Identification of Regulatory Domains in ADP-ribosyltransferase-1 That Determine Transferase and NAD Glycohydrolase Activities*

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Mono-ADP-ribosyltransferases (ART1–7) transfer ADP-ribose from NAD$^+$ to proteins (transferase activity) or water (NAD glycohydrolase activity). The mature proteins contain two domains, an α-helical amino terminus and a β-sheet-rich carboxyl terminus. A basic region in the carboxyl termini is encoded in a separate exon in ART1 and ART5. Structural motifs are conserved among ART molecules. Successive amino- or carboxyl-terminal truncations of ART1, an arginine-specific transferase, identified regions that regulated transferase and NAD glycohydrolase activities. In mouse ART1, amino acids 24–38 (ART-specific extension) were needed to inhibit both activities; amino acids 39–45 (common ART coil) were required for both. Successive truncations of the α-helical region reduced transferase and NAD glycohydrolase activities; however, truncation to residue 106 enhanced both. Removal of the carboxyl-terminal basic domain decreased transferase, but enhanced NAD glycohydrolase, activity. Thus, amino- and carboxyl-terminal regions of ART1 are required for transferase activity. The enhanced glycohydrolase activity of the shorter mutants indicates that sequences, which are not part of the NAD binding, core catalytic site, exert structural constraints, modulating substrate specificity and catalytic activity. These functional domains, defined by discrete exons or structural motifs, are found in ART1 and other ARTs, consistent with conservation of structure and function across the ART family.

Post-translational modification of proteins by mono- or poly-(ADP-ribosylation) has been implicated in the regulation of a variety of biological processes (e.g., T-cell activation, cytoskeleton reorganization, apoptosis). In several systems, the effect on function could be reversed by removal of the ADP-ribose moiety by ADP-ribosylprotein hydrolases or lysases, consistent with the existence of ADP-ribosylation/de-ADP-ribosylation cycles. In the case of mono-ADP-ribosyltransferases (ARTs),¹ a single ADP-ribose (ADPR) moiety from NAD is transferred to a specific amino acid in a target protein. In addition to using proteins or amino acids as acceptors, most of these enzymes can also transfer ADPR to water. The latter activity results in NAD hydrolysis (NAD glycohydrolase (NADase) activity) (1).

Mono-ADP-ribosylation was first recognized as the mode of action of diphtheria toxin, cholera toxin, and other bacterial toxins (2). Subsequently, vertebrate mono-ADP-ribosyltransferases, sharing structural homology with prokaryotic toxin counterparts, were identified. To date, the family of known vertebrate ARTs consists of seven members (ART1–7); all appear to be glycosylphosphatidylinositol (GPI)-anchored or secreted proteins (1, 3). Although they share less than 10% similarity at the level of amino acid sequence, there are several common structural features that define the family: four conserved cysteines, a consensus “ART signature” sequence, an α-helix-rich amino-terminal region, and a carboxyl-terminal region folded into β-sheets. The latter, by analogy to the structural organization of bacterial ADP-ribosylating enzymes, forms their NAD-binding and catalytic sites (4). The mammalian art genes have exons encompassing signal sequences for ER transfer (amino terminus) and addition of the GPI-anchor (carboxyl terminus). The region responsible for catalysis is found in one exon. In some ARTs, however, a basic region is encoded by a separate exon at the carboxyl-terminal end of the coding region (1, 3). Thus, in general, across ART family members, different exons encode functional regions of the protein; within the coding region exon, different structural motifs may be responsible for function.

ART1, the first cloned and characterized vertebrate ART, is an arginine-specific ADP-ribosyltransferase (3, 5–8) that is relatively conserved across species with deduced amino acid sequence of the mouse protein 77% and 73% identical to those of the human and rabbit, respectively (9). This cell-surface, GPI-anchored protein modifies integrin α₇ in mouse skeletal muscle cells (10). An increase in arginine-specific ADP-ribosylation was observed during the process of differentiation into myotubes (11, 12). In vitro, ART1 ADP-ribosylates human defensin-1, leading to changes in its biological properties; ADP-ribosylated human defensins were also detected in vitro, consistent with a modulatory role for mono-ADP-ribosylation in innate immunity (13). In a T-cell lymphoma line stably transfected with ART1, the function of the TCR was altered in the presence of NAD through modification of LFA-1 and other co-receptor proteins (14).

Structure/function studies of ART isoforms have thus far focused on the β-sheet-rich domain of the protein and characterization of the catalytic regions. In rabbit ART1, glutamate (Glu-240) was identified as the catalytic amino acid, and even the conservative E240D substitution abolished ADP-ribose transfer to arginine. Nearby Glu-238 was also critical for ADP-ribose transfer (15). In several ARTs, replacement of this Glu altered the ability of the transferases to use arginine or other guanidine compounds as acceptors, supporting the hypothesis that this region is involved in acceptor recognition (16). Little is known about the role of the α-helical region of the protein in ART activity. The fact that the carboxyl-terminal

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The abbreviations used are: ART, mono-ADP-ribosyltransferase; GPI, glycosylphosphatidylinositol; NADase, NAD glycohydrolase; ADPR, ADP-ribose.
basic region is a separate exon raised the possibility that it could play a role in regulating catalytic actions. Since motifs appear to be responsible for function and targeting elsewhere in ART enzymes, we hypothesized that within the coding region, these motifs may regulate activity. By using truncated mutants of ART1 protein, we identified amino- and carboxy-terminal regions that influence ART1 catalytic activity. Based on these data, it appears that conserved motifs within the amino- and carboxy-terminal regions increase the specificity of the catalysis by exerting some constraints on the active site.

MATERIALS AND METHODS

FLAG-tagged Deletion Mutants of ART1—Several truncated forms of mART1 were generated by PCR amplification (17) using the forward (F) and reverse (R) primers listed in Table I. PCR products were subcloned into the prokaryotic expression vector pFLAG-MAC (Sigma), using HindIII and KpnI restriction enzymes, and expressed in Escherichia coli (DH5α, Invitrogen). Plasmid sequences were verified by DNA sequencing of the entire open reading frame. The amino-terminal FLAG-tagged recombinant proteins were purified from bacterial lysates using M2 affinity chromatography according to the manufacturer’s instructions (Sigma), and enzymatic activity was measured.

NAD Glycohydrolyase and ADP-ribosyltransferase Assays—Nicotinamide release (standard assay) was measured with (ADP-ribosyltransferase activity) or without (NAD glycohydrolyase activity) 20 μM agmatine, in Dulbecco’s phosphate-buffered saline containing 7 μM [carbonyl-14C]NAD (0.05 μCi/sample, Amersham Biosciences) at 30 °C for the indicated time (total volume, 300 μL). Samples (100 μL) were applied to columns (0.4 × 4 cm) of AG1-X2 (Bio-Rad), equilibrated, and eluted with water for radioassay of [14C]nicotinamide (18). Transferase activity was assayed similarly with or without 20 μM agmatine as an ADP-ribose acceptor and with adenosine-14C[NAD substituted for [carbonyl-14C]NAD so that [adenine-14C]ADP-ribose-adenylate was generated. Activity was normalized to the amount of recombinant protein present in each preparation by densitometric analysis of Coomassie Blue-stained gels (see under “Quantification of Truncated Mutants”).

Nicotinamide release

| ART1 (24-288) | 277 | 222 | 31 | 7.1 |
| ART1 (39-288) | 277 | 538 | 111 | 4.8 |
| ART1 (46-288) | 277 | 103 | 47 | 2.2 |
| ART1 (52-288) | 277 | 40 | 17 | 2.4 |
| ART1 (67-288) | 277 | 97 | 42 | 2.3 |
| ART1 (106-288) | 277 | 157 | 213 | 0.7 |

RESULTS AND DISCUSSION

Structure/function studies of mammalian ARTs have, thus far, focused on the carboxy-terminal half of the molecules, a predicted β-sheet-rich region which, based on crystallographic structure of the bacterial toxins (19–26) and rat ART2 (27), contains the catalytic site. Its secondary structure is analogous to that of the active site of prokaryotic ARTs (4); it is formed by the interaction of three regions (I, II, and III). Regions I and II, involved in stabilizing the NAD binding, are defined, respectively, by a conserved arginine and a consensus “serine X serine” motif (where X is any amino acid). Region III contains the catalytic glutamate required for NAD hydrolysis. A basic region, located at the carboxy-terminal end of the β-sheet-rich region, is, in some ARTs, encoded in a separate exon (amino acids 277–290 in mART1). Enzyme catalytic core and characteristics of deleted regions are shown by different shadings. Nt and Ct, amino and carboxyl termini. Not drawn to scale.

Quantification of Truncated Mutants—Affinity-purified mutant proteins together with 10–4 μg amounts of SDS-PAGE low molecular weight standards (Bio-Rad) were separated by SDS-PAGE in 10–20% gradient gels. After Coomassie Blue staining of the gels, optical density measurements of the bands (Epson scanner, Scion densitometry soft-

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the functions of the conserved domains within the amino- and carboxyl-terminal regions adjacent to the catalytic core of the ARTs, we monitored transferase and NADase activities of ART1 mutants generated by progressive deletions from the amino or carboxyl termini of the protein (Fig. 1 and Fig. 5B, respectively).

Regions Surrounding the NAD-binding Catalytic Core Suppress NAD Hydrolysis and Enhance ADP-ribose Transfer to Acceptor—In mART1-(24–288) and rART1-(24–293) mutants, signal sequences necessary for export from the ER (at the amino terminus) and addition of a GPI-anchor (at the carboxyl terminus) were deleted. The resulting mutants retained the catalytic properties of the mature wild-type ART1. Their NADase activities relative to transferase activities were very low, and nicotinamide release was enhanced in the presence of the ADP-ribose acceptor, agmatine, reflecting, as expected, a preference for a guanidino moiety over water as an ADP-ribose acceptor (Fig. 2 and Fig. 6). The products of the reaction were hydrolyzed by the stereospecific ADP-ribosylarginine hydrolase, consistent with the formation of the α-anomer. These results are consistent with ART1 being primarily an arginine-specific ADP-ribosyltransferase that employs an S2-like mechanism in the formation of α-ADP-ribose-agmatine from β-NAD. Amino acids 24–38 correspond to an amino-terminal extension in the GPI-anchored ART1 transferase, which is slightly longer than those in other NADases and/or arginine-specific ADP-ribosyltransferases of the ART family and relatively conserved across species (Fig. 3) (9). On the contrary, region 39–45 is similar among these isoforms (Fig. 3), and comparison of its predicted secondary structure with rat ART2 crystal structure (27) suggests that these amino acids form a coil just amino-terminal to a long α-helical region that is conserved among ART isoforms. Deletion of residues 24–38 of mART1 enhanced NAD hydrolysis as well as ADP-ribose transfer to agmatine. Further amino-terminal deletions of residues 39–45 resulted in a decrease of transferase activities but not of NADase activity when compared with ART1-(24–288) activities. Measurement of ADP-ribose-agmatine formation confirmed the inhibitory influence of amino acids 24–38 on ADP-ribose transfer (data not shown); additional deletions from the amino terminus completely abolished transferase activity (Fig. 4). These results suggest that ART1-specific amino acids 24–38 are inhibitory for both enzymatic activities, whereas ART-conserved amino acids 39–45 are necessary for proper transfer of ADP-ribose to agmatine but do not affect NADase activity. Thus, the ART1-specific extension and the ART-common structural motif appear to have different functional effects on ART activity.

In contrast, region 67–105 appears to be inhibitory for NADase activity. The shortest mutant, mART1-(106–288), was primarily an NADase, consistent with the lack of transferase activity of the core fragment (Figs. 3 and 4). Residues 52–105 include a cysteine (Cys-53 in mART1) conserved among all isoforms (Fig. 3). According to the rat ART2.2 crystal structure (27), Cys-53 is predicted to form a disulfide bond with another conserved cysteine (Cys-272) at the carboxyl terminus of the molecule, which stabilizes folding of the α-helix-rich domain. This region appears not to be required for NAD hydrolysis.

Effect of the Basic Region Encoded by the Small Exon—ART1 proteins contain a short basic region at the end of the catalytic core (Fig. 5A) that is encoded by a small exon, found also in the art5 gene, but not in the art2 or art4 genes. In ART1 proteins, the amino acid stretch encoded by this exon is rather conserved across species. In the ART family, exons appear to encode functional domains (amino-terminal signal sequence for ER transfer, carboxyl-terminal signal sequence for addition of the GPI anchor). Therefore, we investigated if this exon as well encodes a motif with functional effects on catalytic activity. To determine the influence of this basic region on ART1 enzyme activity, mutants were generated by deletion from the carboxyl terminus of mouse ART1 (Fig. 5B). Since the equivalent region in rabbit ART1 differs in pI (Fig. 5A), ART1 rabbit deletion mutants were also generated for comparison. In both mART1-(24–276) and rART1-(24–281), deletion of the small carboxyl-terminal basic region increased NAD hydrolysis. NADase activity of mART1-(24–265) was further enhanced by deletion of amino acids 276 to 266 (Fig. 6). In contrast, nicotinamide release in the presence of agmatine was either not affected.
significantly by the additional deletions from the carboxyl terminus (mART1-(24–276)) or was decreased (mART1-(24–284) and rART1-(24–281)) (Figs. 6 and 7). Therefore, carboxyl-terminal truncations resulted in an overall relative loss of transferase activity, reflecting an increase in acceptor-independent nicotinamide release. Region 266–288 contains the highly conserved Cys-272 (Fig. 5B). Results obtained with the carboxyl-terminal truncations are in agreement with the conclusion that in the amino-terminal truncated mutants, the predicted disulfide-bridge between Cys-53 and Cys-272 appears important for ADP-ribose transfer but not for NAD\(^+\) hydrolysis.

**[FIG. 5.](#) A, amino acid sequences of the small basic domain adjacent to the catalytic site in mouse and rabbit ART. Amino acid charge is indicated by + and −. Net charge for each basic region is on the right. B, structures of mouse and rabbit ART1 carboxyl-terminal deletion mutants. Residues at the amino and carboxyl termini (Nt and Ct) of the molecules are numbered, and the exon/intron junctions are marked by separations. Residues 277–290 in mART1 and residues 282–295 in rART1 are encoded by a short exon of the respective genes. Enzyme catalytic core and characteristics of deleted regions are shown by different shadings. Rabbit ART1 putative exon/intron junctions were drawn by comparison with the gene structure of mouse ART1 as rabbit art1 gene structure is not known. Not drawn to scale.**

**[FIG. 6.](#) Nicotinamide release catalyzed by mouse and rabbit ART1 carboxyl-terminal deletion mutants measured without or with agmatine.** Affinity-purified mutant proteins were incubated for 14 h at 30°C with 7 µM [adenine-\(^{14}\)C]NAD and 1 mM ADP-ribose, with (+Ag) or without (−Ag) 20 mM agmatine, (final volume, 300 µl), before quantification of nicotinamide release and calculation of release per mg of mutant protein in each preparation. Similar results were obtained using 14.5 µM NAD. Data presented are from one experiment representative of results obtained with two different protein purifications. Each activity measurement was performed in duplicate.

![Image](image-url)

**[FIG. 7.](#) Synthesis of ADP-ribose-agmatine by mouse and rabbit ART1 carboxyl-terminal deletion mutants.** Affinity-purified mutant proteins were incubated for 4 h at 30°C with 7 µM [adenine-\(^{14}\)C]NAD, 1 mM ADP-ribose, and 20 mM agmatine (final volume, 300 µl). ADP-ribose-agmatine was separated by anion exchange chromatography and quantified by scintillation counting. Data are expressed as nicotinamide release per mg of mutant protein in each preparation. Similar results were obtained using 100 µM NAD. Data presented are from one experiment representative of results obtained with two different protein purifications. Each activity measurement was performed in duplicate.

![Image](image-url)

**[FIG. 8.](#) Protein ADP-ribosylation by mART1 and rART1 mutants.** Affinity-purified mutant proteins (1 µg) were incubated for 1 h at 30°C with 5 µM [\(^{32}\)P]NAD (10 µCi) and 1 mM ADP-ribose (final volume, 190 µl). After precipitation with 10% trichloroacetic acid, separated by SDS-PAGE in 10–20% gradient gels, and either stained with Coomassie Blue (lower panel) or transferred to Immobilon P membrane that was exposed to x-ray film for 17 h (upper panels). Data presented are from one experiment representative of results obtained with two different protein purifications.
identifying bands not readily seen in Coomassie Blue-stained gels. Prior studies have shown that the availability of arginine acceptors for ADP-ribose differs across proteins and increases with denaturation of the protein. Activities of rabbit ART1 mutants were similar to those of their mouse counterparts. All other deletion mutants lacked transferase activity. These results are consistent with the agmatine-specific transferase activities determined for each of these mutants (Figs. 4 and 7).

By functional characterization of ART1 molecules that were truncated from the amino- and carboxyl-terminal ends, we identified several regions that influence the transfer of ADP-ribose to an acceptor amino acid or to water. At the amino terminus, an ART1-specific extension, amino acids 24–38, and a coil region common to ARTs, amino acids 39–45, modulated both types of transfer but with opposite effects; ART1-specific amino acids 24–38 were inhibitory, and ART-conserved residues 39–45 necessary, for ADP-ribose transfer. In contrast, residues 46–106 were required for ADP-ribose-agmatine formation, as were regions encoded by the small basic exon specific of ART1 isoforms (amino acids 276–288 in mART1 or 281–293 in rART1) near the carboxyl terminus. Enhanced NADase activity of the shortest deletion mutants suggests that the β-sheet-rich carboxyl-terminal domain can maintain a structure adequate for catalysis of NAD hydrolysis independent of the α-helix-rich region. Thus, the two structurally distinct domains of ART1, the α-helix-rich amino terminus and the mainly β-sheet carboxyl terminus, appear to contribute to different catalytic functions with the catalytic core alone retaining NAD hydrolysis activity. The ART1-specific and ART-conserved regions from amino-terminal α-helical and carboxyl-terminal basic domains may modulate the catalytic activity of a predominantly β-sheet catalytic core, in particular, its substrate specificity. It may be relevant that in some ARTs, the carboxyl-terminal basic domain is encoded in a separate exon. Our data suggest that this exon could play a regulatory role in modulating substrate specificity.

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