Hydrolysis of O-Acetyl-ADP-ribose Isomers by ADP-ribosylhydrolase 3*

Atsushi Kasamatsu1,2, Motoyuki Nakao4, Brian C. Smith5, Lindsay R. Comstock5, Tohru Ono4,3, Jiro Kato4, John M. Deni5, and Joel Moss5

From the 1Cardiovascular and Pulmonary Branch, NHLBI, National Institutes of Health, Bethesda, Maryland 20892 and the 2Department of Biomolecular Chemistry, University of Wisconsin, Madison, Wisconsin 53706

O-Acetyl-ADP-ribose (OAADPr), produced by the Sir2-catalyzed NAD+-dependent histone/protein deacetylase reaction, regulates diverse biological processes. Interconversion between two OAADPr isomers with acetyl attached to the C-2” and C-3” hydroxyl of ADP-ribose (ADPr) is rapid. We reported earlier that ADP-ribosylhydrolase 3 (ARH3), one of three ARH proteins sharing structural similarities, hydrolyzed OAADPr to ADPr and acetate, and poly(ADPr) to ADPr monomers. ARH1 also hydrolyzed OAADPr and poly(ADPr) as well as ADP-ribose-arginine, with arginine in α-anomeric linkage to C-1” of ADP-ribose. Because both ARH3- and ARH1-catalyzed reactions involve nucleophilic attacks at the C-1” position, it was perplexing that the ARH3 catalytic site would cleave OAADPr at either the 2”- or 3”-position, and we postulated the existence of a third isomer, 1”-OAADPr, in equilibrium with 2”- and 3”-isomers. A third isomer, consistent with 1”-OAADPr, was identified at pH 9.0. Further, ARH3 OAADPr hydrolysis activity was greater at pH 9.0 than at neutral pH where 3”-OAADPr predominated. Consistent with our hypothesis, IC50 values for ARH3 inhibition by 2”- and 3”-N-acetyl-ADPr analogs of OAADPr were significantly higher than that for ADPr. ARH1 also hydrolyzed OAADPr much more rapidly at alkaline pH, but cleavage of ADP-ribose-arginine was faster at neutral pH than pH 9.0. ARH3-catalyzed hydrolysis of OAADPr in H218O resulted in incorporation of one 18O into ADP-ribose by mass spectrometric analysis, consistent with cleavage at the C-1” position. Together, these data suggest that ARH family members, ARH1 and ARH3, catalyze hydrolysis of the 1”-O linkage in their structurally diverse substrates.

Mono-ADP-ribosylation is a post-translational modification, in which the ADP-ribose (ADPr) moiety of NAD is transferred to an acceptor protein (1). This modification serves as the mechanism by which several bacterial toxins (e.g. Pseudomonas exoenzyme S, cholera toxin, diphtheria toxin) exert their effects on mammalian cells (2, 3). Mammalian cells also produce endogenous ADP-ribose transferases that catalyze reactions similar to the bacterial toxins, specifically, the ADP-ribosylation of arginine residues in proteins (4). In addition, mammalian cells possess hydrolases that cleave the ADPr-protein linkage, releasing ADPr and regenerating the unmodified protein (5, 6). An ADP-ribosyl(arginine) hydrolase, termed ARH1, catalyzes in a stereospecific manner, hydrolysis of the α-linkage of arginine-ribose found in ADP-ribo<wbr/>syl(arginine)-protein to ADPr and (arginine)-protein (7, 8), consistent with the regulation of ADP-ribo<wbr/>syl(arginine)-protein levels by opposing activities of transferases and hydrolases, participating in an ADP-ribosylation cycle (4, 9).

Three known members (ARH1–3) of the ARH family of proteins are similar in molecular size (~39 kDa) and amino acid sequence (10). As noted above, ARH1 catalyzes the hydrolysis of ADP-ribose-arginine and also hydrolyzes ADP-ribose linkages to guanidine. The reaction is stereospecific, and only the α-anomer at the C-1” position of ADP-ribose-arginine is hydrolyzed (8). ARH1 also hydrolyzed the stereospecific C-1”-C-2” linkage in poly(ADP-ribose). The ARH1 reaction is inhibited by ADP-ribose, but not by phosphoribose, suggesting that the catalytic site recognizes the adenosine moiety (8). The fact that only the guanidine group of arginine is necessary for hydrolysis further supports a critical role for the ADP-ribose as opposed to the arginine in catalysis (8). ARH2, which has considerable structural similarity to ARH1, has no reported activity. In this regard, the vicinal aspartate residues that were critical for ARH1 activity are replaced by an aspartate-asparagine, which may explain the lack of activity.

We previously reported that ARH3 possesses poly(ADPr) glyco<wbr/>hydration activity (10). The linkage hydrolyzed by ARH3 and ARH1 in poly(ADP-ribose) is also stereospecific at the C-1” position with the α-anomer present in the polymer (Fig. 1). As with the ARH1-catalyzed reaction, ADP-ribose is a potent inhibitor. ARH3 also hydrolyzed O-acetyl-ADP-ribose (OAADPr) in a Mg2+-dependent manner to produce ADPr, which inhibited these two ARH3-catalyzed reactions (10, 11). Both poly(ADPr) and OAADPr participate in important biological pathways. Poly(ADPr) is involved in carcinogenesis, DNA repair, and cell differentiation (12–14). OAADPr is the product of NAD+-dependent protein deacetylation reactions catalyzed by Silent information regulator 2 (Sir2) proteins that contribute to diverse operations such as gene silencing, DNA repair, chromosomal stability, and lifespan extension (12,
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13). OAADPr may act independently of protein deacetylation in the stabilization of chromatin, formation of silencing complexes, ion-channel gating, and energy metabolism (15–19). Thus, ARH3 can participate in distinct signal transduction pathways that involve polyADPr and OADPr.

During the Sir2-catalyzed reaction, 2'-OADPr has been reported to be the reaction product released by the enzyme (20, 21) (see Fig. 1 for structures). After release, 2'-OADPr rapidly interconverts at pH 7.5 with 3'-OADPr, with which it exists in equilibrium, via acetyl migration, in a ratio of about 1:1 (20, 21). In contrast, 2'- and 3'-N-acetyl-ADP-ribose analogs of OADPr do not undergo acetyl migration, although they do mimic OADPr binding to macro H2A1.1 (22).

Both ARH1 and ARH3 appear to catalyze stereospecific hydrolysis of ADP-ribose-arginine and poly(ADP-ribose), respectively. In both cases, the linkage subject to hydrolysis is at the C-1' position (Fig. 1). In addition, both the ARH1- and ARH3-catalyzed reactions are inhibited by ADP-ribose. For ARH1, ADP-ribose, but not phosphoribose, is an inhibitor, and ADP-ribose-arginine, but not phosphoribose-arginine, is a substrate, demonstrating that the adenosine moiety is important for substrate recognition. Therefore, the ribose linkage to arginine is insufficient for substrate recognition. We observed that ARH1 as well as ARH3 hydrolyzed O-acetyl-ADP-ribose.

We thought it unlikely that ARH3 and ARH1 would hydrolyze the 1''-linkage in poly(ADPPr) and ADP-ribose-arginine, respectively, and also act on 2''- or 3''-linkage in OADPr. Further, in our current studies, ADP-ribose was a potent inhibitor, but not 2''-, 3''-N-acetyl-ADP-ribose. In addition, ARH1 hydrolyzed the C-1'' derivative of ADP-ribose(arginine) as well as OADPr, suggesting that the C-1'' position is the preferred site of cleavage. Here, we propose that, analogous to its poly(ADPPr)-glycohydrolase activity, ARH3 hydrolyzes 1''-OADPr, rather than the 2''- or 3''-isomer, and that 1''-, 2''-, and 3''-OADPr exist in equilibrium at neutral and basic pH values, thus generating 1''-OADPr for hydrolysis by ARH3. These studies demonstrate that ARH1 and ARH3 show a similar preference for substrates and that hydrolysis proceeds with attack at the C-1'' position. Prior studies that showed the presence of C-2'' and C-3'' O-acetyl-ADP-ribose are in agreement with our current findings that at neutral pH, the equilibrium favors these two isomers and not C-1'' O-acetyl-ADP-ribose.

EXPERIMENTAL PROCEDURES

Materials—β-NAD, ADPr, DTT, TFA, and acetonitrile were purchased from Sigma. Adenine [U-13C]NAD (252 mCi (1 Ci = 37 GBq)) was from GE Healthcare. Acetylhistone peptide H3 (acetylated at Lys-14) was from Millipore. SIRT1 was from Enzo Biochem (New York, NY). [18O]Water was from Cambridge Isotope Laboratories, Inc. (Andover, MA). Recombinant human ARH3 and ARH1 were prepared as described previously (10, 23).

Generation and Analysis of O-Acetyl-[14C]ADP-ribose and Other Small Molecules—O-Acetyl-[14C]ADP-ribose was prepared and purified as described by Ono et al. (11). Briefly, 100 μM [14C]β-NAD (2,500 cpm/pmol) and acetylhistone peptide H3 (100 μg) were incubated for 4 h at 30°C with SIRT1 (25 units, 6.1 μg) in 50 mM Tris-HCl (pH 7.0) buffer containing 2.7 mM KCl, 1 mM MgCl2, and 0.2 mg of BSA (total volume, 200 μl). Product and substrate were separated on a Vydc C18 column (4.6 mm × 250 mm; W. R. Grace & Co., Columbia, MD) using reverse-phase high-performance liquid chromatography (RP-HPLC) (Hewlett-Packard, series 1100, with a diode array spectrophotometric detector set at 259 nm). Isocratic elution (1 ml/min) with 100% buffer A (0.05% (v/v) TFA in water) from 0 to 5 min was followed by a linear gradient to 60% buffer A and 40% buffer B (0.05% (v/v) TFA in acetonitrile) from 5 to 45 min. Eluate fractions (30 s, 500 μl) were collected for quantification of radioactivity using liquid scintillation counting (TriCarb 1600TR, PerkinElmer Life Sciences). Eluted solvent was fractionated each 30 s (500 μl) using fraction collector and was subjected to a liquid scintillation counter (Liquid Scintillation Analyzer TriCarb 1600TR, PerkinElmer Life Sciences) for measurement of radioactivity.

Hydrolysis of O-Acetyl-ADP-ribose Catalyzed by ARH3 or ARH1—Samples of 1 μM OADPr and purified recombinant ARH3 (1.5 pmol) or ARH1 (375 pmol) were incubated in 50 mM potassium phosphate buffer (pH 5.0, 7.0, and 9.0), 10 mM MgCl2, and 5 mM DTT (total volume, 200 μl) for 60 min at 30°C. Substrate and products were separated by RP-HPLC, as described above.

Identification of Isomers of O-Acetyl-ADP-ribose—For identification of OADPr isomers, 1 μM OADPr in 50 mM potassium phosphate (pH 9.0), 10 mM MgCl2, and 5 mM DTT (total volume, 200 μl) were incubated (5 min at room temperature), before separation of isomers using RP-HPLC and analysis by MALDI-TOF mass spectrometry as described previously (11). Briefly, before mass spectrometry, 10 mg of α-cyano-4-hydroxycinnamic acid (Bruker Daltonics, Billerica, MA) was mixed in 1 ml of water/acetonirole (50:50, v/v) containing TFA. Then, 40 pmol of OADPr in 1 μl was added to 1 μl of matrix solution (described above) on a stainless steel target plate and
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air-dried. Negative ion MALDI-TOF mass spectra were acquired using the 4700 Proteomics analyzer (Applied Biosystems, Foster City, CA) operated in reflection mode.

Kinetics of ARH3 Hydrolysis—Rates of OAAADPr hydrolysis by purified recombinant ARH3 (1.5 pmol) were determined using the indicated concentrations of OAAADPr in 50 mM potassium phosphate (pH 7.0), 10 mM MgCl₂ and 5 mM DTT (total volume, 200 μl) incubated for 20 min at 30 °C. ADPr was quantified by RP-HPLC. A, B, C, and D, as indicated, were incubated for the indicated times at 30 °C, before separation of substrate and products by RP-HPLC.

Nonenzymatic Hydrolysis of O-Acetyl-ADP-ribose—To evaluate nonenzymatic hydrolysis of OAAADPr, 1 μM OAAADPr in 50 mM potassium phosphate (pH 9.0 or 7.0), 10 mM MgCl₂, and 5 mM DTT (total volume, 200 μl) were incubated for the indicated time at 30 °C before separation of substrate and products by RP-HPLC.

Preparation of [3H]O-Acetyl-ADP-ribose and N-Acetyl-ADP-ribose Analogs—[3H]OAAADPr was generated enzymatically using published methods with [3H]acetylated histone H3 peptides (21, 24). Synthesis of N-acetyl-ADP-ribose analogs was performed as described (22).

Charcoal Binding Assay—[3H]Acetate produced by ARH3-catalyzed hydrolysis of [3H]OAAADPr was quantified using a charcoal binding assay (24). Reaction mixtures (200 μl) containing 50 mM potassium phosphate (pH 7.0), 10 mM MgCl₂, 5 mM DTT, 16.7 pmol of purified human ARH3, 12.5 μM [3H]OAAADPr, and either 2° or 3°-N-acetyl-ADPr, as indicated, were incubated for 30 min at 30 °C. Reactions were terminated by the addition of a 50-μl charcoal slurry (one-third volume of charcoal solid in two-thirds volume of PBS, pH 7.0). Nonenzymatic [3H]acetate production without ARH3 was used as a control. Charcoal was sedimented by centrifugation, and 200 μl of supernatant containing [3H]acetate was transferred to a clean tube. After centrifugation to remove residual charcoal, 195 μl of the supernatant was transferred to scintillation vials containing counting solution. Supernatant radioactivity represents [3H]acetate produced by ARH3-catalyzed hydrolysis of [3H]OAAADPr (1 pmol/mol). Differences between amounts of [3H]acetate (pmol) accumulated with and without (control) ARH3 was plotted against inhibitor concentration, and data were fit to an inhibition equation using KaleidaGraph (Synergy Software, Reading, PA) to calculate IC₅₀.

Hydrolysis of α-ADP-ribosyl-arginine Catalyzed by ARH1—ADP-ribosyl-[14C]arginine was generated and purified as described by Kato et al. (23). α-ADP-ribosyl-arginine was isolated by RP-HPLC. Assays containing 25 μM α-ADP-ribosyl-arginine and purified recombinant ARH1 (3 pmol) in 50 mM potassium phosphate (pH 5.0, 7.0, or 9.0), 10 mM MgCl₂, and 5 mM DTT (total volume, 200 μl) were incubated for the indicated time at 30 °C. Substrate and products were separated by RP-HPLC, as described above, using Affi-Gel boronate columns (Bio-Rad) (23).

18O Incorporation into ADP-ribose—Assays containing OAAADPr (5 nmol), purified recombinant ARH3 (1 nmol in 100 μl), or heat-inactivated ARH3 (1 nmol, incubated at 95 °C for 20 min) in 50 mM pyridine-formic acid (pH 7.0) and 1 mM MgCl₂ with [18O]water (total volume, 100 μl) were incubated (30 min, 30 °C) before the addition of 1 ml of 100% methanol and analysis. Negative ion electrospray ionization mass spectra were acquired using the Shimadzu LCMS-2020 (Shimadzu, Kyoto, Japan). Ion chromatograms were analyzed with Shimadzu LCMSsolution software.

RESULTS

pH-dependent Hydrolysis of OAAADPr by ARH3—We compared ARH3 hydrolysis of OAAADPr (produced from [14C]NAD and acetylated histone H3 peptide by human SIRT1 (see “Experimental Procedures”) at pH 5.0, 7.0, and 9.0. At pH 5.0, RP-HPLC analysis revealed two peaks of OAAADPr, consistent with 2°- (Fig. 2A, peak B) and 3°-OAAADPr (Fig. 2A, peak A) (20, 21). At pH 5.0, OAAADPr hydrolysis in the presence of ARH3 was not above background (Fig. 2A). After incubation with ARH3 at pH 7.0, the remaining substrate contained not only 2°- and 3°-OAAADPr, but also an additional isomer (Fig. 2B, peak C). In OAAADPr hydrolase assays containing ARH3 at pH 7.0, 119

FIGURE 2. pH-dependence of O-acetyl-ADP-ribose hydrolysis by ARH3. A–E, [14C]OAAADPr (1 μM) (total volume, 200 μl) was incubated at pH 5.0 (A), 7.0 (B), or 9.0 (C) with (open circles) or without (filled circles) recombinant human ARH3 (1.5 pmol) for 1 h at 30 °C before separation of substrate and products as described under “Experimental Procedures.” Nonenzymatic hydrolysis of [14C]OAAADPr was assayed in the same conditions at pH 7.0 (D) and 9.0 (E). Substrates and products were separated by RP-HPLC and radioactivity quantified by liquid scintillation counting. F, 1 μM [14C]OAAADPr was incubated with ARH3 (1.5 pmol), 50 mM potassium phosphate (pH 5.0), 10 mM MgCl₂, 5 mM DTT (total volume, 200 μl) for 35 min at pH 7.0 or 30 min at pH 5.0 (C) followed by 5 min at pH 7.0 (pH was adjusted by the addition of 100 mM dipotassium hydrogen orthophosphate). Substrates and products were quantified as described above. The experiment was repeated three times with similar results.
pmol of OADPr, containing all three isomers, were hydrolyzed, with accumulation of 127 pmol of ADPr (Fig. 2B). At pH 9.0, ARH3 activity was greater than that at pH 7.0, and amounts of all three isomers were reduced, with the expected increase in ADPr (Fig. 2C). Peak C, not observed at pH 5.0, was present at low levels at pH 7.0 and higher abundance at pH 9.0 (Fig. 2, A–C). To assess nonenzymatic OADPr hydrolysis and determine whether the greater activity at pH 9.0 was enzymatic or nonenzymatic, OADPr was incubated at pH 9.0 and 7.0. At pH 9.0 (Fig. 2E), nonenzymatic OADPr hydrolysis was clearly faster than at pH 7.0 (Fig. 2D), and all three peaks were reduced. After nonenzymatic OADPr hydrolysis, ARH3 activity at pH 9.0 was still higher than at pH 5.0 or pH 7.0 (subtracted nonenzymatic hydrolysis of OADPr was significant only at pH 9.0). The mass of 558 m/z nonenzymatic product (data not shown) was consistent with ADPr. As shown in Fig. 2A, there was no ARH3 activity at pH 5.0. Two possible causes of deactivation of ARH3 at pH 5.0 were (i) denaturation of ARH3 due to low pH, and (ii) a pH below the optimal range for OADPr hydrolysis by ARH3. To assess denaturation, ARH3 was incubated at pH 5.0 for 30 min and then at pH 7.0 for 5 min, revealing dramatically greater hydrolyase activity than that at pH 5.0 (Fig. 2F). Activity of ARH3 that had been incubated at pH 5.0 for 30 min followed by 5 min of incubation at pH 7.0 was the same as that measured at pH 7.0 (Fig. 2F), indicating that the lower activity at pH 5.0 was not due to irreversible ARH3 denaturation. Thus, ARH3 activity correlated with the relative abundance of peak C at different pH values, and all three isomers were decreased by ARH3 activity, consistent with their interconversion.

Inhibition of ARH3 Activity by ADP-ribose—Because the activity and pH-dependence of ARH3 correlated with the relative abundance of peak C, we further characterized that material. Peak A (3′-OADPr) predominated under pH 2.0 (Fig. 3A), whereas at pH 9.0 three peaks (A, B, and C) were present (Fig. 3B) even if isolated peak C was used as the starting material. Upon addition of 0.05% (v/v) TFA to a solution of OADPr after being incubated at pH 9.0, peak C was completely converted to peaks A and B (Fig. 3C). This indicated that peak C was in equilibrium with peaks A and B, and, importantly, formation of peak C was reversible. The data are consistent with pH-dependent interconversion of the OADPr isomers. The mass of each of the three peaks was 600 m/z (Fig. 4, A–C), consistent with peak C being an isomer of OADPr, with acetate perhaps at the 1′-position.

Inhibition of ARH3 Activity by ADP-ribose, 2′-N-Acetyl-ADP-ribose, and 3′-N-Acetyl-ADP-ribose—Inhibition of ARH3 hydrolysis of [3H]OADPr (Km = 4.3 ± 0.3 μM) by 2′- and 3′-N-acetyl-ADPr was assessed by quantification of the [3H]acetate product. The IC50 values of 2′-N-acetyl-ADPr and 3′-N-
acetyl-ADPr were ~60 and ~200-fold that of ADPr (Table 1 and Fig. 5). Neither 2- nor 3-N-acetyl-ADPr was a substrate for ARH3 (data not shown). If the preferred substrate of ARH3 is 1-OADPr, it is predicted that ADPr would be better accommodated within the active site than either 2- or 3-OADPr. Therefore, inhibition data are consistent with ARH3 preferentially binding to and hydrolyzing 1-OADPr.

**pH Dependence of Hydrolysis of O-Acetyl-ADP-ribose and ADP-ribose-arginine by ARH1**—Given that the reactions catalyzed by ARH3 and ARH1 are stereospecific at the C-1 position and prefer the α-anomer of poly(ADP-ribose) (ARH3, ARH1) and ADP-ribose-arginine (ARH1), we hypothesized that ARH1 would also hydrolyze O-acetyl-ADP-ribose at the C-1 position, and we expected that for ARH1, as for ARH3, conditions favoring the abundance of the C-1 would enhance hydrolysis. In contrast, hydrolysis of ADP-ribose-arginine by ARH1 should not depend on the alkaline conditions that favor the C-1 form of O-acetyl-ADP-ribose. Indeed, effects of pH on OADPr hydrolyase activity of ARH1 and ARH3 were similar (Fig. 6). As the hydrolysis we saw with ARH1 was less than a single turnover, the observed pH dependence of OADPr hydrolysis was not a steady-state phenomenon, whereas differences with ARH3 were at steady state, thus no direct comparisons of rate are possible.

ARH1 also hydrolyzes ADP-arginine, which exists as both α- and β-anomers of the C-1 guanidine linkage. The α-anomer predominated at pH 5.0 and amounts of α- and β-anomers were similar at pH 9.0, consistent with pH-dependent anomerization (Fig. 7A). ARH1 activity was minimal at pH 5.0, but in contrast to the findings with OADPr, its hydrolysis of ADP-arginine at pH 7.0 appeared greater than that at pH 9.0 (Fig. 7).
At pH 7.0, anomerization was relatively slow, and preferential hydrolysis of the α-anomer by ARH1 was seen with preservation of β-anomer. At pH 9.0, anomerization was relatively rapid, with depletion of both α- and β-anomers, because hydrolysis of α-anomer was accompanied by continuing anomerization. ARH1 activities at pH 7.0 and 9.0 were constant with time (Fig. 7B), and rates of ARH1-catalyzed hydrolysis correlated with the abundance of the α-anomer at the 1′-position for ADPr-arginine. Thus, as expected, hydrolysis of O-acetyl-ADP-ribose and ADP-ribose-arginine appears to be dependent in part on pH conditions that favor the substrate form of the molecule.

18O Incorporation into ADP-ribose—To obtain evidence that ARH3 cleaved OAADPr at the C-1′ position, OAADPr was hydrolyzed by ARH3 in H218O. The reaction product, ADPr, was analyzed by mass spectrometry to quantify 18O incorporation into ADPr. The major ADPr product (85% of total m/z 560.15) of ARH3-catalyzed hydrolysis incorporated one 18O (560.15 m/z) (Fig. 8A). Reactions with heat-inactivated ARH3 yielded nonenzymatically hydrolyzed ADPr (m/z 558.15) with no significant 18O incorporation above natural abundance (Fig. 8B). As expected, a 30-min reaction of heat-inactivated ARH3 displayed some nonenzymatic 18O incorporation (14% of total 558.15/560.15 m/z) (Fig. 8C). When the [18O]ADPr sample formed in Fig. 8A was acidified by 0.1% formic acid and incubated for 5 min, a [18O] ADPr (560.15 m/z) was diminished, with a corresponding increase of ADPr (558.15 m/z) (data not shown). These data suggested that ARH3 cata-
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lyzes attack of [18O]water at the 1°-carbon of OAADPr, with incorporation of 18O into ADPPr at the 1°-position. Since only the 1°-position is exchangeable, the 18O must be at the 1°-position.

DISCUSSION

Herein, we found not only 2°- and 3°-OADPr at pH 9.0, but also substantial quantities of a third peak, of mass (600 m/z) that was consistent with 1°-OADPr. At acidic pH 5.0, 3°-OADPr predominated, whereas at higher pH, all three forms of OAADPr were observed. Rapid interconversion of 2°- and 3°-OADPr had been reported (21), and the amount of peak C at pH 9.0 was greater than that at pH 7.0, whereas peak C was absent at pH 5.0. All three peaks appeared to be in rapid equilibrium at pH 9.0. ARH3 hydrolysis of OAADPr at pH 9.0 was significantly faster than that at pH 7.0. Thus, ARH3 activity correlated with peak C concentration, although other factors related to protein stability and active site chemistry may also affect hydrolyase activity at acidic or alkaline pH.

ARH3 activity was not inhibited by d-ribose 5-phosphate, AMP, ADP, or β-NAD (11). ADPr was an effective inhibitor, likely due to competition for substrate binding within the ARH3 catalytic active site (11). In contrast to ADPr (11), 2°- and 3°-N-acetyl-ADPr were poor ARH3 inhibitors. These data suggested that the 2°- and 3°-acetyl-ribose rings were, relative to ADPr, accommodated poorly in the ARH3 active site, as might be expected if the preferred substrate were 1°-OADPr.

ARH3 hydrolyzes not only OAADPr, but also poly(ADPr) (10, 11). However, ARH3-catalyzed generation of ADPr from OAADPr was significantly faster than that from poly(ADPr) (11). Poly(ADPr) is a homopolymer of ADPr molecules, linked by ribose-ribose (C-1° to C-2°) glycosidic bonds in a linear or branched structure (25, 26). Again, ARH3 catalyzes hydrolysis at the C-1° position within poly(ADPr).

ARH3 and ARH1 molecules share substantial amino acid sequence similarity (10) and stereospecificity (6–8). ARH1 catalyzes hydrolysis of the α-anomer of ADP-ribose-arginine at the ribose C-1° position (6–8). ARH1 also cleaved OAADPr to produce ADPr but at <1% the rate of ARH3 (11). In the experiments reported here, ARH1 activity was pH-dependent; pH 7.0 was preferred for ADPr-arginine hydrolysis, whereas pH 9.0 was optimal for OAADPr hydrolysis. These data are consistent with the hypothesis that the apparent pH optimum for ARH1 is, in part, determined by substrate availability.

If ARH3 hydrolyzed OAADPr at C-1°, 18O should be incorporated into the OAADPr product at the 1°-hydroxyl. Otherwise, 18O attack occurred on a carbon atom of an acetyl group, resulting in no 18O incorporation into ADPr (scheme in Fig. 8). Indeed, one 18O was incorporated into ADPr (560.15 m/z) in contrast to the result of control experiments with inactive ARH3, which showed basal uncatalyzed 18O incorporation. In addition, when ADPr (560.15 m/z) was acidified in natural abundance water, 18O exchanged out rapidly, in agreement with previous research (27). These data are consistent with OAADPr hydrolysis catalyzed by ARH3 occurring at the C-1° position.

In summary, we report that three isomers of OAADPr are in pH-dependent equilibrium: under acidic conditions 3°-OADPr is most abundant, 3°- and 2°-OADPr predominate at neutral pH, and a third isomer that is consistent with 1°-OADPr predominates in a basic milieu. ARH3 activity with OAADPr as substrate was optimal at pH 9.0, where the putative 1°-isomer is the greatest. ARH3 activity was not observed at pH 5.0, where the 3°-isomer predominates. Consistent with the conclusion that hydrolysis occurs at the 1°-position of OAADPr, 2°- and 3°-N-acetyl-ADPr were much less effective inhibitors of ARH3 activity than ADPr. Moreover, the alkaline pH optimum for OAADPr hydrolysis was similar for ARH1 and ARH3, but ARH1 cleavage of ADPr-arginine, which does not have 2°- and 3°-isomers, was optimal at neutral pH. Based on these data, we propose that ARH3 specifically hydrolyzes the 1°-OADPr isomer, i.e. a bond similar to those hydrolyzed by ARH3 in poly(ADPr). As the OAADPr produced in the Sir2-catalyzed NAD-dependent histone/protein deacetylase reaction is reported to participate in several biological processes, including formation of silencing complexes, ion channel gating, and energy metabolism (15–19), ARH3 may influence several signaling pathways through degradation of OAADPr.

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