Transition-State Analysis of 2-O-Acetyl-ADP-Ribose Hydrolysis by Human Macrodomain 1

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Supporting Information

ABSTRACT: Macrodomains, including the human macrodomain 1 (MacroD1), are erasers of the post-translational modification of monoadenosinediphospho-ribosylation and hydrolytically deacetylate the sirtuin product O-acetyl-ADP-ribose (OAADPr). OAADPr has been reported to play a role in cell signaling based on oocyte microinjection studies, and macrodomains affect an array of cell processes including transcription and response to DNA damage. Here, we investigate human MacroD1 by transition-state (TS) analysis based on kinetic isotope effects (KIEs) from isotopically labeled OAADPr substrates. Competitive radiolabeled-isotope effects and mass spectrometry were used to obtain KIE data to yield intrinsic KIE values. Intrinsic KIEs were matched to a quantum chemical structure of the TS that includes the active site residues Asp184 and Asn174 and a structural water molecule. Transition-state analysis supports a concerted mechanism with an early TS involving simultaneous nucleophilic water attack and leaving group bond cleavage where the breaking C−O ester bond = 1.60 Å and the C−O bond to the attacking water nucleophile = 2.30 Å. The MacroD1 TS provides mechanistic understanding of the OAADPr esterase chemistry.

Macrodomains are an evolutionarily conserved family of enzymes and protein domains that recognize NAD⁺-derived metabolites. These include mono- and poly-ADP-ribose (ADPr), ADP-ribosylated proteins, and the product of sirtuin deacetylase reactions, 2-O-acetyl-ADP-ribose (OAADPr). ADP-ribosylation is a reversible protein post-translational modification impacting cellular processes including transcription, neuronal signaling, and response to stresses such as infection and DNA damage. ADP-ribosyltransferases also influence biological pathways through poly- and mono-ADPr polymerases including diphtheria toxin-like ADP-ribosyltransferases and the clostridial toxin-like ADP-ribosyltransferases. MacroD1 and other macrodomain family members including human MacroD2, C6orf130, and archaeal Af1521 have esterase activity toward the acetyl group of OAADPr and hydrolytic activity for mono-ADP-ribosylated proteins.

Macrodomains related to MacroD1 bind OAADPr and hydrolyze the 2-ester bond to form ADPr and acetate as products. The OAADPr product of sirtuin NAD⁺-dependent deacetylases has been implicated as a signaling molecule. Thus, macrodomains may function in the regulation of cellular OAADPr levels and downstream signaling in the sirtuin pathways (Figure 1). A functional link is also suggested by reports that macrodomains are physically or genetically linked to histone deacetylases. We investigated the TS structure of human MacroD1 in its esterase activity toward OAADPr. Isotopically labeled OAADPr molecules were synthesized and used in kinetic isotope effect (KIE) studies. Macrodomains related to MacroD1 have been discovered in all kingdoms of life including yeast (enzyme POA1p) and Archaeoglobus fulgidus (enzyme Af1521). MacroD1 is one of 11 annotated human macrodomains whose founding member is histone protein macroH2A1. MacroD1 overexpression has been linked to the progression of...
breast cancer, and a loss-of-function mutation of macrodomain C6orf130 is responsible for a lethal neurodegeneration.27–29

The classic mechanism of ester hydrolysis proceeds in two steps including a kinetically reversible tetrahedral intermediate. M.L. Bender in 1951 observed 18O-exchange from the carbonyl of ethyl benzoate into solvent during the reaction, supporting a diol intermediate.30 Our analysis of the TS for MacroD1 is different and supports a concerted mechanism. Thus, the TS of MacroD1 shows significant bond order to the attacking water nucleophile and significant bond loss for the acetate leaving group.

Kinetic isotope effects provide experimental guides to computational chemistry for the understanding of enzymatic TSs. Enzymatic TS analysis based on $k_{cat}/k_{m}$ competitive KIEs provides a two state analysis. It compares the structure and geometry of free reactant to that of the TS and includes all steps between reactant and the first chemically irreversible step. At the TS, bond orders, angles, and molecular electrostatic potentials can be extracted from the wave function.31,32 Transition state analysis does not provide information on steps after the TS, but these can often be deduced from likely paths to product from the detailed knowledge of the TS. Experimentally determined KIEs are paired with density function theory (DFT) to determine the TS structure of the enzymatic reaction, here applied to the deacetylation of OAADPr by MacroD1.

We used two independent methods of obtaining KIE data at mechanistically critical atomic positions. The combination of radio-isotope labeling and mass spectrometry in competitive assays confirmed the data obtained by both methods. The intrinsic isotope effects were used to generate an electrostatic potential surface (ESPS) map, a tool for understanding the electron distribution at the transition state. In other systems, this approach has provided a starting point for the design of TS analogues. This approach has been successful in inhibiting ribosyl transfer enzymes including purine nucleosidase phosphoryles. Transition state analogues of MacroD1 would be useful in exploring functions of OAADPr.33,34 Here, the structure of the MacroD1 TS provides chemical insight into the mechanism of MacroD1 ester hydrolysis.

### RESULTS AND DISCUSSION

**Purification and Activity of Protein.** Human Macrodomain I was produced from its cDNA, overexpressed in *E. coli* with an N-terminal 6xHis tag and purified to homogeneity based on SDS-PAGE analysis. The DNA sequence encoding MacroD1 was validated by nucleotide sequencing. The MacroD1 structure contains a macrodomain and an N-terminal region. The macrodomain portion is made of a distinct fold containing a six stranded $\alpha$-helices.

Steady-state parameters for OAADPr hydrolysis to ADPr and acetate were determined in reactions containing MacroD1, OAADPr, and pH 6.8 sodium phosphate at 25 °C (Table 1).

At pH 6.8, catalytic rates are near-optimal and base-catalyzed, nonenzymatic hydrolysis of the ester bond is minimized. Under these conditions, nonenzymatic hydrolysis was insignificant for at least 1 h (SI Figure S3).

Kinetic parameters determined under these conditions were similar to reported values, and the $k_{cat}$ of the hydrolysis rates were equivalent at pH values from 6.5 to 8.0 (SI Figure S4).

The specific rate constant for the nonenzymatic 3- to 2-transesterification reaction of the acetate moiety ($k_{3-2}$) was determined as previously described.35 Under these conditions, transesterification had a first-order rate constant of $1.81 \times 10^{-2}$ s⁻¹. Thus, 3-O-AADPr was found to nearly equilibrate after 3 min under these conditions (SI Figure S5).

**Commitment to Catalysis ($C_C$ and $C_I$).** Intrinsic KIEs are required to provide TS information. They are obtained from experimental KIEs by correction for the forward and reverse commitment to catalysis. Forward commitment ($C_F$) is the probability for the substrate-enzyme complex to form products rather than return to free enzyme and substrate. Reverse commitment is the probability of the enzyme bound products to form substrate rather than dissociating from the enzyme.

The forward commitment can be determined by isotope-trapping experiments pioneered by I. Rose.36 Substrate trapping experiments with MacroD1 and labeled 2-O-AADPr, gave a $C_F$ of less than 1% (SI Figure S6). Competitive KIEs measure all enzymatic steps from free OAADPr to the first kinetically irreversible step. For MacroD1, reverse commitment is expected to be negligible, as MacroD1, such as most hydrolases, is kinetically irreversible under our experimental conditions.

**Intrinsic KIEs.** The family of intrinsic KIE values associated with labeled 2-O-AADPr and MacroD1 show significant differences to the reported values, and the $k_{cat}$ of the hydrolysis rates were equivalent at pH values from 6.5 to 8.0 (SI Figure S4).

Table 1. Steady-State Parameters of MacroD1

| pH 6.8 | pH 7.3 |
|--------|--------|
| $K_m$ (μM) | 1400 ± 400 | 370 ± 50 |
| $k_{cat}$ (s⁻¹) | 0.72 ± 0.01 | 0.20 ± 0.04 |
| $k_{cat}/K_m$ (s⁻¹ M⁻¹) | $(4.9 ± 0.28) \times 10^2$ | $(5.3 ± 1.2) \times 10^2$ |

*Values represent catalyzed hydrolysis of OAADPr at pH 6.8 and 7.3.

Table 2. Intrinsic and Calculated KIEs for the Hydrolysis of 2-O-AADPr Catalyzed by MacroD1

| heavy isotope | light isotope | KIE type | intrinsic KIE | calculated KIE |
|---------------|--------------|----------|---------------|---------------|
| 1-[^13C]-acetyl | 5-[^1]H-ribose | primary | 1.033 ± 0.006 | 1.034 |
| 1-[^13C]-acetyl | 5-[^1]H-ribose | primary | 1.059 ± 0.012 | 1.064 |
| 2-[^1]H1-acetyl | 5-[^1]H-ribose | $\beta$-secondary | 0.976 ± 0.003 | 0.976 |
| 2-[^1]H1-acetyl | 5-[^14C]-ribose | $\beta$-secondary | 0.971 ± 0.013 | 0.982 |
| 2-[^1]H1-acetyl | 5-[^14C]-ribose | $\beta$-secondary | 1.062 ± 0.008 | 1.060 |
| 2-[^1]H1-ribose | 5-[^14C]-ribose | $\beta$-secondary | 1.169 ± 0.044 | 1.087 |
| 2-[^18O]-ribose | 5-[^14C]-ribose | primary | 1.039 ± 0.004 | 1.037 |

*Atomic position and value of all intrinsic KIEs with standard deviations compared to KIEs for the best fit transition state. Light isotope refers to remote labels reporting on the reaction rate of the light isotopic reactant in competitive radio-isotope experiments. Intrinsic KIE values have been corrected for commitments ($C_C < 0.1\%$) and remote label KIE contributions. The model for the TS included solution-phase (water) reactant states and an in vacuo TS. Calculated KIEs were determined from a reactant state model containing an average of the four variants known in the solution geometry for 2-end and 3-end ribose and $\alpha$- and $\beta$-anomers.
intrinsic KIE values from both the acetyl and ribosyl groups (Table 2). For radioisotope-labeled OAADPr, intrinsic KIEs were obtained by measuring the observed KIE for the remote reporting labels 5-[3H] or 5-[14C] and correcting to give the intrinsic KIEs.

The relationships between 2H and 3H or 13C and 14C intrinsic KIEs are defined by the Swain–Schaad equations (eqs 1 and 2, respectively). The KIE values for MacroD1 agree with this relationship, with the exception at the tritiated 2-H-ribose position.\(^{36,37}\)

\[
\frac{k_{lH}}{k_{lD}} = \left( \frac{k_{rH}}{k_{rD}} \right) \times 1.442
\]  

\[
r = \frac{\ln(k_{12}/k_{13})}{\ln(k_{12}/k_{14})} \quad \text{where} \quad 1.8 \geq r \leq 2.0
\]

The primary 1-[13C/14C]-acetyl KIEs, of 1.033 and 1.059, respectively, provide information on the TS by reporting on the hybridization change of the acetyl carbonyl carbon from sp\(^2\) toward sp\(^3\) as the nucleophile attacks. Asp\(^{184}\) has been proposed as the base for activation of the water nucleophile. The primary 2-[\(^{18}\)O]-ribose KIE, 1.039, reports on the degree to which the primary KIE values were not significant to the 

\[\frac{\ln(k_{12}/k_{13})}{\ln(k_{12}/k_{14})} \quad \text{where} \quad 1.8 \geq r \leq 2.0\]

The TS to match the intrinsic KIEs was found by iterating the bond lengths for both the forming and breaking C–O bonds as well as exploring conformations of the ribose ring pucker and anomeric conformations. All calculated TS structures were optimized to local energy minima. Theoretical KIE values were calculated for each optimized structure in ISOEFF98\(^{42}\) for comparison to the intrinsic KIEs. The breaking and forming bond lengths and the distance between the ribose and aspartate mimic were the sole constraints imposed to generate the TS model. All other parameters were unconstrained. The TS that best matched the intrinsic KIEs was subject to additional analysis using polarizable continuum models (PCM) with dielectric constants of water or acetone as solvents. Atomic coordinate data for the reactant and transition-state optimizations can be found in the Supporting Information (Figure S7).

**Generating the Reactant State.** The two-state nature of TS analysis requires an accurate reactant state. The reactant state of OAADPr is complicated by the near-equal distributions of the 2-endo and 3-endo ribose puckerers in both \(\alpha\)- and \(\beta\)-anomeric positions. Thus, four reactant state optimized geometries were used to generate four sets of KIE values for each transition-state. The average of the reactant states provided an unbiased representation of the actual reactant state structures and these calculated values were averaged to provide the final KIE values (Table 2). This process was needed to match the geometry-dependent \(\beta\)-secondary KIE values. However, primary KIE values were not significantly affected by ribose conformation in the reactant state.

Reactant state analysis also considered the effects of 2-O and 3-O-acetate chemical equilibrium. Transesterification equilibration on reactant states from 3-O-AADPr were evaluated through QM models and KIE calculations. The modeled KIE values from a 3-O-AADPr reactant state do not agree at the 2-\[^{18}O\] ribose or 1-[\(^{13}\)C] acetyl positions. The calculations establish that equilibration of the 3-OH to the reactive 2-O-AADPr species does not contribute significantly to the observed KIE values.

**Properties of the Transition State.** The computed TS structure provided a good match of calculated and intrinsic KIEs at four isotopically substituted positions (Table 2). The TS included significant bond order (\(R\)) to the attacking nucleophile (\(R_{C_{\alpha}-O} = 0.264\)) and departing acetate (\(R_{C_{\alpha}-O} = 0.58\)).
The mechanism for MacroD1 catalysis in a stepwise reaction with formation of a tetrahedral intermediate as the highest barrier (TS1, Scheme 1B) was eliminated. The calculated 2-[18O]-ribose KIEs for any chemically reasonable TSs for diol formation were 0.997–1.013, well outside the experimental error of the intrinsic KIE value of 1.039. Small 2-[18O]-ribose KIEs are a consequence of little change in bond order to the oxygen from reactant to transition-state, off the path to intermediate formation (Scheme 1).

The best fit of the intrinsic 2-[2H/3H]-ribose KIE required a TS with a 2-endo ribose pucker, whereas the 3-endo TS did not match well at either secondary hydrogen KIE position. The 2-endo ribosyl geometry is similar to that in crystal structures obtained for ADPr in macrodomains with a active site residue homology to human MacroD1, also supporting the TS analysis.

**Consideration of Stepwise TSs.** The mechanism for MacroD1 catalysis in a stepwise reaction with formation of a tetrahedral intermediate as the highest barrier (TS1, Scheme 1B) was eliminated. The calculated 2-[18O]-ribose KIEs for any chemically reasonable TSs for diol formation were 0.997–1.013, well outside the experimental error of the intrinsic KIE value of 1.039. Small 2-[18O]-ribose KIEs are a consequence of little change in bond order to the oxygen from reactant to intermediate.

The TS2 mechanism predicted KIE values for 1-[13C]-acetyl from 1.048 to 1.064 and 2-[18O]-ribose values from 1.054 to 1.057, well outside the experimental errors of intrinsic KIE values (1.034 and 1.039, respectively) for both positions (Table 2). The relatively large KIEs for TS2 result from large bond order changes to both ribosyl oxygen and acetyl carbon as the ester bond breaks at TS2. Thus, neither TS1 nor TS2 along a reaction coordinate to or from a diol intermediate agreed with the intrinsic KIE values.

**MacroD1 Elements in TS Structure.** Transition state analysis to match the intrinsic KIEs required an Asn174 mimic or second water molecule to be included in the QM calculations. These interactions mediate a shift of electron density at the carbonyl oxygen at the TS (Figure 3). The natural negative charge on the carboxyl oxygen increased from −0.598 to −0.630 between the reactant and the TS. The main function of Asn174 is to coordinate the water molecule for attack on the acetyl C-1 and to stabilize the TS. Catalytic roles for Asp184 and Asn174 have been previously reported in mutational studies where a 93% loss of activity in OAADPr hydrolysis occurs with alanine at these positions.

**Electrostatic Potential Map.** Wave function analysis provides an electrostatic potential surface (ESPS) map for the TS of MacroD1 (Figure 3). The ESPS map also provides information for the design of TS analogues. This information has been useful for inhibitor design in other systems. A MacroD1 inhibitor would be useful for dissecting the biological functions of OAADPr and related proteins. MacroD1 and related macrodomains also hydrolyze mono-ADPribosylated (MARylated) proteins. These include the automodified mono-ADP-ribose transferase ARTD10 and glycogen synthase kinase 3β (GSK3β). Here, we selected OAADPr as the substrate because of the accessibility of isotopically substituted reactants and because of the link to SirTuin pathways.

**Macrodomain Mechanisms.** Mechanisms of MacroD1 ester hydrolysis are debated. Denu and co-workers proposed a mechanism in which the ester bond is broken in a nucleophilic attack assisted by Asp184 and Asn174 (Asp182 and Asn172 in MacroD2). Rosenthal and colleagues examined macrodomain mechanisms through molecular dynamics models and mutagenesis of MacroD2, altering its activity toward ADPribosylated-GSK3β and ARTD10. Their studies, extrapolated to MacroD1 and OAADPr, showed a drastic decrease in activity when the proposed catalytic amino acids Asp182 and His108 were mutated to alanine. However, the mutants retained a small fraction of the wild-type activity suggesting participation of other residues in the active site. A molecular dynamics model of enzyme activity based on a MacroD1 crystal structure (PDB 2 × 47) agreed with the concerted mechanism supported by our TS structure. This mechanism proposed Asp184 as the general base to deprotonate the nucleophilic water.
MacroD2-ADPr crystal structure (PDB 4IQY), the conformation of the distal ribose could accommodate a 1-O-ester linkage to a MARylated protein substrate, and this 1-hydroxyl is within the van der Waals radius of a water molecule situated between two glycine-rich loops and is coordinated by the ribose α-phosphate. In molecular dynamics simulations, the ADPr α-phosphate was proposed as a base to deprotonate water for attack directly onto the ribose C1 atom in a substrate-assisted, catalytic mechanism. Although OAADPr exists predominately in the 2- or 3-conformation, it is possible for the acetyl group to transesterify to the C1 position.

**Tests of the C1 Mechanism.** A test of the C1-linked mechanism by MacroD2 was hydrolysis of ARTD10-MARylated substrate in H18O2. The assay showed incorporation into ADPr; however, a control experiment of ADPr with H18O2 also showed a significant, albeit decreased, nonenzymatic incorporation. Thus, oxygen exchange at 1-O-ribose during the macrodomain catalyzed reaction is not a rigorous consequence of the proposed 1-O-hydrolysis mechanism.

The intrinsic KIE values from OAADPr hydrolysis by MacroD1 can also be used to test the 1-hydrolysis mechanism. Transition states for the C1 hydrolysis mechanism were generated, and the KIEs calculated (SI Figure S9). The labeled atoms in the 2-ribosyl position are more distant from the reaction’s chemical center in a C1-mechanism and give KIEs near-unity. These computational data do not support a C1 hydrolysis mechanism.

We also tested the mechanism experimentally by running the MacroD1 assay in 5% methanol. Methanol incorporation into ADPr would be expected to occur if the MacroD1 mechanism operated through a C1-ribocation, as proposed in the 1-O-ester hydrolysis mechanism. This experiment is related to the H18O2 incorporation experiment, but with methanol as the alternative nucleophile. Methanol reacts with carbocations 24-fold faster than water based on the Mayr-Patz equation. With a 20:1 ratio of water/methanol, a 1:1.2 ratio of ADPr/methoxy-ADPr would be expected as product, assuming methanol has access to the catalytic site. Mass spectrometric analysis of the reaction products detected no significant methoxy-ADPr in product or in controls lacking the MacroD1 enzyme (Figure 4). Analysis by HPLC confirmed that >50% of OAADPr was converted to products under these conditions. Thus, experimental and computational analyses make the 1-O-ester mechanism unlikely for MacroD1.

**Test of Phosphate Assistance.** Catalysis through an α-phosphate substrate-assisted mechanism requires the phosphate to coordinate water. Chelation of phosphate with high Mg2+ ion concentration in the MacroD1 reaction would prevent the ADPr α-phosphate from activating a water molecule. At a concentration of 10 mM MgCl2 and 1 μM MacroD1, there was a 6% increase in MacroD1 activity (SI Figure S8). Intrinsic KIEs and mechanism-based studies are consistent with a concerted 2-ester hydrolysis mechanism. However, we studied MacroD1’s activity toward OAADPr, and are extrapolating some to results from MacroD1, MacroD2, and C6orf130 with MARylated protein substrates. It is possible that
other macrodomain enzymes may operate by distinct chemical mechanisms.

Conclusion. Macrodomains are candidates for erasers of the mono-ADP-ribosylation of proteins and regulators of cellular OAADPr. Here, we investigate the MacroD1 TS based on intrinsic KIE values. The TS for MacroD1 catalyzed OAADPr ester hydrolysis is an early transition-state for the concerted hydrolysis of the acetyl ester by an activated water molecule. Analysis of KIEs combined with chemical experiments establish the transition state and the likely mechanism of MacroD1. Concerted ester hydrolysis catalyzed by an aspartate-activated water is the most likely mechanism of action. This mechanism may also apply to MARylated protein substrates. The electrostatic potential map of the TS may provide information for design of analogues matching features of the TS.

**METHODS**

**Materials.** 1-[^14]C-Acetyl and 2-[^3H]-acetyl acetic acid as well as 6-[^14]C and 6-[^3H] D-glucose were purchased from American Radiolabeled Chemicals Inc. or Moravek Biochemicals. 2-[2H]-D-ribose, 1-[13C]-acetyl, and 2-[2H3]-acetyl acetic acids were obtained from Cambridge Isotope Laboratories. All other reagents were purchased in the highest purity from Fisher Scientific, Sigma-Aldrich, or other industrial sources and used without further purification.

**Expression and Purification of MacroD1.** cDNA containing the sequence of human MacroD1 (BC003188.1) was obtained (Origene) and used for expression of a 6xHis-MacroD1 protein in *E. coli* as described in the Supporting Information.

**Synthesis of OAADPr.** A one-step enzymatic reaction was used to convert NAD*+ into 2,3-O-AADPr. The residue Glu179 from NAD-glycosylhydrolase of *Aplysia californica* is crucial for catalysis and the E179G mutation prevents NAD*+ hydrolysis.45 NAD*+ (50 mM) was added to 1 M sodium acetate at pH 5.5. Mutant E179G NAD*-glycosylhydrolase (25 μM) was added to the solution and reacted overnight at 25 °C to quantitatively provide 1-[14C]-acetyl, 2-[2H]-acetyl, and 2-[3H]-OAADPr by HPLC purification, lyophilized to dryness, and stored at −80 °C. A ThermoFisher Orbitrap Velos mass spectrometer was used to precisely determine sample isotope ratios. Samples were dissolved in 30–50 μL of solvent (6:12:1, acetonitrile/water/acidic acid), centrifuged at 15 000 rpm for 5 min, and directly injected into the spectrometer at a rate of 4–6 μL min−1. Sample data was collected over 10 min to obtain the integrated peak area for both labeled and unlabeled OAADPr peaks. This information allowed the determination of observed KIE, as shown in eq 3, where *f* is the fractional conversion of product over initial substrate and *r* and *r′* are the ratios of detected peak intensities for the unreacted and partially reacted samples respectively, corrected for natural isotope abundance.49 Six individual experiments were completed for each isotopically substituted position.

**Radiolabel Determination of KIEs.** Radioisotopically labeled OAADPrs were used to determine KIEs by the competitive method. Reactions containing 100 mM sodium phosphate at pH 6.8, 250 μM of isotopically labeled OAADPr (heavy), 250 μM of unlabeled OAADPr (light), and 50 μM of MacroD1 and allowed to react at 25 °C for 20 min to achieve approximately 50% conversion to ADPr. OAADPr was isolated by HPLC purification, lyophilized to dryness, and stored at −80 °C. A PerkinElmer Tritos 2910 TR mass spectrometer was used to precisely provide the observed KIE extrapolated back to 0%.

**Figure 4.** C1-methoxy-ADPriboside detection. (A) The red arrow indicates where the C1-methoxy-ADPriboside species would appear as a methanolysis product ([M–H]) m/z = 572.09, if the mechanism proceeds through a ribocation ion mechanism. (B) Expected peak height of methoxy-ADPriboside is represented in red based on observed ADPrib peak response (m/z = 558.06).

**Synthesis of Isotopically Labeled OAADPrs.** 1-[14C]-Acetyl, 1-[14C]-acetate, 2-[2H]-acetate, and 2-[3H]-OAADPr were synthesized from NAD*+ and the corresponding acetic acid or sodium acetate with E179G NAD*-glycosylhydrolase. All syntheses contained 1 M MES Buffer pH 5.5, 100 mM NAD*+, 10 μM NAD*+-glycosylhydrolase, and 70 mM of the labeled acetic acid or acetate. For radioactive OAADPrs, 0.1–0.2 μCi of labeled acetate was used in each synthesis along with carrier.

5-[^3H] and 5-[^14C] OAADPrs were synthesized from commercially available isotopically labeled glucose and 2-[^3H]-OAADPr from available 2-[^3H] labeled ribose as described in the Supporting Information and shown in Figure S2.

2-[^3H]-OAADPr was synthesized from 2-[^3H]-nicotinamide mononucleotide (NMN) whose synthesis has been previously described.47 Labeled NMN was converted to labeled NAD*+ and then into 2-[^3H]-OAADPr using the protocol described above.

Labeled products were purified by HPLC and lyophilized to dryness to afford a white, fluffy solid. 1-[^14C]-acetyl, 2-[^3H]-acetyl, and 2-[^3H]-OAADPr were confirmed by mass spectrometry analysis (SI Figure S1), while radioactive 1-[[^14C]]-acetyl, 2-[[^3H]]-acetyl, 5-[^3H] ribose, and 5-[^14C]-ribos OAADPr were confirmed by HPLC coelution with unlabeled OAADPr.

2-[^18O]-OAADPr Synthesis. 2-[[^18O]]-Uridine was synthesized as the precursor of 2-[^18O]-OAADPr and then converted to 2-[^18O]-OAADPr as described in the Supporting Information.48 The final product was confirmed by mass spectrometry (SI Figure S1).

**Mass Spectrometry Determination of KIEs.** The kinetic isotope effects were determined using the competitive method in which isotope ratios of the OAADPr substrate were measured by mass spectrometry before and after depletion by the MacroD1 catalyzed reaction. Reactions contained 100 mM of sodium phosphate pH 6.8, 250 μM of isotopically labeled OAADPr (heavy), 250 μM of unlabeled OAADPr (light), and 30 μM of MacroD1 and allowed to react at 25 °C for 20 min to achieve approximately 50% conversion to ADPr.

OAADPr was isolated by HPLC purification, lyophilized to dryness, and stored at −80 °C. A ThermoFisher Orbitrap Velos mass spectrometer was used to precisely determine sample isotope ratios. Samples were dissolved in 30–50 μL of solvent (6:12:1, acetonitrile/water/acidic acid), centrifuged at 15 000 rpm for 5 min, and directly injected into the spectrometer at a rate of 4–6 μL min−1. Sample data was collected over 10 min to obtain the integrated peak area for both labeled and unlabeled OAADPr peaks. This information allowed the determination of observed KIE, as shown in eq 3, where *f* is the fractional conversion of product over initial substrate and *r* and *r′* are the ratios of detected peak intensities for the unreacted and partially reacted samples respectively, corrected for natural isotope abundance.49 Six individual experiments were completed for each isotopically substituted position.

\[
\text{KIE}_{\text{obs}} = \frac{\ln\left(1 - f \left(1 + r\right) / \left(1 + r′\right)\right)}{\ln\left(1 - f \left(1 + r\right) / \left(1 + r′\right)\right)}
\]

(3)

**14C**-Only Control, as previously described.50 Once the corrected isotope ratios of the OAADPr substrate were measured by mass spectrometry analysis (SI Figure S2), while radioactive 1-[^14C]-acetyl, 2-[^3H]-acetyl, 5-[^3H]-OAADPr were synthesized from NAD*+ and the corresponding acetic acid or sodium acetate, and used for expression of a 6xHis-MacroD1 protein in *E. coli*.
reaction. Experiments were performed a minimum of 12 times over 4 independent experiments at each atomic position.

\[
KIE = \ln(1 - f)/\ln(1 - (f_0/r_0))
\]  

(4)

Computational Methods. The TS of OAADPr ester hydrolysis was determined by Gaussian 09 quantum mechanics optimizations using density functional theory calculations with m062x in a 6-31g(d,p) basis set. The reactant state of reactants was first determined and optimized at a global energy minimum with zero imaginary frequencies present. The transition state was determined by iterating the distances of the breaking C–O ester bond and the forming C–O nucleophile bond by 0.2 and then 0.05 Å and comparing intrinsic, experimental, and computational KIEs as determined by frequency data in ISOEFF98. KIE values are calculated from vibrational frequency differences between both the transition and reactant states. The TS was found when sets of KIE values matched, and the resulting structure contained only one imaginary frequency for ester hydrolysis.

ASSOCIATED CONTENT

Supporting Information

This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes
The authors declare no competing financial interest.

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