

Identification of Critical, Conserved Vicinal Aspartate Residues in Mammalian and Bacterial ADP-ribosylarginine Hydrolases*

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NAD:arginine ADP-ribosyltransferases and ADP-ribosylarginine hydrolases catalyze opposing arms of a putative ADP-ribosylation cycle. ADP-ribosylarginine hydrolases from mammalian tissues and *Rhodospirillum rubrum* exhibit three regions of similarity in deduced amino acid sequence. We postulated that amino acids in these consensus regions could be critical for hydrolase function. To test this hypothesis, hydrolase, cloned from rat brain, was expressed as a glutathione S-transferase fusion protein in *Escherichia coli* and purified by glutathione-Sepharose affinity chromatography. Conserved amino acids in each of these regions were altered by site-directed mutagenesis. Replacement of Asp-60 or Asp-61 with Ala, Gln, or Asn, but not Glu, significantly reduced enzyme activity. The double Asp-60 → Glu/Asp-61 → Glu mutant was inactive, as were Asp-60 → Gln/Asp-61 → Gln or Asp-60 → Asn/Asp-61 → Asn. The catalytically inactive single and double mutants appeared to retain conformation, since they bound ADP-ribose, a substrate analogue and an inhibitor of enzyme activity, with affinity similar to that of the wild-type hydrolase and with the expected stoichiometry of one. Replacing His-65, Arg-139, Asp-285, which are also located in the conserved regions, with alanine did not change specific activity. These data clearly show that the conserved vicinal aspartates 60 and 61 in rat ADP-ribosylarginine hydrolase are critical for catalytic activity, but not for high affinity binding of the substrate analogue, ADP-ribose.

Mono-ADP-ribosylation is a post-translational modification of proteins, in which the ADP-ribose moiety of β -NAD is transferred to specific amino acid residues in target proteins (1). This reaction has been well characterized for bacterial toxins, which thereby alter the activity of critical regulatory proteins in mammalian cells (1). Cholera toxin, for example, ADP-ribosylates an arginine in the α -subunit of the stimulatory guanine nucleotide-binding (G) protein of the adenyl cyclase system, resulting in its activation and an increase in intracellular cAMP (2). Pertussis toxin modifies a cysteine in another family of G proteins, blocking the action of inhibitory agonists on adenyl cyclase (3, 4). Other toxins use different proteins, and in some instances, different acceptor amino acids (e.g. asparagine) as substrates for ADP-ribosylation (2–7).

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Enzymes that catalyze reactions similar to bacterial toxins have been identified in mammalian and avian tissues, including turkey erythrocytes, chicken neutrophils, rat neurons, and mammalian cardiac and skeletal muscle cells (8–10). These NAD:arginine ADP-ribosyltransferases vary in cellular localization, some being intracellular, others secreted, and a third group linked to the cell surface by glycosylphosphatidylinositol anchors (8). These transferases catalyze the stereospecific transfer of ADP-ribose from β -NAD to the guanidino group of arginine (protein), producing the α -ADP-ribosylarginine (protein) (11).

In some instances, substrates and/or effectors of ADP-ribosylation have been defined. In murine cytotoxic T cells, a glycosylphosphatidylinositol-anchored transferase regulates proliferation and cytotoxic activity, possibly by modulating activity of the protein tyrosine kinase p56^{lck}, through ADP-ribosylation of a regulatory protein, p40 (12). Integrin α_7 is a major substrate of a glycosylphosphatidylinositol-linked transferase in skeletal muscle cells (C2C12) (13), which, based on inhibitor studies, was proposed to play a role in muscle cell differentiation (14). Integrin $\alpha_7\beta_1$, a laminin-binding protein, may be involved in cell adhesion and communication between myoblasts and extracellular matrix (15–17).

ADP-ribosylation of arginine residues may be a reversible modification of proteins. ADP-ribosylarginine hydrolases cleave the ADP-ribose-arginine (protein) linkage, regenerating the arginine guanidino group (18). The hydrolase-catalyzed reaction is stereospecific and utilizes α -ADP-ribosylarginine, a product of the transferase-catalyzed reaction (19). Hydrolases thus complete an ADP-ribosylation cycle that could reversibly regulate the function of substrate proteins (19).

In the photosynthetic bacterium *Rhodospirillum rubrum* (20), an ADP-ribosylation cycle plays an important role in nitrogen fixation, which is controlled by the reversible ADP-ribosylation of dinitrogenase reductase. An ADP-ribosyltransferase (termed DRAT for dinitrogenase reductase ADP-ribosyltransferase) inactivates dinitrogenase reductase by ADP-ribosylation, and DRAG or dinitroreductase ADP-ribosylarginine glycohydrolase regenerates the active form by releasing ADP-ribose from the enzyme (20). This ADP-ribosylation cycle is regulated by environmental signals (e.g. nutrients, light) that determine the requirement for nitrogen fixation and hence the need for active or inactive dinitrogenase reductase (21, 22).

In eukaryotes, ADP-ribosylarginine hydrolases have been identified in mammalian and avian tissues. ADP-ribosylarginine hydrolase cDNAs cloned from human, rat, and mouse tissues share >82% similarity of deduced amino acid sequences (23, 24). Consistent with this degree of similarity, rabbit anti-rat brain hydrolase polyclonal antibodies cross-react with hydrolases from turkey erythrocytes, and calf, mouse, and human brains (24). Despite their functional similarities, ADP-ribosylarginine hydrolases from human, rat, and mouse tissues and *R.*

rubrum exhibit only limited regions of similarity in deduced amino acid sequences (24). We postulated, therefore, that amino acids in these areas could represent consensus motif residues critical for hydrolase function. We report here that mutation of rat brain ADP-ribosylarginine by replacement of the conserved Asp-60 and/or Asp-61 with alanine, glutamine, or asparagine significantly reduced enzyme activity, but not the affinity for a substrate analogue ADP-ribose, consistent with an essential role for both residues in catalysis, but not structure of the substrate site.

EXPERIMENTAL PROCEDURES

Materials—[adenine- U - ^{14}C]NAD (252 mCi/mmol) was purchased from Amersham Pharmacia Biotech; [U- ^{14}C]L-arginine (320 mCi/mmol) from NEN Life Science Products; β -NAD from Sigma; Affi-Gel 601 (boronate) and prestained protein SDS-polyacrylamide gel electrophoresis standards from Bio-Rad; plasmid DNA isolation QIAprep Spin Miniprep Kit from Qiagen; QuikChange site-directed mutagenesis kit from Stratagene; ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction Kit with AmpliTaq DNA Polymerase FS from Perkin-Elmer; glutathione-Sepharose 4B from Amersham Pharmacia Biotech; cholera toxin A subunit from List Biological Laboratories Inc.; and isopropyl-1-thio- β -D-galactopyranoside from Gold Biotechnology, Inc. Restriction enzymes were purchased from Roche Molecular Biochemicals. Other reagents were of analytical grade.

Construction of Wild-type and Mutant Rat Brain ADP-ribosylarginine Hydrolase Expression Vectors—Wild-type hydrolase cDNA was amplified from the λ ZAP clone (23) with forward and reverse primers 5'-ACGTACGTGGATCCATGGGTGGGGCCCTGA-3' and 5'-ACGTACGTGAATTCCTAGGAACCTAGTATAGTG-3', respectively, digested with *Bam*HI and *Eco*RI, and cloned into a pGEX-2T expression vector (Amersham Pharmacia Biotech), to produce the rWT plasmid. *Pfu* polymerase was used for polymerase chain reaction (Perkin-Elmer Thermal Cycler) amplification according to the manufacturer's protocols for 25 cycles of 96 °C, 45 s; 62 °C, 1 min; 72 °C, 2 min. Mutants were generated by the QuikChange site-directed mutagenesis method (Stratagene) according to the manufacturer's protocol. The following oligonucleotides and the complementary primers (not shown) were used to generate hydrolase mutants. The modified codons are underlined, and the mutant is identified in parenthesis: 5'-GGAGAGTCAGCGCCGAC-ACCATCATGC-3' (D60A), 5'-GAGAGTCAGCGTGCCACCATCATGC-AC-3' (D61A), 5'-GATGACACCATCATGGCCCTGGCTACAGC-3' (H65A), 5'-GGAGCGCCATGGCCGCATGTGCATCG-3' (R139A), 5'-CCATGATTGCCTATGGCGCCCTCTGGC-3' (D285A), 5'-GGAGAGTCAGCGAGGACACCATCATGC-3' (D60E), 5'-GGAGAGTCAGCAATG-ACACCATCATGC-3' (D60N), 5'-GGAGAGTCAGCCAGGACACCATCATGC-3' (D60Q), 5'-GGAGAGTCAGCGATGAGACCATCATGC-3' (D61E), 5'-GGAGAGTCAGCGATAATACCATCATGC-3' (D61N), 5'-GGAGAGTCAGCGATCAGCCATCATGC-3' (D61Q), 5'-GGAGAGTCAGCGCCGCAACCATCATGCAC-3' (D60A/D61A), 5'-GGAGAGTCAGCGAGGAGACCATCATGCAC-3' (D60E/D61E), 5'-GGAGAGTCAGCAAC-ACACCATCATGCAC-3' (D60N/D61N), 5'-GGAGAGTCAGCCAGCA-GACCATCATGCAC-3' (D60Q/D61Q).

After amplification, parental, supercoiled dsDNA was digested with *Dpn*I endonuclease (2.5 units for 2 h at 37 °C in 50 μ l of reaction mix). *Dpn*I endonuclease, which is specific for methylated and hemimethylated DNA, was used to digest the parental DNA template and thereby select for DNA containing the desired mutation. DNA isolated from almost all *Escherichia coli* strains is *dam*-methylated and therefore susceptible to *Dpn*I digestion (25). The nicked vector DNA containing the hydrolase mutations was transfected into Epicurian Coli XL1-Blue supercompetent cells. Transformed Epicurian Coli XL1-Blue supercompetent cells were plated on LB-ampicillin agar plates that were incubated at 37 °C overnight. 20 colonies of each mutant were grown overnight in 3 ml of LB/Amp at 37 °C. Plasmid cDNA was isolated by QIAprep Spin Miniprep Kit (Qiagen), and the entire coding region was sequenced (ABI PRISM 377, Perkin-Elmer). Proteins were synthesized in *E. coli* BL21(DE3) with incubation at 30 °C for 12 h in the presence of 0.2 mM isopropyl-1-thio- β -D-galactopyranoside (100 ml total volume). Cells were harvested, washed with ice-cold phosphate-buffered saline, and suspended in 5 ml of lysis buffer (10 mM sodium phosphate, 10 mM EDTA, pH 8.0). After freezing (dry ice, 5 min) and thawing at room temperature (a total of three times) followed by sonicating for 1 min on ice, the cell lysate was centrifuged (4000 \times g, 20 min), and the supernatant was incubated with gentle agitation at room temp for 30 min with a slurry of glutathione-Sepharose 4B equilibrated with phosphate-

TABLE I

Similarities in deduced amino acid sequences of ADP-ribosylarginine hydrolases from rat brain and *R. rubrum*

Differences in amino acid sequences (in single letter code) are noted by asterisks; a gap is indicated by a dash. Sequences for *R. rubrum* are from Ref. 20 and those for rat from Ref. 23. In human hydrolase, leucine 287 is replaced by tyrosine. There are no differences between rat and mouse hydrolases in these three regions.

Source	Sequence
A Rat brain <i>R. rubrum</i>	⁶⁰ DDTIMHSLA ⁶⁷ ⁹⁸ DDTEMSLA ¹⁰⁵ * * * *
B Rat brain <i>R. rubrum</i>	¹³³ GCGAAMR ¹³⁹ ¹⁶³ GNGAAMR ¹⁶⁹ *
C Rat brain <i>R. rubrum</i>	²⁸⁵ DALLA-AG ²⁹¹ ³²³ DALLALAG ²³⁰

buffered saline. The matrix was washed before fusion proteins were eluted with 10 mM reduced glutathione in 50 mM Tris-HCl, pH 8.0, and concentrated in Microcon microconcentrators to a final volume of 500 μ l. Samples (20 μ l) were mixed with 10 μ l of 2 \times SDS/sample buffer (4% SDS, 10% glycerol, 0.02% bromphenol blue, 1% β -mercaptoethanol, 125 mM Tris, pH 6.8), boiled for 3 min, and subjected to electrophoresis in 4–20% gradient Tris-glycine gels (Novex), which were then stained with 0.05% Coomassie Blue. Fusion proteins were estimated to be ~95% pure. Protein concentration was quantified using bovine serum albumin as standard.

ADP-ribosylarginine Hydrolase Assay—The substrate was synthesized by incubation at 30 °C overnight in 2 mM β -NAD, 2 mM [^{14}C]arginine (50 μ Ci), 50 μ g of cholera toxin A subunit, 50 μ g of recombinant human ADP-ribosylation factor (rARF 1; gift from Walter Patton, NHLBI, National Institutes of Health, Bethesda, MD), 30 μ g of ovalbumin, and 5 μ g of cardiolipin, 20 mM dithiothreitol, 100 μ M GTP, 50 mM potassium phosphate, and 10 mM MgCl₂, pH 7.5 (total volume 300 μ l). ADP-ribosyl- ^{14}C arginine was purified by HPLC (Hewlett-Packard series 1100 equipped with a diode-array spectrophotometric detector set at 260 nm) using an anion exchange perfusion chromatography column (Zorbax Sax 4.6-mm inner diameter \times 25 cm, Rockland Technologies, Inc.) and elution with a linear gradient of NaCl (0–1 M) in 20 mM sodium phosphate buffer, pH 4.5, for 30 min at a flow rate of 1 ml/min. ADP-ribosyl- ^{14}C arginine was eluted at 12 min, well separated from arginine and nicotinamide (which eluted between 2 and 5 min), NAD (16–18 min), and ADP-ribose (40 min). ADP-ribosyl- ^{14}C arginine was lyophilized, dissolved in 100 μ l of H₂O, and stored at –20 °C. Samples (50 ng) of purified rat brain hydrolase (wild-type or mutants) synthesized as a GST¹ fusion protein were assayed in 50 mM potassium phosphate, pH 7.5, containing 5 mM dithiothreitol, 10 mM MgCl₂, 100 μ M ADP-ribosyl- ^{14}C arginine (78,000 cpm) (total volume 100 μ l). After 1 h at 37 °C, a sample (90 μ l) was applied to a column (0.5 \times 4 cm) of Affi-Gel 601 (boronate) equilibrated and eluted with five 1-ml portions of 0.1 M glycine, pH 9.0, 0.1 M NaCl, and 10 mM MgCl₂. The total eluate was collected for liquid scintillation counting. If activity was not detected, the experiment was repeated with 10 μ g of fusion protein and an incubation time of 5 h.

Inhibition of ADP-ribosylarginine Hydrolase—Samples (50 ng) of purified rat brain, wild-type hydrolase (synthesized as a GST fusion protein) in 50 mM potassium phosphate, pH 7.5, containing 5 mM dithiothreitol, 10 mM MgCl₂, 100 μ M ADP-ribosyl- ^{14}C arginine (78,000 cpm) with ADP-ribose (Sigma) as indicated (total volume 100 μ l) were incubated for 1 h at 37 °C. A sample (90 μ l) was applied to a column (0.5 \times 4 cm) of Affi-Gel 601 (boronate) equilibrated and eluted with 0.1 M glycine buffer, pH 9.0, containing 0.1 M NaCl, and 10 mM MgCl₂ (five 1-ml portions). The total eluate was collected for liquid scintillation counting. Activity is expressed as micromoles of free arginine formed during the incubation.

ADP-ribose Binding Assay— ^{14}C ADP-ribose was generated in a mixture (300 μ l) containing 50 mM Tris-HCl, pH 7.5, 30 μ g of ovalbumin, 20 mM dithiothreitol, 2 mM (50 μ Ci) of [adenine- U - ^{14}C]NAD, 100 μ M GTP, 5 μ g of cardiolipin, 10 mM MgCl₂, 50 μ g of cholera toxin A subunit, and 50 μ g of ADP-ribosylation factor (rARF 1) and incubated for 1 h at 37 °C. After incubation, [^{14}C]ADP-ribose was purified by

¹ The abbreviations used are: GST, glutathione S-transferase; HPLC, high performance liquid chromatography; WT, wild-type.

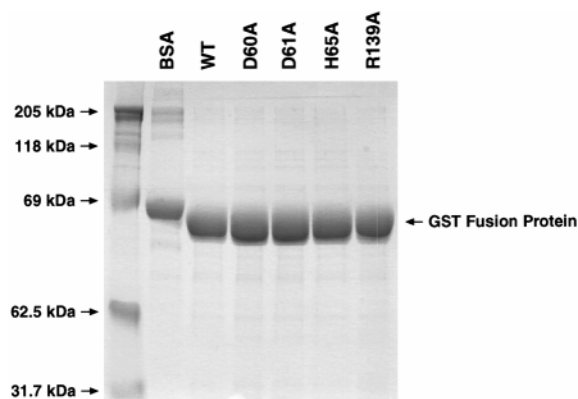


FIG. 1. **Purification of recombinant ADP-ribosylarginine hydrolases.** ADP-ribosylarginine hydrolases (5 μg), purified on glutathione-Sepharose, were subjected to electrophoresis in SDS-polyacrylamide gel electrophoresis using 4–20% gradient gels, followed by staining with 0.05% Coomassie Blue. 1 μg of bovine serum albumin was loaded as a control. This experiment was repeated three times with similar results.

HPLC as described for purification of ADP-ribose- ^{14}C arginine. To assay binding of ^{14}C ADP-ribose, 10 μg of wild-type or mutant rat hydrolases or bovine serum albumin were incubated (total volume 100 μl) in 50 mM potassium phosphate, pH 7.5, containing 5 mM dithiothreitol, 10 mM MgCl_2 . After 1 h at 37 $^\circ\text{C}$, the mixture was dialyzed in a Microdialyzer100 (Pierce) for 48 h at 4 $^\circ\text{C}$ against semipermeable membranes (Spectra/Por Membrane MWCO 6–8,000 Spectrum Medical Industries) in the presence of 0.25 M sucrose, 0.15 M NaCl, 20 mM Tris-HCl, pH 8.0, 5 mM dithiothreitol, 1 mM NaN_3 , 0.1 mM EDTA. After dialysis, samples and 100 μl from the dialysis chamber (total volume 100 ml) were counted using a liquid scintillation counter for calculations of bound and free ADP-ribose.

RESULTS AND DISCUSSION

The mammalian ADP-ribosylarginine hydrolase gene is, thus far, unique in the genome. To identify structural features necessary for activity, evolutionary conservation of sequence was investigated. Deduced amino acid sequences of the mammalian hydrolases contained only limited regions of identity to the hydrolase from *R. rubrum* (Table I) (20). Amino acids 60–67, 133–139, and 285–291 of rat, mouse, and human brain hydrolase showed significant identity with bacterial ADP-ribosylarginine hydrolase. Conceivably, conserved amino acids in these areas could represent consensus domains critical for hydrolase function. Of possible importance with regard to hydrolase function, Asp-60, Asp-61, His-65, Arg-139, and Asp-285 were deemed possible candidates for active site residues. His-65 is not present in the *R. rubrum* enzyme, where it is replaced by serine.

A second domain in the hydrolase, considered potentially crucial in catalysis, is Ala-185-X-X-X-Gly-Lys-Ser-192 in rat and mouse enzymes, which is a consensus NTP binding sequence. It differs, however, in human hydrolase, in which -Gly-Lys-Ser-192 is replaced by -Ser-Arg-Pro-192. Since the enzymes presumably act by similar mechanisms, this region may not play as important a role in hydrolase activity as do the corresponding regions in protein kinases and nucleotide-binding proteins and was not mutated here (26).

Site-directed mutagenesis was used to replace Asp-60, Asp-61, His-65, Arg-139, and Asp-285 codons with alanine in the ADP-ribosylarginine hydrolase cDNA. The GST-linked fusion proteins (GST-hydrolase) were purified to homogeneity as described under "Experimental Procedures" (Fig. 1). H65A, R139A, and D285A mutants had activities similar to that of the wild-type, whereas the activities of D60A and D61A were three orders of magnitude lower (Table II). Both D60A and D61A mutant proteins exhibited the same immunoreactivity as wild-

TABLE II

Specific activities of ADP-ribosylarginine hydrolase constructs: WT, D60A, D61A, H65A, R139A, and D285A

Purified rat brain hydrolase (as a GST fusion protein) (50 ng) was assayed in 50 mM potassium phosphate, pH 7.5, 5 mM dithiothreitol, 10 mM MgCl_2 , 50 μM ADP-ribose- ^{14}C arginine (39,000 cpm) (total volume 100 μl). If enzymatic activity was not detected (mutants D60A and D61A), the experiment was repeated with 10 μg of hydrolase and an incubation time of 5 h. Specific activity is recorded as in $\mu\text{mol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$ GST fusion protein. Data are expressed as means of values from three experiments \pm half the range with assays performed in duplicate.

Mutant	ADP-ribosylarginine hydrolase activity	
	$\mu\text{mol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$	
WT	0.55 \pm 0.06	
D60A	0.0006 \pm 0.00021	
D61A	0.00054 \pm 0.0011	
H65A	0.52 \pm 0.08	
R139A	0.49 \pm 0.09	
D285A	0.52 \pm 0.1	

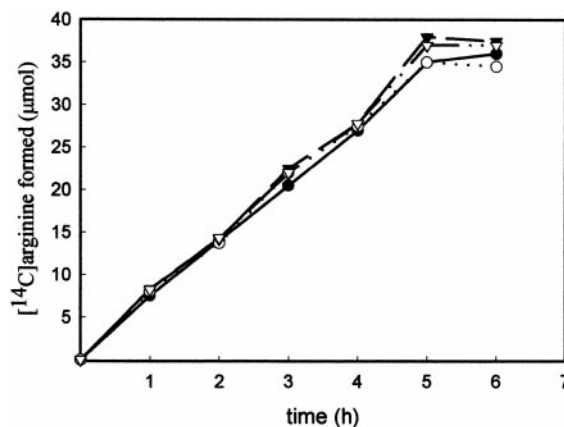


FIG. 2. **Time course of ADP-ribosylarginine hydrolase activity of mutants.** Purified recombinant rat brain hydrolase (as a GST fusion protein) (50 ng) was assayed as noted under "Experimental Procedures." After incubation (1–6 h), a sample was applied to a column (0.5 \times 4 cm) of Affi-Gel 601 (boronate), equilibrated, and eluted with five 1-ml portions of 0.1 M glycine, pH 9.0, 0.1 M NaCl, 10 mM MgCl_2 . The total eluate was collected for liquid scintillation counting. Activity of the hydrolases is defined as the ability to generate free arginine from ADP-ribosyl- ^{14}C arginine. Data are means \pm S.D. of values from three experiments, each with assays in duplicate. S.D. bars within symbols are not shown. \bullet , R139A; \circ , D285A; \blacktriangle , H65A; ∇ , WT. Substitution of Ala for His-65, Arg-139, or Asp-285, which are located in conserved regions, did not change specific activity.

type on Western blots (data not shown). Activities of WT hydrolase and active mutants, which were constant for the first 5 h of assay (Fig. 2), were essentially equal and similar to that of the native rat brain enzyme (24). These data suggest that native and recombinant hydrolase have similar catalytic properties and that recombinants produced in *E. coli* can be used as an appropriate model for the native enzyme. Replacement of Asp-60 or Asp-61 with Ala, Asn, or Gln resulted in a loss of enzymatic activity. Mutants D60E and D61E, however, had activity comparable with that of wild-type (Table III). Double mutants in which both aspartates were replaced with asparagine, glutamine, or glutamic acid were inactive (data not shown).

Because it was crucial to demonstrate that the catalytically inactive wild-type and mutant proteins were structurally intact, advantage was taken of the fact that ADP-ribose (a substrate analogue) is a potent inhibitor of the enzyme (Fig. 3). GST fusion proteins were incubated with ^{14}C ADP-ribose fol-

TABLE III

Specific activities of ADP-ribosylarginine hydrolase constructs: WT, D60A, D60E, D60N, D60Q, D61A, D61E, D61N, and D61Q

Experiments were carried out as described for Table II. Specific activity is recorded as in $\mu\text{mol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$ of GST fusion protein. Data are expressed as means of values from three experiments \pm half the range with assays performed in duplicate.

Construct	ADP-ribosylarginine hydrolase activity $\mu\text{mol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$
WT hydrolase	0.56 \pm 0.12
D60A	0.0006 \pm 0.00021
D60E	0.56 \pm 0.09
D60N	0.00065 \pm 0.00018
D60Q	0.00062 \pm 0.00017
D61A	0.00054 \pm 0.00011
D61E	0.70 \pm 0.08
D61N	0.00035 \pm 0.0002
D61Q	0.00040 \pm 0.00022

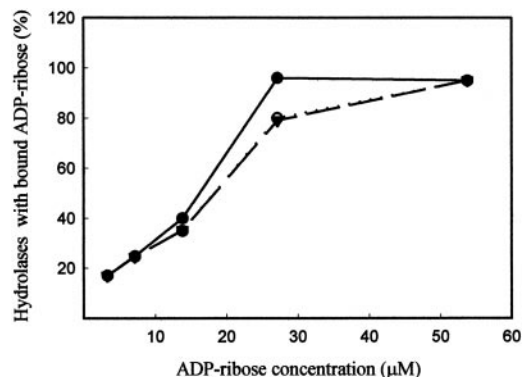


FIG. 3. **Binding of ADP-ribose to hydrolase.** Binding of ADP-ribose to hydrolase was determined as described under "Experimental Procedures." Data are means of values from duplicate assays and representative of three for each of the hydrolases. Each experimental point was determined in three duplicate assays. Mutants (○) D60A and (▼) D61A, despite their differences in activity, bound ADP-ribose with an affinity similar to that of the WT enzyme (●).

lowed by removal of unbound substrate analogue by dialysis. All mutants, despite their extreme differences in activity, bound ADP-ribose in a manner similar to that of the wild-type (Figs. 3 and 4). Affinities of the wild-type and mutant (D60A) ADP-ribosylarginine hydrolases for ADP-ribose were determined from Scatchard analysis and were similar ($K_D = 16 \mu\text{M}$ in both instances) (Fig. 4).

The concentration of ADP-ribose that decreased enzyme activity by 50% (IC_{50}) is not equivalent to the K_D for the competitor, but depends on the concentration of the radioligand present in the incubation. For conditions described under "Experimental Procedures," $\text{IC}_{50} = 17 \mu\text{M}$ (Fig. 5) and corresponds well to the concentration of ADP-ribose that saturates 50% of the ADP-ribosylarginine hydrolase under the same conditions (Figs. 3 and 4).

Catalysis by some hydrolases involves coordinate action of a proton donor and a nucleophile (26). For ADP-ribosylarginine hydrolase, the conserved Asp-60 and Asp-61 may be positioned within the proposed catalytic cleft and could serve as proton donor and nucleophile, respectively. The positioning of the two aspartic acids would need to allow sufficient room for an attacking water molecule. In the single mutants D60E and D61E, sufficient space and flexibility would accommodate a water molecule, whereas in the double mutant, D60E/D61E, catalysis could no longer proceed. This study represents the first identi-

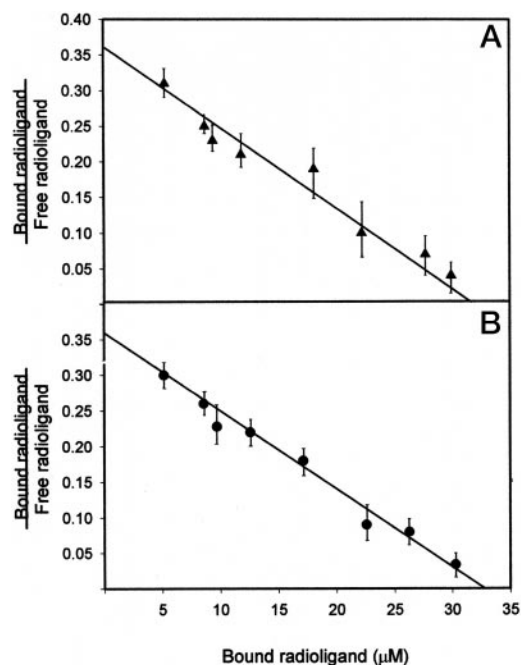


FIG. 4. **Scatchard analysis of ADP-ribose binding to WT and mutant ADP-ribosylarginine hydrolase D60A.** Binding of [^{14}C] ADP-ribose was quantified as described under "Experimental Procedures." After incubation for 1 h and dialysis against semipermeable membranes, hydrolase samples and 100 μl from the dialysis chamber (total volume 100 ml) were counted using a liquid scintillation counter for calculations of bound and free ADP-ribose. In the Scatchard plots for wild-type hydrolase (A) and D60A (B), mean values \pm half the range are given for duplicate assays performed in two separate experiments. The results are representative of those obtained for D61A and "double" mutants. All mutants, despite their differences in activity, bound ADP-ribose with affinity similar to that of the wild type.

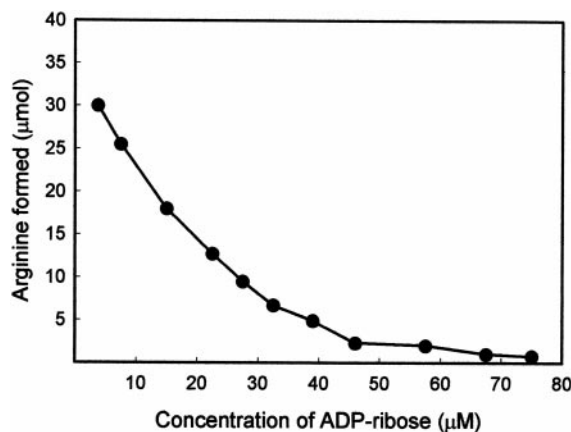


FIG. 5. **Inhibition of wild-type ADP-ribosylarginine hydrolase activity by ADP-ribose.** Inhibition of hydrolase activity by ADP-ribose was determined as described under "Experimental Procedures." Activity of the WT ADP-ribosylarginine hydrolase was expressed as ability to release free arginine. Data are means of values from two experiments, each with triplicate assays.

fication of critical, active site residues in the ADP-ribosylarginine hydrolases that appear to be conserved from bacteria to humans.

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