 (11.3%)

The presumed product of the cleavage reaction, [^{14}C]arginine, was generated from ADP-ribose-[^{14}C]arginine by action of the organomercurial affinity column-purified cleavage enzyme, as described in the legend to Table 1. To convert the putative [^{14}C]arginine to its ADP-ribosylated derivative, the radiolabeled compound (10,400 cpm; 66.6 μM) was incubated with the indicated additions in 25 mM Hepes (pH 8.0) with ovalbumin (1 mg/ml) and 100 mM NaCl in a total vol of 0.2 ml; NAD:arginine ADP-ribosyltransferase, 1 milliunit; 1 mM NAD; additional arginine, 10 mM. After 2 hr at 30°C, 0.02-ml aliquots were applied to a boronate affinity column. Unadsorbed cpm (pH 8 "run-through" and wash) and adsorbed cpm eluted with 50 mM sodium acetate (pH 5.0) are termed in the table [^{14}C]arginine and ADP-ribose-[^{14}C]arginine, respectively.

*The percentage of the total [^{14}C]arginine cpm applied is in parentheses.

proceed through phosphodiesterases (24, 25), working on the pyrophosphate linkage to yield phosphoribosyl-AMP and poly(ADP-ribose) glycohydrolases (33–35), which act on the ribose–ribose linkages to yield ADP-ribose. Hayaishi and co-workers (36, 37) demonstrated that the terminal ADP-ribose-protein linkage is degraded by a specific lyase that catalyzes the release of ADP-ribose in a complex elimination reaction; congenital absence of lyase activity may result in progressive neurological degeneration and renal failure (38). A second class of enzymes catalyzes the formation of mono(ADP-ribosyl)-arginine linkages, using both the free amino acid and proteins as acceptors (15–19). Using ADP-ribose-histone generated by NAD:arginine ADP-ribosyltransferase A from turkey erythrocytes, Smith *et al.* (26) found evidence for degradation of the linkage in cultured murine SVT2 cells. Due to the presence of contaminating activities, multiple degradation products including ADP-ribose and phosphoribose were generated. In the present study, a series of purification steps was used to obtain an enzyme preparation free of the contaminating phosphodiesterases that act on the pyrophosphate moiety and phosphatases that degrade 5′-AMP and phosphoribosylarginine. To circumvent the problem posed by the presence of proteases, a model compound, ADP-ribose-[^{14}C]arginine, was used to survey turkey erythrocyte fractions for ribose-arginine cleavage enzymes.

The chemical techniques used to identify the products might not differentiate between compounds having minor differences in structure with ADP-ribose and arginine. Action of a specific rat liver lyase on the ADP-ribose-histone, which was formed by degradation of poly(ADP-ribose)–protein, results in the synthesis of an elimination product distinct from ADP-ribose (37). It is clearly difficult to determine whether the glutamate residue of the histone is altered; from a mechanistic viewpoint, elimination of the ADP-ribose should proceed with regeneration intact of the glutamate moiety. Degradation of ADP-ribose-arginine by the cleavage enzyme appears to generate an arginine moiety; by using the model substrate in place of a protein acceptor, it was possible to characterize more fully the amino acid product. The compound appears to be arginine, based on identification and quantification by amino acid analysis and its mobility on thin-layer chromatograms. Furthermore, this compound formed by cleavage enzyme degradation of ADP-ribose-arginine served as a substrate for the NAD:arginine ADP-ribosyltransferase; in a reaction dependent on NAD and transferase, a compound was generated having the characteristics of ADP-ribose-arginine. Thus, it would appear that the presence of both transferase and cleavage enzyme may lead to an ADP-ribosylation/de-ADP-ribosylation cycle, lending further support to the hypothesis that this covalent modification may have a regulatory function in animal cells. Identification of the natural substrates for these enzymes will be necessary to define the physiological role of arginine-specific ADP-ribosylation.

We thank D. Marie Sherwood for expert secretarial assistance, Dr. John J. Pisano for the amino acid analysis, and Dr. Martha Vaughan for critical review of the manuscript.

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