Purification and Characterization of ADP-ribosylarginine Hydrolase from Turkey Erythrocytes

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ABSTRACT: ADP-ribosylation of arginine appears to be a reversible modification of proteins with NAD:arginine ADP-ribosyltransferases and ADP-ribosylarginine hydrolases catalyzing the opposing arms of the ADP-ribosylation cycle. ADP-ribosylarginine hydrolases have been purified extensively (>90%) (150 000–250 000-fold) from the soluble fraction of turkey erythrocytes by DE-52, phenyl-Sepharose, hydroxylapatite, Ultrogel AcA 54, and Mono Q chromatography. Mobilities of the hydrolase on gel permeation columns and on sodium dodecyl sulfate–polyacrylamide gel electrophoresis under reducing conditions are consistent with an active monomeric species of ~39 kDa. Insertion of an organomercurial agarose chromatographic step prior to Ultrogel AcA 54 resulted in the isolation of a hydrolase exhibiting ~35-fold greater sensitivity to dithiothreitol ($K_{a,sensitive}$ = 41 ± 16.7 μM, n = 4; $K_{a,resistant}$ = 1.44 ± 0.12 mM, n = 3). A similar dithiothreitol-sensitive hydrolase was generated by exposure of the purified resistant enzyme to $HgCl_2$. At 30 °C, both thiol-sensitive (H_S) and thiol-resistant (H_R) hydrolases were relatively resistant to *N*-ethylmaleimide (NEM); incubation with dithiothreitol prior to NEM resulted in complete inactivation. Both H_S and H_R required Mg^{2+} and thiol for enzymatic activity. Mg^{2+} stabilized both H_S and H_R against thermal inactivation in the absence and presence of thiol. A purified NAD:arginine ADP-ribosyltransferase, in the presence of NAD, inactivated both H_S and H_R; Mg^{2+} and to a greater extent Mg^{2+} plus dithiothreitol protected both H_S and H_R from NAD- and transferase-dependent inactivation. Thus, activation of the hydrolase enhanced its resistance to inactivation by transferase. These observations are consistent with the coexistence in animal cells of active enzymes that catalyze opposing arms of the ADP-ribosylation cycle.

Mono-ADP-ribosylation of proteins is the mechanism by which certain bacterial toxins alter the activity of critical metabolic pathways (Stryer & Bourne, 1986). In the case of cholera and pertussis toxins, agents involved in the pathogenesis of cholera and pertussis (whooping cough), respectively, the targets of the ADP-ribosylation reactions are regulatory guanine nucleotide binding proteins of the adenylate cyclase system, and in the case of pertussis toxin other guanine nucleotide binding proteins as well, apparently unrelated to the cyclase (Gilman, 1987; Moss & Vaughan, 1988; Stryer & Bourne, 1986). ADP-ribosylation is also utilized by diphtheria toxin (Pappenheimer, 1977), *Pseudomonas* exotoxin A (Iglewski & Kabat, 1975), *Escherichia coli* heat-labile enterotoxin (Moss et al., 1979), and botulinum toxins (Aktories et al., 1986) to alter cellular metabolism. In *Rhodospirillium rubrum*, a nitrogen-fixing microorganism, ADP-ribosylation of an arginine residue in a nitrogenase inhibits its activity (Pope et al., 1985). Inactivation of the nitrogenase can be reversed by a hydrolase which cleaves the ADP-ribose–arginine bond (Saari et al., 1986a,b).

Animal cells contain both ADP-ribosyltransferases (Godeau et al., 1984; Moss & Vaughan, 1978, Moss et al., 1980; Yost & Moss, 1983; Soman et al., 1984; Tanigawa et al., 1984; West & Moss, 1986) and ADP-ribosylarginine hydrolases (Chang et al., 1986; Moss et al., 1985, 1986; Smith et al., 1985) that could catalyze the opposing arms of an ADP-ribosylation cycle in which arginine(s) is (are) modified and regenerated. The ADP-ribose acceptor protein substrates in this potential regulatory pathway have not been defined. Several members of a family of NAD:arginine ADP-ribosyltransferases have been purified from turkey erythrocytes. These are localized in nuclear, soluble, and membrane compartments and classified according to their regulatory and kinetic properties (Moss et al., 1980; West & Moss, 1986; Yost & Moss, 1983). Recently, ADP-ribosylarginine hydrolases have been identified in erythrocytes and their enzymatic properties partially characterized (Moss et al., 1985, 1986; Smith et al., 1985). To define further their interaction with the ADP-ribosyltransferases, the hydrolases have been purified >90% from erythrocytes.

Experimental Procedures

Materials

NAD, ovalbumin, and arginine were purchased from Sigma; *N*-ethylmaleimide was from Pierce; dithiothreitol was from Bethesda Research Laboratories; DE-52 was from Whatman; Mono Q and phenyl-Sepharose CL-4B were from Pharmacia; hydroxylapatite, Affi-gel 501, Affi-gel 601, and sodium dodecyl sulfate were from Bio-Rad; propylene glycol and $HgCl_2$ were from J. T. Baker; Ultrogel AcA 54 was from LKB; $MgCl_2$ and potassium phosphate were from Mallinckrodt; NaCl was from Fisher; glycine was from Schwarz/Mann; turkey blood was from Pel-Freez; L-[U-^{14}C]arginine (348 mCi/mmol) and [*carbonyl*-^{14}C]NAD (52 mCi/mmol) were from Amersham. Sources of other materials are published (Moss et al., 1985, 1986).

Methods

NAD:arginine ADP-ribosyltransferase and ADP-ribosylarginine hydrolase were assayed as described (Moss et al.,

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1976; Moss & Vaughan, 1977). The ADP-ribosylarginine hydrolase assay (total volume 100 μL) contained 50 μM ADP-ribosyl[^{14}C]arginine (~6000 cpm), 50 mM potassium phosphate (pH 7.5), 10 mM $MgCl_2$, 5 mM dithiothreitol (DTT),[1] and 1 mg/mL ovalbumin. Reaction was initiated with hydrolase (0.07 unit; 1 unit equals 1 nmol of ADP-ribosylarginine hydrolyzed per minute at 30 °C). Protein was determined by a dye binding procedure from Bio-Rad. SDS–PAGE was performed as described previously (Laemmli, 1970). The NAD:arginine ADP-ribosyltransferase was isolated from turkey erythrocytes (Moss et al., 1980).

Purification of Thiol-Resistant ADP-ribosylarginine Hydrolase (H_R). Packed cells (600 mL) from 1.5 L of turkey blood were washed with 1.2 L of 0.154 M NaCl, suspended in 10 mM potassium phosphate, pH 7.5 (400 mL), and freeze-thawed. After addition of 1600 mL of 10 mM potassium phosphate, pH 7.5, the suspension was centrifuged at 27000*g* for 30 min. The precipitate was washed with 1.2 L of the same buffer, and after centrifugation, the wash was added to the initial supernatant (final volume 2820 mL) which was then applied to a column (15.5 × 10.1 cm) of DE-52 equilibrated with 10 mM potassium phosphate, pH 7.5, and washed with 12 L of the same buffer. Elution was carried out with 0.5 M NaCl/20 mM potassium phosphate, pH 7.5. The eluate (3560 mL) was applied to a column (300 mL) of phenyl-Sepharose, which was washed with 0.5 M NaCl/20 mM potassium phosphate, pH 7.5 (1 L), and eluted with 40% propylene glycol in 40 mM potassium phosphate, pH 7.5 (915 mL). After addition of an equal volume of water, the eluate was applied to a column (2.5 × 33 cm) of hydroxylapatite which was eluted with a linear gradient of 20–150 mM potassium phosphate, pH 7.5, in 20% propylene glycol/50 mM NaCl (300 mL/300 mL); 8.3-mL fractions were collected. Active fractions were desalted on a Sephadex G-25 column (2.5 × 40 cm) and applied to a DE-52 column (2.5 × 14 cm) which was eluted with a continuous gradient of 20% propylene glycol/20 mM potassium phosphate, pH 7.5 (250 mL), to 20% propylene glycol/20 mM potassium phosphate (pH 7.5)/0.2 M NaCl (250 mL); active fractions were desalted on a G-25 column. After concentration of the DE-52 fractions on another DE-52 column (1 × 7 cm) and elution with 0.2 M NaCl, 20% propylene glycol, and 20 mM potassium phosphate (4.2 mL), the hydrolase was applied to an Ultrogel AcA 54 column (1.5 × 90 cm) in 0.1 M NaCl, 20% propylene glycol, and 20 mM potassium phosphate, pH 7.5; 1.2-mL fractions were collected. The most active fractions (9 mL) were combined and concentrated by centrifugation in a Centricon 10 microconcentrater to 0.66 mL. A sample (0.25 mL) was chromatographed on a Mono Q HR 5/5 column as described in Figure 1A. The peak of enzymatic activity cochromatographed with protein (Figure 1A). The major protein exhibited a mobility on SDS-PAGE consistent with a molecular weight of 39000 (data not shown). Purification is described in Table I.

Purification of Thiol-Sensitive ADP-ribosylarginine Hydrolase (H_S). Washed and freeze-thawed turkey erythrocytes (1 L) were centrifuged for 30 min at 37000*g*; the particulate was washed with 1350 mL of 10 mM potassium phosphate, pH 7.5. The pooled supernatant (730 mL) and wash (1350 mL) were applied to a DE-52 column (15.6 × 9 cm) which was washed with 10 mM potassium phosphate, pH 7.5 (12 L), and eluted with 0.5 M NaCl/20 mM potassium phosphate, pH 7.5 (2560 mL). The DE-52 eluate was then applied to phenyl-Sepharose (300 mL) which was washed with 0.5 M NaCl/20 mM potassium phosphate, pH 7.5 (1.2 L), and eluted with 40% propylene glycol/40 mM potassium phosphate, pH 7.5 (469 mL). The eluate was diluted 1:1 with water and applied to a hydroxylapatite column (2.5 × 29 cm) which was eluted with a continuous gradient of 20% propylene glycol/20 mM potassium phosphate (pH 7.5)/50 mM NaCl (300 mL) to 20% propylene glycol/150 mM potassium phosphate (pH 7.5)/50 mM NaCl (300 mL); 11.1-mL fractions were collected. Active fractions (122 mL) were placed on an Affi-gel 501 column (2.5 × 7.6 cm) which was washed with 75 mL of 20% propylene glycol/20 mM potassium phosphate, pH 7.5, and eluted with 5 mM DTT/20% propylene glycol/20 mM potassium phosphate, pH 7.5 (110 mL). The eluate was ap-

[1] Abbreviations: DTT, dithiothreitol; NEM, *N*-ethylmaleimide; H_R and H_S, dithiothreitol-resistant and -sensitive forms, respectively, of ADP-ribosylarginine hydrolase; SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

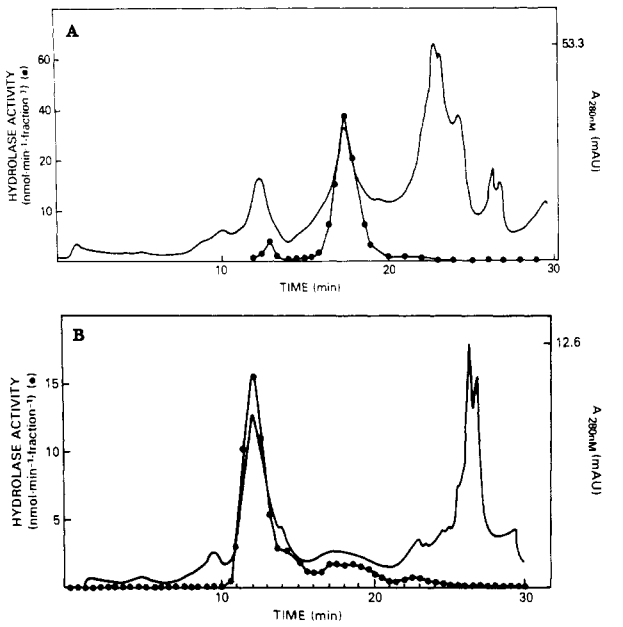

FIGURE 1: Chromatography of thiol-resistant (A) and thiol-sensitive (B) forms of ADP-ribosylarginine hydrolase on Mono Q. Samples of H_R (179.1 units, 450 μg) and H_S (47.2 units, 72 μg) were applied to a Mono Q HR5/5 column (Pharmacia; Piscataway, NJ). The volume of each injection was 0.2 mL. Elution was performed at a flow rate of 1.0 mL·min^{-1}, at room temperature, with two buffers (buffer A, 20 mM potassium phosphate plus 20% propylene glycol, pH 7.5; buffer B, buffer A plus 0.5 M NaCl, pH 7.5) in a gradient of the following program: t (minutes) = 5, $\%B = 0$; $t = 20$, $\%B = 20$; $t = 28$, $\%B = 100$; $t = 30$, $\%B = 0$. Fractions (0.5 mL) were collected at 0.5-min intervals. Recoveries of activity in (A) and (B) were 117.0% and 87.9%, respectively.

Table I: Purification of Thiol-Resistant ADP-ribosylarginine Hydrolase[a]

purification step	total protein (mg)	total act. (units × 10^{-3})	recovery (%)	sp act. (units·mg^{-1})	x-fold purification
(1) supernatant	492000	8.10	100	0.016	
(2) DE-52	10500	5.40	66	0.51	31
(3) phenyl-Sepharose	1050	4.20	52	4.0	244
(4) hydroxylapatite	262	2.46	30	9.4	570
(5) Sephadex G-25	268	2.15	26	8.0	490
(6) DE-52	34	2.06	25	61	3700
(7) Ultrogel AcA 54	1.20	0.77	9.5	640	39000
(8) Mono Q	0.05	0.20	2.4	4000	250000

[a] Details of the purification procedure are given in the text. Assays were performed as noted under Methods.

Table II: Purification of Thiol-Sensitive ADP-ribosylarginine Hydrolase[a]

purification step	total protein (mg)	total act. (units × 10^{-3})	recovery (%)	sp act. (units·mg^{-1})	x-fold purification
(1) supernatant	383000	9.89	100	0.026	1
(2) DE-52	13300	6.90	70	0.52	22
(3) phenyl-Sepharose	891	3.31	34	3.72	143
(4) hydroxyl-apatite	121	2.61	26	21.6	831
(5) Affigel 501	86	1.67	17	19.5	750
(6) DE-52	7.5	2.26	23	301	11600
(7) Sephadex G-25	7.9	2.12	22	270	10400
(8) DE-52	4.6	1.70	17	367	14100
(9) Ultrogel AcA 54	0.42	0.43	4.4	1030	39700
(10) Mono Q	0.036	0.14	1.4	3890	150000

[a] Details of the purification procedure are given in the text. Assays were performed as noted under Methods.

plied to DE-52 (1.5 × 14.3 cm) which was eluted with a continuous gradient of 20 mM potassium phosphate (pH 7.5)/20% propylene glycol (200 mL) to 0.2 M NaCl/20 mM potassium phosphate (pH 7.5)/20% propylene glycol (200 mL); 4.8-mL fractions were collected. Active fractions were desalted on a Sephadex G-25 column and applied to a DE-52 column (1.5 × 2.3 cm) to concentrate to a final volume of 5 mL, of which 4.5 mL was placed on an Ultrogel AcA 54 column (1.2 × 90 cm) equilibrated and eluted with 20% propylene glycol/20 mM potassium phosphate (pH 7.5)/0.5 M NaCl; 1.2-mL fractions were collected. Further purification by Mono Q HPLC is described in Figure 1B. The mobility of H_S activity on Mono Q differed from that of H_R; however, as with purified H_R, peaks in activity and protein comigrated (Figure 1B). The major protein band observed on SDS–PAGE had a mobility consistent with a protein of 39 000 Da (data not shown). The purification summarized in Table II was performed 3 other times with similar results.

Both H_R and H_S exhibited similar molecular weights by SDS–PAGE and gel permeation chromatography under nondenaturing conditions (data not shown). It appears that the hydrolase exists as a monomer of 39 kDa. H_S appears to be a $HgCl_2$-induced form of H_R and not a different cell protein. For both H_S and H_R, the K_m for ADP-ribosylarginine with the purified enzyme was very similar to that of the previously reported hydrolase preparations (Moss et al., 1986). Maximal catalytic activity is given in the purification tables for both hydrolases (Tables I and II).

Results

Immediately after homogenization of erythrocytes and centrifugation, hydrolase activity in the soluble fraction was primarily thiol independent, perhaps due to endogenous thiols (Figure 2). As noted previously, following chromatography on DE-52 and phenyl-Sepharose, the hydrolase was resistant to dithiothreitol (Moss et al., 1985). The hydrolase was purified extensively by the procedures outlined under Methods and in Tables I and II, yielding thiol-resistant and thiol-sensitive species.

The thiol-sensitive and thiol-resistant forms of the purified ADP-ribosylarginine hydrolase exhibited a difference in the apparent K_a of 35-fold ($K_{a,sensitive}$ = 41 ± 16.7 μM, n = 4; $K_{a,resistant}$ = 1.44 ± 0.12 mM, n = 3) (Figure 2). The form present in the supernatant of an erythrocyte homogenate appears to be independent of thiol (Figure 2). An enzyme

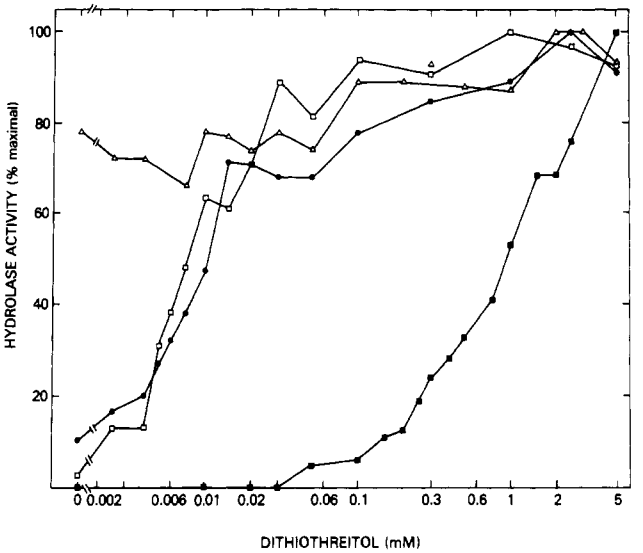

FIGURE 2: Effect of dithiothreitol concentration on activities of the supernatant, Hg^{2+}-treated and purified thiol-resistant, and thiol-sensitive forms of ADP-ribosylarginine hydrolases. Samples of Ultrogel AcA 54 purified H_R (■) (0.073 unit, 2.1 μg), Ultrogel AcA 54 purified H_S (●) (0.029 unit, 0.3 μg), a freshly prepared turkey erythrocyte supernatant (△) (0.02 unit, 558 μg), and $HgCl_2$-treated hydrolase (□) (see below) (0.029 unit, 0.3 μg) were assayed under standard conditions as noted under Methods except that the final concentrations of DTT were as indicated. The average of experiments gave a $K_{a,sensitive}$ = 41 ± 16.7 μM (n = 4) and a $K_{a,resistant}$ = 1.43 ± 0.12 mM (n = 3). To prepare the Hg-treated hydrolase, a sample of Ultrogel AcA 54 purified H_R (68.1 units, 2.8 mg) was incubated with 1 mM $HgCl_2$ and ovalbumin (1 mg/mL) at 30 °C for 15 min in a total volume of 0.8 mL before addition of 0.2 mL of 75 mM DTT. After 15 min at 30 °C, 0.32 mL of 50% propylene glycol was added, and 1.2 mL of the mixture was chromatographed on Ultrogel AcA 54 (1.2 × 90 cm), which was equilibrated and eluted with 20% propylene glycol/20 mM potassium phosphate (pH 7.5)/0.1 M NaCl; 1.2-mL fractions were collected. Active fractions, corresponding to the predicted K_{av} of the hydrolase, were combined; 61.9 units were applied to the Ultrogel AcA 54 column, and 9.4 units were recovered (15.2%). This preparation was then assayed as noted previously.

Table III: Thiol Sensitivity of ADP-ribosylarginine Hydrolase before and after Chromatography on Organomercurial Agarose[a]

[DTT] (μM)	ADP-ribosylarginine hydrolase act. (pmol·min^{-1}) step 4	step 6
0	0	5.5
2.5	ND[b]	10.5
5	ND	18.8
10	ND	26.3
50	1.0	31.1
100	2.8	30.9
1000	16.6	30.3
3000	22.3	29.9
5000	29.5	30.1

[a] Hydrolase purified through step 4 (1.5 μg) or through step 6 (0.16 μg) was assayed with the indicated concentration of DTT. [b] ND, not determined.

partially purified through the hydroxylapatite step (Tables I and II, step 4) is thiol resistant (Table III). Following chromatography of the hydrolase on organomercurial agarose (Table II, step 5) and removal of the endogenous thiol used for elution by purification on DE-52 (Table II, step 6), a thiol-sensitive form was generated (Table III). Consistent with a role for Hg in the generation of the thiol-sensitive hydrolase was the finding that incubation of the thiol-resistant species with $HgCl_2$ and then with thiol followed by gel permeation chromatography resulted in a thiol-sensitive form (Figure 2).

Table IV: Effect of Dithiothreitol and *N*-Ethylmaleimide on the Activity of the Thiol-Resistant and -Sensitive Hydrolases[a]

incubation		ADP-ribosyl-arginine hydrolase act. (pmol·min^{-1})	
I	II	H_R	H_S
		48.7	39.5
DTT		59.1	39.8
	NEM	38.9	27.8
DTT	NEM	3.9	0

[a] Ultrogel AcA 54 purified H_R (0.98 unit, 28 μg) or Ultrogel AcA 54 purified H_S (1.04 units, 0.7 μg) was incubated (I) at 30 °C in a reaction mix containing 4% propylene glycol, 4 mM potassium phosphate, pH 7.5, 0.1 mg of ovalbumin, 10 mM $MgCl_2$, and 10 mM DTT, as indicated, in a total volume of 0.08 mL. After 10 min, the samples were cooled to 4 °C, and 0.02 mL of 200 mM NEM or water was added to the indicated samples which were then incubated (II) at 4 °C for 10 min. Samples (0.02 mL) were assayed under standard conditions as noted under Methods.

Table V: Effect of NEM and DTT at 4 °C on Activity of the Thiol-Sensitive and Thiol-Resistant Hydrolases[a]

	incubation		hydrolase act. (pmol·min^{-1})	
enzyme	I	II	-DTT	+DTT
H_S			4.8	24.1
	DTT		19.7	22.6
		NEM	0.5	24.1
	DTT	NEM	0.5	0.9
H_R			0.4	61.6
	DTT		52.1	68.6
		NEM	0.7	53.5
	DTT	NEM	1.2	42.2

[a] Ultrogel AcA 54 purified H_S (0.7 μg) or H_R (0.3 μg) was incubated in 5 mM potassium phosphate, pH 7.5, containing 5% propylene glycol, ovalbumin (1 mg/mL), and 12.5 mM $MgCl_2$ with or without 12.5 mM DTT in a total volume of 0.08 mL for 30 min at 4 °C (incubation I), followed by addition of 0.02 mL of 0.2 M NEM or water and incubation for 10 min at 4 °C (incubation II). Samples (0.01 mL) were then assayed with or without an additional 10 mM DTT.

Table VI: Effect of Mg^{2+} and DTT on the Stability of the Thiol-Sensitive Hydrolase[a]

additions	hydrolase act. (% of zero time) at 1 h	2 h
none	87	80
$MgCl_2$, 10 mM	102	98
DTT, 20 mM	77	47
$MgCl_2$ plus DTT	96	98

[a] Ultrogel AcA 54 purified H_S (4.2 μg) was incubated at 30 °C in 2.5 mM potassium phosphate, pH 7.5, containing 2.5% propylene glycol with or without 10 mM $MgCl_2$ and/or 20 mM DTT in a total volume of 1.5 mL. At 0, 1, or 2 h, 60-μL samples were added to an assay mix modified so that final concentrations in all cases were 16 mM Mg^{2+} and 17 mM DTT. Activity is expressed as a percentage of that at zero time which was 68.7, 67.7, 76.5, and 75.1 pmol/min, respectively, for no additions, +Mg^{2+}, +DTT, and +Mg/DTT.

Table VII: Effect of Mg^{2+} and Dithiothreitol on Inactivation of the Thiol-Resistant and Thiol-Sensitive ADP-ribosylarginine Hydrolase by NAD:Arginine ADP-ribosyltransferase and NAD[a]

	ADP-ribosylarginine hydrolase act. (pmol·min^{-1})			
	H_R		H_S	
additions	-NAD	+NAD	-NAD	+NAD
none	36.5	6.6	17.7	10.8
Mg^{2+} (10 mM)	43.3	22.4	36.3	26.9
dithiothreitol (5 mM)	32.9	4.3	27.7	16.6
Mg^{2+}, dithiothreitol	39.8	39.7	35.1	37.0

[a] HPLC-purified H_R (0.49 unit, 0.33 μg) and Ultrogel AcA 54 purified H_S (0.99 unit, 0.66 μg) were incubated with NAD:arginine ADP-ribosyltransferase (107 milliunits, 6 μg) in a reaction mix containing 12% propylene glycol, 14.5 mM potassium phosphate, pH 7.5, and 0.1 M NaCl with 100 μM NAD, 10 mM $MgCl_2$, and/or 5 mM DTT as indicated (final volume 0.2 mL). After 30 min at 30 °C, samples (0.02 mL) were assayed under standard conditions; all assays contained 10 mM Mg^{2+} and 5 mM DTT. Excess transferase was associated with some inactivation even under conditions noted here as giving protection from inactivation.

Neither the thiol-sensitive nor the thiol-resistant form of the hydrolase was inactivated by NEM at 4 °C (Table IV). After incubation with dithiothreitol at 30 °C, however, both forms were inactivated by NEM at 4 °C. As might be expected, conditions for activation of the resistant species with thiol were much more extreme. Exposure to dithiothreitol at 4 °C was sufficient to permit subsequent inactivation of H_S but not H_R by NEM (Table V).

Mg^{2+}, as noted previously, protected the thiol-resistant species from thermal inactivation (Moss et al., 1986). A protective effect of Mg^{2+} was also observed with the thiol-sensitive enzyme (Table VI). Under similar conditions, Mg^{2+} or Mg^{2+} plus dithiothreitol reduced the extent of inactivation of both H_S and H_R by NAD and an erythrocyte NAD:arginine ADP-ribosyltransferase (Table VII). The protective effect of Mg^{2+} or Mg^{2+} plus dithiothreitol at limiting transferase was associated with reduced [^{32}P]ADP-ribosylation of a 39 000-Da protein, presumably the hydrolase. Under these assay conditions, Mg^{2+} in the absence of DTT did not activate the hydrolase; reduced ADP-ribosylation therefore did not appear to result from removal of the ADP-ribose moieties from ADP-ribosylarginine hydrolase by active enzyme. Incubation of hydrolase partially inactivated by ADP-ribosylation with Mg^{2+} and DTT did not restore enzymatic activity, again consistent with the conclusion that the ADP-ribose–hydrolase linkage was not readily cleaved. In addition, Mg^{2+} and dithiothreitol did not reduce the ADP-ribosylation of agmatine (data not shown), consistent with the conclusion that these agents affected the hydrolase, not the ADP-ribosyltransferase.

Discussion

An ADP-ribosylarginine hydrolase was purified extensively (>90%) from turkey erythrocytes. Depending on the procedure used for purification, either a thiol-resistant or a thiol-sensitive species was isolated. Organomercurial chromatography apparently resulted in the formation of the thiol-sensitive species of hydrolase as did exposure of H_R to $HgCl_2$; H_R thus appears to be the parent protein of H_S. The two species of purified hydrolase differed in their dithiothreitol requirement for activation by ~30-fold. The mobilities of hydrolase on gel permeation columns under nonreducing, native conditions and on SDS-polyacrylamide gels under reducing, denaturing conditions are consistent with the conclusion that the native hydrolase exists as a ~39-kDa monomer.

Of concern is the fact that the use of organomercurial chromatography in protein purification may alter the regulatory properties of the isolated enzyme. It is generally accepted that reaction of proteins with mercurials may lead to alterations in protein configuration; gradual unfolding of the protein following the primary reaction may lead to secondary exposure of internal or masked residues (Madsen, 1963; Rothstein, 1973). Although both hydrolases are activated by thiol after organomercurial agarose chromatography, it is not clear, for lack of an appropriate assay, that the conformations of the thiol-activated species of H_S and H_R are identical.

Both H_R and H_S were relatively resistant to NEM unless previously treated with thiol. With H_R, as might be expected,

it was far more difficult to fully reduce the enzyme to a form readily inactivated by NEM. Incubation with thiol and activation of the hydrolase thus appear to be a reversible event. With H_R, it leads to the formation of a species exhibiting the same thiol dependency as the parent enzyme. Thus, a reduced hydrolase present in erythrocyte, when stored under nonreducing conditions, should return to the thiol-resistant conformation. These data may explain why the thiol-independent activity in the supernatant generates during purification a thiol-resistant form. In view of the difficulty of activating H_R with thiol, it may be that the native hydrolase (as present in supernatant) is activated.

NAD:arginine ADP-ribosyltransferases have been noted to catalyze the ADP-ribosylation of many proteins, presumably due to the presence of a readily accessible arginine (Moss et al., 1984; Moss & Vaughan, 1978). On the basis of studies with glutamine synthetase, it would appear that certain arginine residues are more susceptible to ADP-ribosylation and that, in some cases, these arginines may play a critical role in function (Moss et al., 1984). Presumably in the cell, the amounts of ADP-ribosylated proteins then reflect a balance between transferase, hydrolase, and the availability of acceptor.

To determine if transferase might alter hydrolase activity, hydrolase was incubated under ADP-ribosylation conditions in the presence or absence of Mg^{2+} and DTT. Hydrolase incubated with NAD and transferase in the absence of Mg^{2+} and DTT exhibited a significantly lower activity in a subsequent assay containing Mg^{2+} and DTT. In contrast, when the incubation with transferase contained Mg^{2+} and DTT, no inactivation was observed; ADP-ribosylation of hydrolase was significantly less. There are at least two plausible explanations for the protective effect of Mg^{2+} and DTT on inactivation by transferase. First, Mg^{2+} and DTT, by promoting an active conformation, reduce the accessibility of arginine residues on the hydrolase to ADP-ribosylation. Second, in the presence of Mg^{2+} and DTT, but not in their absence, the ADP-ribose residues are continually being excised by active hydrolase. The latter hypothesis appears to be less likely on the basis of the observations that (1) Mg^{2+} which does not activate the hydrolase did decrease the extent of ADP-ribosylation, implying that the mechanism for decreased modification involved protection from ADP-ribosylation rather than excision of ADP-ribose residues from inactive hydrolase, and (2) partially ADP-ribosylated and inactivated hydrolase was incapable of significantly restoring activity upon addition of Mg^{2+} and DTT. These studies are consistent with the coexistence in the cell of active species of both hydrolase and transferase, leading to modulation of the level of ADP-ribosylation of substrate proteins. Since, as noted earlier, a larger percentage of the hydrolase appears to exist in an active state when initially examined following cell disruption, it may be that, intracellularly, transferase and hydrolase operate as opposing arms of a dynamic ADP-ribosylation cycle.

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