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Biochemistry

Isolation of an avian erythrocyte protein possessing ADP-ribosyltransferase activity and capable of activating adenylate cyclase

(NAD/choleragen/*Escherichia coli* heat-labile enterotoxin)

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ABSTRACT **An ADP-ribosyltransferase was purified ~500-fold from the supernatant fraction of turkey erythrocytes. The enzyme hydrolyzed [*carbonyl*-^{14}C]NAD to ADP-ribose and [*carbonyl*-^{14}C]nicotinamide at a low rate. Nicotinamide formation from NAD was enhanced by arginine methyl ester > D-arginine ~ L-arginine > guanidine; lysine, histidine, and citrulline were ineffective. Incubation of [*adenine-U*-^{14}C]NAD and arginine methyl ester or arginine with the purified enzyme resulted in the formation of new compounds that contained ^{14}C, reacted with ninhydrin, and quenched background fluorescence of thin-layer plates viewed in ultraviolet light. Their mobilities on thin-layer chromatograms were indistinguishable from those of ADP-ribosylarginine methyl ester and ADP-ribosylarginine formed during incubation of choleragen with NAD and arginine methyl ester or arginine, respectively [Moss, J. & Vaughan, M. (1977) *J. Biol. Chem.* 252, 2455–2457]. The purified transferase also catalyzed the incorporation of label from [*adenine*-^{14}C]-NAD into lysozyme, histones and polyarginine. When the ^{14}C-labeled lysozyme was incubated with snake venom phosphodiesterase, the radioactivity was released and, on thin-layer chromatograms, exhibited a mobility indistinguishable from that of 5′-AMP, as would be expected of an ADP-ribosylated protein, but not of a poly(ADP-ribosylated) product. The purified transferase activated rat brain adenylate cyclase and, as is the case with choleragen, activation was absolutely dependent on NAD. The presence in the avian erythrocyte of a protein that, like choleragen and *Escherichia coli* heat-labile enterotoxin, apparently activates adenylate cyclase and possesses ADP-ribosyl transferase activity is consistent with the view that the mechanisms through which the bacterial toxins produce pathology are not entirely foreign to vertebrate cells, at least some of which may possess and employ an analogous mechanism for activation of adenylate cyclase.**

The activation of adenylate cyclase in vertebrate cells by choleragen, an enterotoxin of *Vibrio cholerae*, is dependent on NAD as originally demonstrated by Gill (1, 2). The observation that choleragen catalyzes the hydrolysis of NAD (3, 4) and the transfer of ADP-ribose from NAD to the guanidino group of arginine (5) led to the suggestion that the mechanism of action of choleragen is analogous to that of diphtheria toxin and *Pseudomonas* exotoxin A, which catalyze the NAD-dependent ADP-ribosylation of elongation factor II and thereby inhibit protein synthesis (6–9). It seems probable that choleragen activates adenylate cyclase by catalyzing the ADP-ribosylation of a specific protein component of the cyclase system. We carried out the studies reported here in an attempt to determine whether animal cells might themselves possess a similar mechanism for regulation of adenylate cyclase activity. We have found that the supernatant fraction from turkey erythrocytes catalyzes the NAD-dependent ADP-ribosylation of arginine, and we have extensively purified from this source an ADP-ribosyltransferase that activates adenylate cyclase in an NAD-dependent reaction.

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EXPERIMENTAL PROCEDURE

Materials. Choleragen, guanidine, and dithiothreitol were purchased from Schwarz/Mann; ATP, GTP, pyruvate kinase [465 units/mg in 2.2 M $(NH_4)_2SO_4$], L-lysine (free base), L-arginine methyl ester di-HCl, phospho*enol*pyruvate (sodium salt), cyclic AMP (sodium salt), ADP-ribose, L-arginine·HCl, ovalbumin, lysozyme (18,000 units/mg), snake venom phosphodiesterase, polyarginine, histone (Type IIA, calf thymus), and NAD from Sigma; L-citrulline and D-arginine·HCl from P-L Biochemicals; Polygram Cel 3000 DEAE (for thin-layer chromatography) from Brinkmann Instruments; glycine from Fisher Scientific; precoated thin-layer cellulose plates from E. M. Laboratories; bovine serum albumin from Armour Pharmaceutical; AG 1-X2 (100–200 mesh, chloride form), AG 50W-X8 (100–200 mesh) and AG 1-X2 (200–400 mesh) from Bio-Rad; EGTA from Eastman; [*adenine-U*-^{14}C]NAD (280 mCi/mmol), [*carbonyl*-^{14}C]NAD (50 mCi/mmol) and L-[^{3}H]arginine (11 mCi/mmol) from Amersham/Searle; [2,8-^{3}H]ATP (29.3 Ci/mmol) from New England Nuclear.

Enzyme Purification. Turkey erythrocytes were generously provided by Allen M. Spiegel. Details of the purification of the supernatant enzyme and its physical and kinetic properties will be described elsewhere. In brief, washed turkey erythrocytes were suspended in 50 mM potassium phosphate (pH 7.0), homogenized, and centrifuged at 100,000 × *g* for 30 min to obtain a supernatant fraction. The enzyme was then purified *ca* 500-fold by acid precipitation and ethanol extraction and subsequent chromatography successively on cellulose phosphate and phenyl-Sepharose. These preparations showed one major protein band after electrophoresis on polyacrylamide gels in the presence and absence of sodium dodecyl sulfate.

The experiments reported here were carried out with a single preparation. All observations have been confirmed with different preparations of the purified transferase. The adenylate cyclase preparation consisted of a washed particulate fraction from rat brain suspended in 50 mM glycine·HCl, pH 8.0/0.5 mM EGTA (10).

Assays. The methods used to measure NAD glycohydrolase and ADP-ribosyltransferase activities have been described (3–5). Unless otherwise noted, assays contained 50 mM potassium phosphate (pH 7.0), 0.3 mg of ovalbumin, 30 μM [*carbonyl*-^{14}C]NAD (40,000 cpm) and, where specified, 75 mM arginine methyl ester in a total volume of 0.3 ml. Assays were initiated with the addition of transferase and incubated for 1 hr at 30°. Duplicate 0.1 ml samples were then transferred to

Table 1. Effect of amino acids and related compounds on [*carbonyl*-^{14}C]nicotinamide release from NAD catalyzed by the ADP-ribosyltransferase

Additions, 75 mM	Nicotinamide released, pmol/assay
None	26
L-Arginine methyl ester	190
L-Arginine	170
D-Arginine	150
Guanidine	130
L-Lysine	11
L-Histidine	9
L-Citrulline	7
Ovalbumin*	99
L-Arginine methyl ester plus ovalbumin*	3600

Purified transferase (0.22 μg in 10 μl of 50 mM potassium phosphate/1 M NaCl, pH 7.0) was added to initiate assays which were carried out in a total volume of 0.3 ml containing 50 mM potassium phosphate (pH 7.0), 32.4 μM [*carbonyl*-^{14}C]NAD (42,600 cpm), and other additions as indicated. After incubation for 1 hr at 30°, duplicate 0.1 ml samples were withdrawn for isolation of [*carbonyl*-^{14}C]nicotinamide. Data are means of values from duplicate incubations.
* Ovalbumin, 1 mg/ml.

columns of AG 1-X2 (100–200 mesh) and [*carbonyl*-^{14}C]nicotinamide was collected. All assays were run in duplicate.

Adenylate cyclase was assayed by a modification of described procedures (10, 11). The enzyme preparation was incubated with "activated" choleragen, purified transferase or buffer, and 10 μl of a solution containing 12.5 mM ATP, 20 mM NAD, 50 mM $MgCl_2$, 185 mM phospho*enol*pyruvate, and pyruvate kinase (1160 units/ml) for 10 min at 30° in a total volume of 50 μl. The adenylate cyclase assay was initiated with the addition of 25 μl of a solution containing [^{3}H]ATP (3.78×10^6 cpm, plus unlabeled ATP if not added earlier), 100 mM glycine·HCl (pH 8.0), 1 mM dithiothreitol, and bovine serum albumin (9 mg/ml). After incubation for 10 min at 30°, sodium dodecyl sulfate was added and [^{3}H]cAMP was isolated.

"Activated" choleragen was prepared by incubation of 0.4 mg of choleragen and 0.4 mg of ovalbumin in 0.4 ml of 20 mM dithiothreitol in 50 mM glycine·HCl, pH 8.0, for 10 min at 30°. Such incubation was unnecessary for the purified erythrocyte transferase.

Protein was determined by the method of Lowry (12).

Table 2. Effect of buffer and pH on activity of ADP-ribosyltransferase

Buffer, 50 mM	Nicotinamide released, nmol/assay
Sodium acetate, pH 6.5	3.2
Potassium phosphate, pH 6.5	3.6
pH 7.0	4.0
Tris·HCl, pH 7.5	2.7
pH 8.0	2.3
Glycine·HCl, pH 8.0	2.1
pH 8.5	1.5

Purified transferase (0.22 μg in 10 μl of 50 mM potassium phosphate/1 M NaCl, pH 7.0) was added to initiate assays which were carried out in a total volume of 0.3 ml containing 75 mM L-arginine methyl ester, 32.4 μM [*carbonyl*-^{14}C]NAD (39,300 cpm), ovalbumin at 1 mg/ml, and the indicated buffer. After incubation for 1 hr at 30°, two 0.1-ml samples were taken from each assay for isolation of [*carbonyl*-^{14}C]nicotinamide. Data are means of values from duplicate incubations.

Table 3. Effect of lysine, arginine methyl ester, and acceptor proteins on activity of ADP-ribosyltransferase

Additions	Products formed, nmol/assay: Nicotinamide	ADP-ribosyl protein
None	0.077	0.002
Lysine, 75 mM	0.067	0.002
Arginine methyl ester, 75 mM	1.1	<0.002
Histone, 5 mg/ml	3.8	2.6
plus lysine	3.3	2.3
plus arginine methyl ester	5.8	0.052
Lysozyme, 5 mg/ml	1.4	1.1
plus lysine	1.4	1.0
plus arginine methyl ester	5.6	0.017
Polyarginine, 5 mg/ml	1.8	1.1
plus lysine	2.2	1.7
plus arginine methyl ester	5.3	0.11

Purified transferase (0.33 μg in 10 μl) was added to initiate assays which were carried out in a total volume of 0.3 ml containing 50 mM potassium phosphate (pH 7.0), 32.4 μM NAD, with 42,000 cpm of [*carbonyl*-^{14}C]NAD or [*adenine*-^{14}C]NAD, and other additions as indicated. After incubation for 1 hr at 30°, duplicate 0.1 ml samples were withdrawn from assays with [*carbonyl*-^{14}C]NAD for isolation of [*carbonyl*-^{14}C]nicotinamide. To assays containing [*adenine*-^{14}C]NAD, 0.1 ml of cold 20% trichloroacetic acid was added and after 30 min at 0° the samples were transferred to 0.45 μm Millipore filters. The assay tubes and filters were washed three times with 2 ml of cold 5% trichloroacetic acid. The dried filters were placed in counting vials with 2 ml of Piersolve (Pierce), and after they had dissolved counting solution was added.

Formation of [*Adenine*-^{14}C]ADP-Ribose-L-[^{3}H]Arginine and [*Adenine*-^{14}C]ADP-Ribose-L-Arginine Methyl Ester. Incubation of [*adenine*-^{14}C]NAD with arginine methyl ester and the supernatant transferase resulted in the formation of a new compound that was readily separable from nicotinamide, ADP-ribose, and NAD by thin-layer chromatography on cellulose. The mobility of the product was indistinguishable from that of the radioactive product isolated after the incubation of [*adenine-U*-^{14}C]NAD and arginine methyl ester with choleragen (5). As was the case with the choleragen reaction product, the compound isolated from the incubation with the erythrocyte enzyme contained ^{14}C, quenched the background fluorescence of the thin-layer plates, and reacted with ninhydrin.

The radioactive reaction product formed during incubation

Table 4. Incubation of lysozyme with [*adenine*-^{14}C]NAD with and without ADP-ribosyltransferase

Fraction	Distribution of ^{14}C, cpm: No transferase	Plus transferase
Supernatant	583,000	156,000
First wash	16,400	19,800
Second wash	288	2,620
Precipitate	135	322,000

Purified transferase (3.3 μg in 10 μl of 50 mM potassium phosphate/1 M NaCl, pH 7.0) was incubated for 1 hr at 30° with 0.75 mg of lysozyme in a total volume of 0.15 ml containing 50 mM potassium phosphate, pH 7.0, and 11.5 μM [*adenine*-^{14}C]NAD (~500,000 cpm). One ml of 6% trichloroacetic acid was then added and after 30 min at 0° the mixture was centrifuged at $1000 \times g$ for 15 min. The supernatant was saved, and the precipitate was washed twice with 1 ml of 5% trichloroacetic acid. The precipitate was dissolved in 0.25 ml of 50 mM Tris·HCl, pH 8.0. Samples of precipitate, washes, and supernatant were taken for radioassay.

Table 5. Effect of snake venom phosphodiesterase on ^{14}C-labeled lysozyme

^{14}C-labeled lysozyme incubated with:	^{14}C radioactivity, cpm, in: Precipitate	Supernatant
No phosphodiesterase	82,100	3,650
Plus phosphodiesterase	8,830	132,000

Samples (0.1 ml, ~129,000 cpm) of the dissolved precipitate from incubation of lysozyme with the transferase (described in Table 4) were incubated with 0.1 ml of snake venom phosphodiesterase (13 units in 50 mM Tris·HCl, pH 8.0) or diluent for 1.5 hr at 30°. Trichloroacetic acid (0.1 ml of 20%) was then added, and after 30 min at 0° the mixtures were centrifuged at 1000 × *g* for 15 min. Samples of supernatant and precipitate were taken for radioassay.

of the erythrocyte transferase with [*adenine*-^{14}C]NAD and L-[^{3}H]arginine was separated from ADP-ribose, NAD, and nicotinamide by thin-layer chromatography on DEAE-cellulose. Its mobility was indistinguishable from that of the product generated from these substrates in the presence of choleragen (5).

RESULTS

The purified enzyme from erythrocyte supernatant catalyzed the formation of [*carbonyl*-^{14}C]nicotinamide from [*carbonyl*-^{14}C]NAD (Table 1). Production of [*carbonyl*-^{14}C]nicotinamide was greatly accelerated by the addition of L-arginine methyl ester > D-arginine ~ L-arginine > guanidine (Table 1). Basic amino acids lacking a guanidino moiety were inactive. Citrulline, in which the guanidino group of arginine is replaced by a ureido function, did not stimulate [*carbonyl*-^{14}C]nicotinamide formation. The products of the reaction of [*adenine*-^{14}C]NAD and arginine methyl ester or arginine were identified as [*adenine*-^{14}C]ADP-ribosyl-L-arginine methyl ester or [*adenine*-^{14}C]ADP-ribosyl-L-[^{3}H]arginine, respectively, as outlined in *Experimental Procedure* (3–5).

ADP-ribosyltransferase activity with arginine methyl ester as an acceptor was greater in potassium phosphate, pH 7.0, than it was in other buffers tested (Table 2) and this buffer was used in all other studies reported here.

As shown in Table 1, the presence of ovalbumin somewhat increased nicotamide release from NAD in the absence of an acceptor and greatly increased it when arginine methyl ester was added. It was found that in the absence of ovalbumin the rate of nicotinamide release (with or without arginine methyl ester) decreased after the first few minutes, but remained relatively constant throughout the 1 hr incubation period when ovalbumin was present (data not shown). Histones, lysozyme, and polyarginine also increased nicotinamide production in the presence of arginine methyl ester (Table 3). In parallel incubations using [*adenine*-^{14}C]NAD, there was essentially no incorporation of ^{14}C into the trichloroacetic acid-precipitated protein and these proteins apparently acted like ovalbumin to stabilize transferase activity. In the absence of arginine methyl ester, on the other hand, histones, lysozyme, and polyarginine enhanced nicotinamide release catalyzed by the purified transferase and, after incubation with [*adenine*-^{14}C]NAD, the protein precipitated from solution with trichloroacetic contained ^{14}C (Table 3). When lysozyme was present in the transferase assay at a concentration of 5 mg/ml, the ratio of [*carbonyl*-^{14}C]nicotinamide released to [*adenine*-^{14}C]adenine incorporated into protein was 1:1 (data not shown). When the trichloroacetic acid-precipitated ^{14}C-labeled lysozyme (Table 4) was dialyzed and incubated with snake venom phosphodiesterase (Table 5), essentially all of the radioactivity was released and migrated on thin-layer chromatography with a mobility indistinguishable from that of 5′-AMP (Table 6). This result is consistent with formation of an ADP-ribosylated, rather than poly(ADP-ribosylated), protein.

As shown in Table 3, although 75 mM arginine methyl ester (a maximally effective concentration) increased nicotinamide production, it markedly inhibited the incorporation of label from [*adenine*-^{14}C]NAD into trichloroacetic acid-precipitable

Table 6. Identification of ^{14}C in supernatant after phosphodiesterase treatment of lysozyme from Table 5

Chromatography system	Recovery of ^{14}C with 5′-AMP, %
Polyethyleneimine	102
DEAE-cellulose paper	100

Samples of the trichloroacetic acid supernatant after incubation of ^{14}C-labeled lysozyme with phosphodiesterase (see Table 5) were chromatographed on polyethyleneimine with 0.9 M acetic acid/0.3 M LiCl (13) or on DEAE-cellulose paper with isobutyric acid: NH_4OH:water, 66:1:33 (vol/vol). The percentage of applied radioactivity that migrated with added known 5′-AMP is recorded.

Table 7. Effect of acceptor proteins and arginine methyl ester on ADP-ribosyltransferase activity of choleragen

Additions	Products formed, nmol/assay: Nicotinamide	ADP-ribosyl protein
None	21	0
Arginine methyl ester, 75 mM	139	0
Histone, 5 mg/ml	39	8.2
plus arginine methyl ester	159	4.1
Lysozyme, 5 mg/ml	38	3.8
plus arginine methyl ester	206	1.8
Polyarginine, 5 mg/ml	55	19.5
plus arginine methyl ester	185	9.6

Choleragen, 50 μg (*Experimental Procedure*), was added to initiate assays which were carried out in a total volume of 0.3 ml containing 20 mM dithiothreitol, 400 mM potassium phosphate (pH 7.0), 2 mM NAD with ~40,000 cpm of [*carbonyl*-^{14}C]NAD or [*adenine*-^{14}C]NAD, and other additions as indicated. After incubation for 1 hr at 30°, products were isolated as described in Table 3.

Table 8. Effect of ADP-ribosyltransferase on adenylate cyclase activity

Additions	Adenylate cyclase activity, pmol/10 min: No transferase	Plus transferase
None	20.9	19.8 (95)*
ATP	31.9	33.1 (104)
NAD	10.1	14.3 (142)
ATP, NAD	19.6	32.3 (165)

Samples of the adenylate cyclase preparation (196 μg protein) were incubated with purified transferase (6.6 μg in 20 μl of 50 mM potassium phosphate/1 M NaCl, pH 7.0) or 20 μl of transferase buffer and 10 μl of the solution described in *Experimental Procedure* (lacking ATP or NAD as indicated) in a total volume of 50 μl for 10 min at 30°. The adenylate cyclase assay solution (25 μl containing unlabeled ATP if it had not been added earlier) was then added, and, after incubation for 10 min at 30°, assays were terminated with the addition of sodium dodecyl sulfate. In this experiment, other samples of adenylate cyclase were incubated with 10 μl of "activated" choleragen solution or the same solution minus choleragen and other additions as described in *Experimental Procedure* (including NAD and ATP) for 10 min at 30° before assay of adenylate cyclase. Activity in the control samples was 83 and in the choleragen-treated samples, 196 pmol/10 min.

* Activity relative to that without transferase = 100.

material. Other amino acids which did not act as ADP-ribose acceptors, such as lysine (Table 3), had minimal effects on the incorporation of label into the trichloroacetic acid-precipitable material. Choleragen, in the presence of lysozyme, also catalyzed the incorporation of [*adenine*-^{14}C] from [*adenine*-^{14}C]NAD into trichloroacetic acid-precipitable material (Table 7). Arginine methyl ester (75 mM) decreased this incorporation to a lesser extent than was observed with the avian transferase. This concentration of arginine methyl ester is, however, not a saturating one for choleragen (data not shown).

The purified erythrocyte transferase activated adenylate cyclase from rat brain (Table 8) and activation, like that produced by choleragen (10), was dependent on NAD.

DISCUSSION

Activation of adenylate cyclase by bacterial toxins, such as choleragen and the heat-labile *Escherichia coli* enterotoxin, is believed to result from the NAD-dependent ADP-ribosylation of an intracellular protein (1–5, 14, 15). The isolation of an avian erythrocyte enzyme with catalytic properties resembling those of the bacterial toxins, as reported above, provides the first evidence that animal cells might employ a similar mechanism to control adenylate cyclase activity. The avian transferase, choleragen, and *E. coli* heat-labile enterotoxin can: (*i*) hydrolyze NAD to ADP-ribose and nicotinamide; (*ii*) transfer the ADP-ribose moiety of NAD to the guanidino moiety of arginine; (*iii*) catalyze the NAD-dependent ADP-ribosylation of model acceptor proteins;* and (*iv*) activate adenylate cyclase. These observations are consistent with the view that the erythrocyte ADP-ribosyltransferase and the two bacterial toxins activate adenylate cyclase by catalyzing the NAD-dependent ADP-ribosylation of a protein that is either the cyclase itself or a regulatory component of the cyclase system.

Both the avian erythrocyte transferase and choleragen can apparently use several proteins as acceptors for ADP-ribose. Although it has not been directly demonstrated that it is the arginine residues that are ADP-ribosylated, the fact that transfer of ADP-ribose to lysozyme by the erythrocyte enzyme is inhibited by arginine methyl ester, which at the same time stimulates nicotinamide release by acting as an alternative acceptor, is highly suggestive that the site of interaction of the protein acceptor with the transferase contains an arginine moiety. The failure of other amino acids to block ADP-ribosylation of these proteins is also consistent with this conclusion. ADP-ribosylation of purified proteins used as model acceptors might occur relatively nonspecifically on arginine moieties that are readily accessible—i.e., suitably situated and exposed with the protein in solution under experimental conditions. It would be expected, on the other hand, that in the adenylate cyclase system there is a specific arginine that is ADP-ribosylated. In the case of the viral transferase studied by Goff (16), it is clear that ADP-ribosylation occurs at only a single arginine in the substrate protein, a bacterial RNA polymerase. Regulation of adenylate cyclase activity by an endogenous ADP-ribosyltransferase would likely not be effectively achieved by the random formation of ternary complexes between enzyme, adenylate cyclase, and NAD. The transferase could, however, be subject to control through, for example, intracellular localization, covalent modification, or effector molecules.

If an ADP-ribosyltransferase functions in the intact erythrocyte to activate adenylate cyclase, then the cell should possess a mechanism for inactivation, perhaps an enzyme to catalyze the removal of ADP-ribose from the protein. No evidence for such a pathway is available. Recent studies of Oppenheimer (17) indicate that the ribosyl-guanidino bond of ADP-ribose-L-arginine is subject to isomerization which, if it occurred in the adenylate cyclase system, would complicate the enzymatic reversal of the activation. Nevertheless, the presence in the avian erythrocyte of a protein that, like choleragen and *E. coli* enterotoxin, activates adenylate cyclase and possesses ADP-ribosyltransferase activity supports the view that the mechanisms utilized by the bacterial toxins to produce cell pathology are not entirely foreign to vertebrate cells, at least some of which may possess and employ an analogous mechanism for physiological activation of adenylate cyclase.

* ADP-ribosylation of proteins has not been demonstrated with *E. coli* enterotoxin.

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