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A Covalent Intermediate in CD38 Is Responsible for ADP-Ribosylation and Cyclization Reactions

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Abstract: Human CD38 is an ectoenzyme expressed on the surface of B-cells that makes cyclic-ADP-ribose (cADPR) and ADP-ribose from NAD^+ (nicotinamide diphosphate ribose, oxidized form). The compound cADPR is a potent second messenger for calcium release inside cells. Nicotinamide guanine dinucleotide (NGD^+) is also cyclized by CD38 to form cGDPR (cyclic guanosine diphosphate ribose) and hydrolyzed to form GDPR (guanosine diphosphate ribose). Kinetic isotope effect studies in the presence of 20 mM nicotinamide gave 1′-^{3}H and 1′-^{14}C isotope effects of 1.02 ± 0.01 and 1.00 ± 0.01, respectively, for the cyclization reaction and 1.23 ± 0.01 and 1.02 ± 0.01, respectively, for the hydrolysis reaction. These values support a covalent intermediate. The existence of a covalent intermediate was established by reaction of *ara*-F-NMN^+ (arabinosyl-2′-fluoro-2′-deoxynicotinamide mononucleotide) with the enzyme. This compound reacted to release 1 mol of nicotinamide/mole of CD38 monomer and to form an inactive covalent intermediate. Reaction with excess nicotinamide rescued catalytic activity with an apparent K_m of 17 ± 5 mM and a V_{max} of 0.023 ± 0.003 s^{-1}. Proof of covalent labeling of the enzyme by this inhibitor was obtained by MS analysis. Treatment of CD38 with *ara*-F-NMN^+ increased mass by 215 amu, consistent with formation of CD38-fluoro-sugar monophosphate. Tryptic digestion in urea, phosphatase treatment, and purification of peptides in combination with MALDI-PSD permitted identification of Glu226 as the amino acid nucleophile. This residue is highly conserved across all ADP-ribosyl (adenosine diphosphate ribosyl) cyclases. The covalent intermediate inherent to the catalytic mechanism of human CD38 provides chemical precedent for related NAD^+ glycosyltransferases.

Introduction

Human CD38[1−3] and *Aplysia californica* ADP ribosyl-cyclase[4−7] share 68% homology in primary sequence[8] and synthesize cyclic-ADP-ribose (cADPR) from NAD^+.[9,10] The compound cADPR is formed by intramolecular ADP-ribosylation at the N1 position of the adenine ring,[11] and is a potent agent for Ca^{2+} release from intracellular Ca^{2+} stores.[12−20] An

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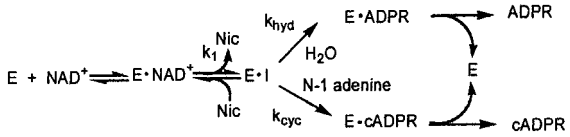
Scheme 1. Catalytic Mechanism for Reaction of CD38 with NAD^+ at pH 7.5 and 37 °C

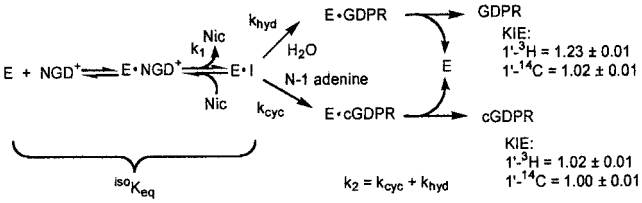


increasing volume of evidence indicates that these enzymes regulate important physiological processes in invertebrates,[12−19] plants,[21] and mammals[1,2,22−25] via their synthesis of cADPR. In mammals, CD38 and cADPR have been implicated in the regulation of cellular processes including insulin release,[22] lymphocyte activation,[2,23] bone homeostasis,[24] and synaptic plasticity.[25] These biological studies have made the cyclases attractive targets for inhibitor design[26,27] and have increased interest in the details of their catalytic mechanism. The catalytic mechanism of the ADP-ribosyl cyclases and CD38 are thought to be similar,[28−32] proceeding through double displacement mechanisms where NAD^+ forms an enzyme-bound ADP-ribosyl electrophile with departure of nicotinamide. Subsequent reaction of this intermediate with nucleophiles, including N1 of adenine, H_2O, or the freely exchangeable nicotinamide, completes the chemical steps leading to formation of products or reformation of substrate (Scheme 1). The atomic structure of this intermediate has not been determined, although it has been proposed to be a noncovalent enzyme-stabilized oxo-carbenium ion.[33−35] The alternative species is a covalent intermediate, attached to an active site residue, possibly a Glu or Asp.[31,35]

Results and Discussion

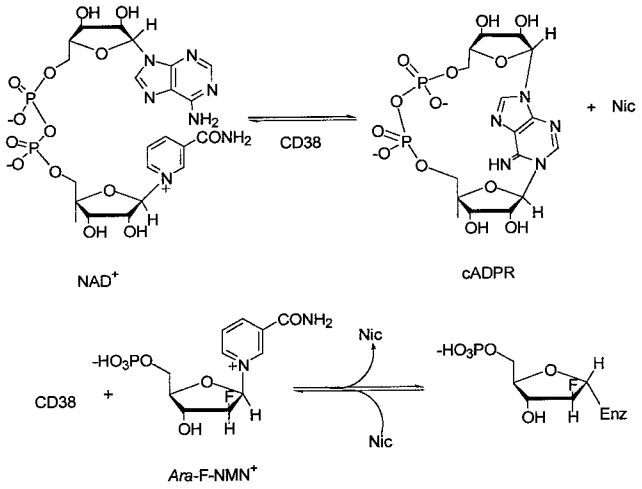
Our previous mechanistic study of CD38 included measurement of the microscopic rate constants for the reaction of the substrate NGD^+ (the guanine analogue of NAD^+) to hydrolytic and cyclized products.[28] The nicotinamide bond-breaking step is fast relative to the forward reaction and product release ($k_1 > k_{hyd} + k_{cyc}$ Scheme 1). The intermediate is relatively long-lived in the catalytic cycle ($t_{1/2} = 6$ ms). This longevity explains an $NGD^+ \leftrightarrow$ nicotinamide base-exchange reaction at elevated nicotinamide concentration.[28] The substrate NGD^+ reforms readily from the intermediate and forward reaction progress in the presence of 20 mM nicotinamide is diminished (reverse/forward = 35:1, $K_m = 7$ mM).[28] Under these conditions, near equilibration of the intermediate with free substrate and free nicotinamide is achieved during the steady state (Scheme 2). Kinetic isotope effects (KIEs, 1′-^{3}H and 1′-^{14}C) were obtained by the competitive method[36] under these conditions for the reaction of NGD^+ to both GDPR and cGDPR. The method reports the KIE on k_{cat}/K_m, encompassing all catalytic steps from free substrate to the first irreversible step.[36] Irreversible steps are the hydrolysis or cyclization of the intermediate. The 1′-^{3}H and 1′-^{14}C isotope effects obtained at 20 mM nicotinamide are small for cyclization (Scheme 2). These KIEs indicate that k_{cyc} for NGD^+ involves rate limiting conformational change ($t_{1/2} = 4-5$ ms) since either nucleophilic or dissociative chemistry as a rate-limiting step would cause larger KIEs.[37] Motion that brings the purine ring to the appropriate attack geometry is consistent with the KIEs. The large KIEs for NGD^+ hydrolysis establish that chemistry is rate-limiting ($t_{1/2} = 1-2$ ms).[28] In consequence, entry of a water molecule to the site appropriate

Scheme 2. Catalytic Mechanism for Reaction of CD38 with NGD^+[a]



[a] The KIEs were determined in the presence of 20 mM nicotinamide. The k_{hyd}/k_{cyc} at pH 7.5 and 37 °C is 0.38.[28]

Scheme 3. Reaction Catalyzed by CD38 for Formation of cADPR and the Reaction Mechanism of *ara*-F-NMN^+ Inhibition of CD38 and Rescue by Nicotinamide



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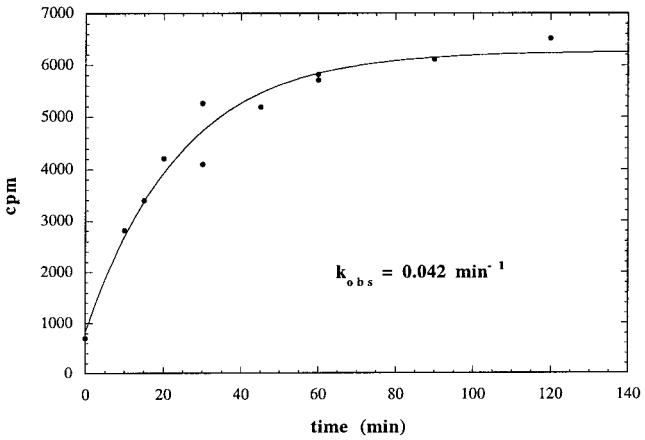

Figure 1. Plot of released [carbonyl-^{14}C]nicotinamide as a function of time where 228 nM CD38 is incubated with 1.8 μM [carbonyl-^{14}C]-*ara*-F-NMN$^+$. Free nicotinamide is isolated by HPLC separation and the radioactivity counted. Counts of nicotinamide were fit according to $N(1 - e^{(-k_{obs}t)}) + C = N(t)$ where $N(t)$ is counts measured in cpm at a given time, C is counts at time = 0, and k_{obs} is the rate constant.

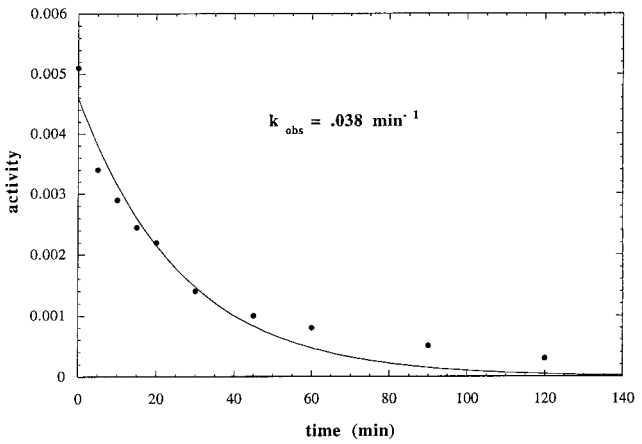

Figure 2. Plot of residual enzymatic activity as a function of time when 228 nM CD38 is incubated with 1.8 μM [carbonyl-^{14}C]*ara*-F-NMN$^+$. Time points reflect residual catalytic activity that converts NGD$^+$ to cGDPR. The solid curve is best fit to the equation $A(t) = Ae^{(-k_{obs}t)}$ for decay of activity, where A is 100% activity, $A(t)$ is activity measured at a given time t, and k_{obs} is the rate constant.

for the hydrolytic reaction is fast ($t_{1/2} \ll 1-2$ ms) since KIEs would be diminished if water entry is partially rate-limiting. The observed KIEs ($KIE_{observed}$) are not intrinsic to the irreversible steps (KIE_{irrev}), but are the products of the isotope effect on the equilibrium of the intermediate with solution substrate, i.e., $KIE_{observed} = {}^{iso}K_{eq} \cdot KIE_{irrev}$ (Scheme 2).[36] Because the cyclization is rate-limited by a nonchemical process, KIE_{irrev} for cyclization is expected to be near unity, thus ${}^{iso}K_{eq}$ must be near unity as well. Calculations for a proposed oxo-carbenium ion intermediate (E•I in Schemes 1 and 2) predict larger values for ${}^{iso}K_{eq}$, approximately 1.02 for 1′-^{14}C and 1.20 for 1′-^{3}H.[37,38] The constraint on ${}^{iso}K_{eq}$ for this enzyme implies that a covalent intermediate exists at the active site during catalysis.

Support for a covalent intermediate at the active site of CD38 was obtained by the mechanism of inactivation of CD38 by *ara*-F-NMN$^+$,[39] a truncated relative of *ara*-F-NAD$^+$ (Scheme 3).[39] *ara*-F-NAD$^+$ is a potent inhibitor of CD38 (K_i = 169 nM), and has been reported to function by a slow-binding mechanism.[30,40] Under the conditions of our studies, *ara*-F-NMN$^+$ was shown to inhibit CD38 with a K_i = of 61 nM (k_{on}= 410 M^{-1} s^{-1}, $k_{off} = 2.5 \times 10^{-5}$ s^{-1} at 310 K). Incubation of 200 μM *ara*-F-NMN$^+$ with 1 μM CD38 in the presence of 10 mM [carbonyl-^{14}C]nicotinamide (50 μCi/μmol) resulted in the complete exchange of radiolabel into the inhibitor over a 24 h period. This result establishes that CD38 catalyzes the exchange reaction with the *ara*-F-NMN$^+$ molecule as the substrate. The radiolabeled [carbonyl-^{14}C]*ara*-F-NMN$^+$ was used to demonstrate that decomposition of the nicotinamide−sugar bond by the enzyme leads directly to inhibition. Treatment of CD38 with [carbonyl-^{14}C]*ara*-F-NMN$^+$ releases [^{14}C]nicotinamide establishing covalent reaction with *ara*-F-NMN$^+$. The rate of [^{14}C]-nicotinamide release could be fit to a single-exponential curve ($k_{obs} = 0.042 \pm 0.006$ min^{-1}, Figure 1) that reached completion at 1:1 mol nicotinamide/mol CD38 subunit. Aliquots of this inactivation mixture were assayed for activity and activity declined in an exponential fashion with a rate of k_{obs} of 0.038 $\pm$ 0.004 min^{-1} (Figure 2). Inactivation of CD38 by *ara*-F-NMN$^+$ is therefore due to formation of an intermediate with

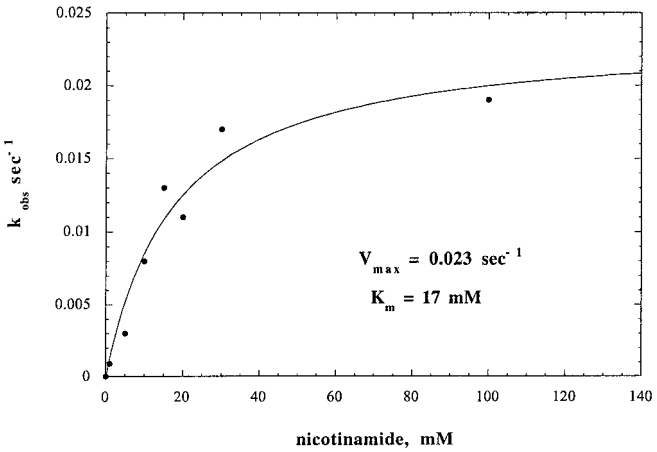

Figure 3. Rate of rescue of catalytic activity of *ara*-F-NMN$^+$ inhibited CD38 as a function of added nicotinamide. The solid line is the best fit of points to the Michaelis−Menten. equation. The value of V_{max} is 0.023 ± 0.003 s^{-1} and the apparent K_m for nicotinamide is 17 ± 5 mM.

release of nicotinamide. Rescue of CD38 activity occurs with added nicotinamide. The maximum rate of rescue is 0.023 ± 0.003 s^{-1} with an apparent K_m for nicotinamide of 17 ± 5 mM (Figure 3). Based on the exchange reaction, rescue occurs through the reverse reaction to reform *ara*-F-NMN$^+$. A chemically similar rescue of catalytic activity after covalent reaction of deoxy-nucleoside transferase with *ara*-F-adenosine is effected by additions of adenine.[41] Adenine rescue exhibits a saturable rate with an appropriate K_m for adenine.[41] Mechanism-based inhibition of deoxy-nucleoside transferase and glycosidases by 2′-F-substituted compounds along with identification of a stable covalent moiety has been used to support the existence of a covalent intermediate along the reaction coordinate of the catalytic mechanism.[42−45]

Proof of covalent modification of CD38 by *ara*-F-NMN$^+$ was obtained by MS analysis. The MW of underivatized CD38

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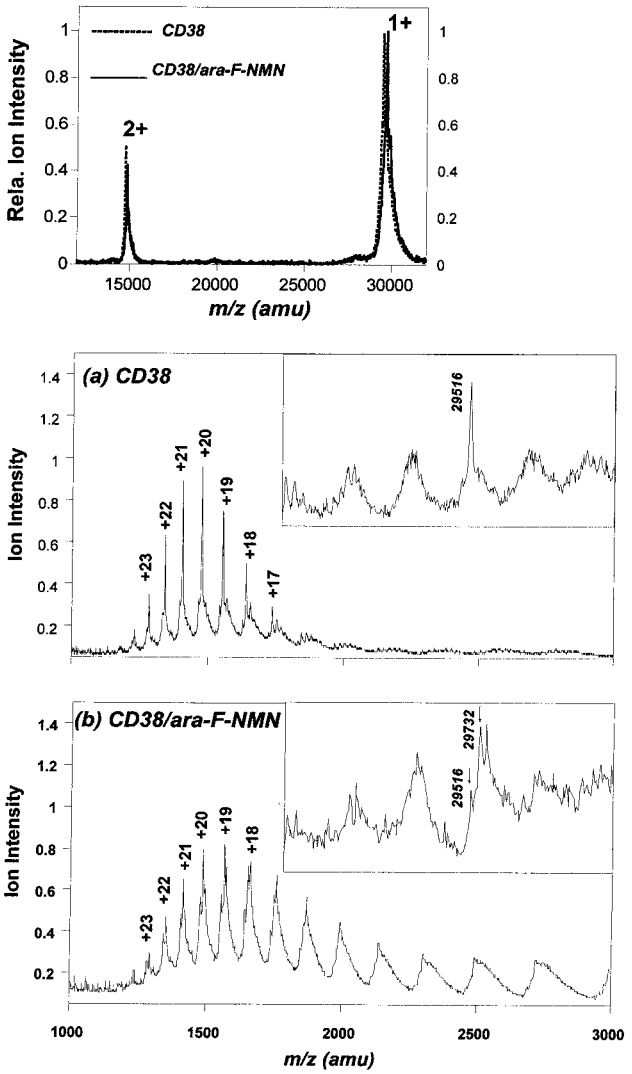

Figure 4. Upper panel: Comparison of MALDI-MS of CD38 before and after treatment with *ara*-F-NMN$^+$. Charge +1 species is the monomer. Lower panel: Electrospray mass spectroscopy of CD38 not treated (a) and treated (b) with *ara*-F-NMN$^+$. The inset represents the mass of the species determined by deconvolution of the raw signal shown in the main figure. The mass difference of 216 is the mass difference expected from covalent attachment of the 2-fluoro-5-phosphoarabinosyl moiety to the monomer.

monomer was determined to be 29516 (Figure 4). The major species in the derivatized sample has a mass of 29732 consistent with addition of the 2-fluoro-5-phosphoarabinosyl moiety (215 amu) to the protein. The MS study shows that most monomers are labeled, consistent with the results of the radiochemical titration. The amino acid that undergoes covalent modification was determined by subjecting the derivatized protein to trypsin digestion in 5 M urea followed by HPLC purification of peptide fragments. The labeled peptide was treated with alkaline phosphatase to remove the 5′-phosphate, repurified, and then examined by MALDI-PSD. A mass analysis demonstrates the mass of peptide fragments at different places along the amino acid chain (Figure 5). Fragmentation analysis identifies a Glu (E226) as the modified amino acid based on the presence of Glu in all fragments containing an extra mass of 134 (the mass addition resulting from the 2-fluoroarabinosyl modification). Homology studies reveal E226 to be highly conserved across all known ADP-ribosyl cyclase sequences,[31] and has been mutagenized resulting in abolition of catalytic activity (Figure 6).[31] It has been localized at the active site of ADP-ribosyl

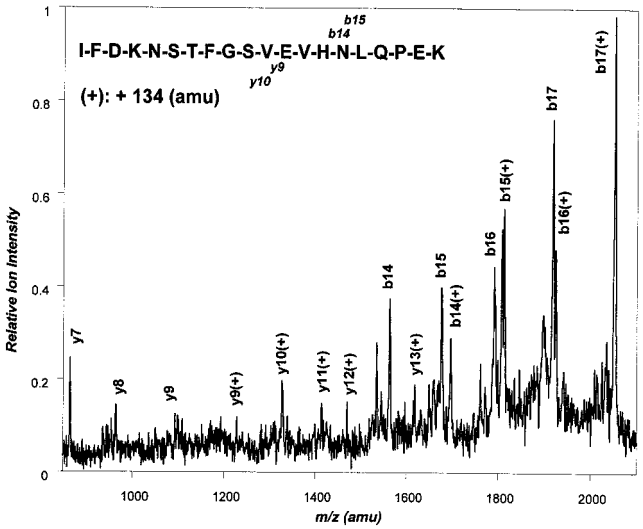

Figure 5. Mass spectrum of the fragmentation in the MALDI-PSD mode of the tryptic peptide derivatized with the 2-fluoroarabinosyl group. The fragments y^n represent charged species whose amino acid residues increase in length starting from the C-terminal end (K). The mass 134 represents the mass increase due to the presence of the 2-F-arabinose moiety on the peptide fragment.

cyclase by X-ray crystallography and is proposed to be an important catalytic residue.[6,31,46] The present result clearly identifies Glu226 as the attachment point of the intermediate with the enzyme, via a ribosyl-ester type bond.

Conclusion

Evidence from KIE data and from the covalent modification of CD38 by *ara*-F-NMN$^+$ establishes the mechanism of CD38 via a covalent intermediate. Covalency stabilizes an ADP-ribosyl moiety on the enzyme that is formed by displacement of nicotinamide by an enzyme glutamate (E226). These results are inconsistent with a stabilized oxo-carbenium ion as the active site electrophile. However, the values of KIE are consistent with an oxo-carbenium transition state for hydrolysis of the covalent intermediate. ADP-ribosyl cyclases and NAD$^+$ glycohydrolases from a variety of species have high homology, catalyze similar reactions, and conserve this active site residue. It is likely that the ADP-ribosyl cyclases and NAD$^+$ glycohydrolases proceed through covalent intermediates to achieve their respective chemistries.

Experimental Section

General Experimental. Solvents and compounds were obtained in the highest purity available from commercial vendors. The inhibitor *ara*-F-NMN$^+$ was made as previously described.[39] Human recombinant CD38 was made and purified according to published procedures.[46] HPLC purifications used a Waters 600/626 pump fitted with a 996 PDA detector controlled by computer using the Millenium[32] software package. UV/vis measurements were obtained on either a HP8453 diode array instrument or a Varian Cary100Bio with temperature control. All enzymatic measurements were done at 37 °C. Radioactivity was counted on a Wallac 1414 scintillation counter.

Kinetic Isotope Effects Measurements. Radiolabeled substrates [1′-^{3}H]NGD$^+$, [1′-^{14}C]NGD$^+$, [5′-^{3}H]NGD$^+$, and [5′-^{14}C]NGD$^+$ were synthesized as previously reported.[28] KIE studies were done by reacting 200 μL of [1′-^{3}H]NGD$^+$ and [5′-^{14}C]NGD$^+$ or [1′-^{14}C]NGD$^+$ and [5′-^{3}H]NGD$^+$ in the presence of CD38 in 50 mM potassium phosphate at pH 7.0 and 20 mM nicotinamide. The reactions were permitted to reach

(46) Munshi, C. B.; Fryxell, K. B.; Lee, H. B.; Branton, W. D. *Methods Enzymol.* **1997**, *280*, 318−330. E226 is the glutamate residue in the full-length CD38 sequence. Recombinant CD38 used in this study lacks the N-terminal membrane spanning domain, which makes the derivatized residue E189 of the recombinant sequence.

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        143                                                                 212
akADPRC ITLEDTLPGY MLNSLVWCGQ RDKPGFNQKV CPDF.KDCPV QARESFWGTA SSSYAHSAEG DVTYMVDGSN
acADPRC ITLEDTLPGY MLNSLVWCGQ RANPGFNEKV CPDF.KTCPV QARESFWGMA SSSYAHSAEG EVTYMVDGSN
ratBST1 MPLCDVLYGK VGDFLSWCRQ ENASGLDYQS CPT.AEDCEN NAVDAYWKSA SMQYSRDSSG VINVMLNGSE
mouBST1 VALCDVLYGK VGDFLSWCRQ ENASGLDYQS CPT.SEDCEN NAVDSYWKSA SMQYSRDSSG VINVMLNGSE
humBST1 MPLSDVLYGR VADFLSWCRQ KNDSGLDYQS CPT.SEDCEN NPVDSFWKRA SIQYSKDSSG VIHVMLNGSE
mouCD38 FTLEDTLLGY IADDLRWCGD PSTSDMNYVS CPHWSENCPN NPITMFWKVI SQKFAEDACG VVQVMLNGSL
ratCD38 FTLEDTLLGY IADDLRWCGD PSTSDMNYDS CPHWSENCPN NPVAVFWNVI SQKFAEDACG VVQVMLNGSL
humCD38 FTLEDTLLGY LADDLTWCGE FNTSKINYQS CPDWRKDCSN NPVSVFWKTV SRRFAEAACD VVHVMLNGSR

        213           *                                                     280
akADPRC PKVPAYRPDS FFGKYELPNL .TNKVTKVKV IVLHQLGQKI IERCGAGSLL DLEMVVKAKK FGFDCVENPK
acADPRC PKVPAYRPDS FFGKYELPNL .TNKVTRVKV IVLHRLGEKI IEKCGAGSLL DLEKLVKAKH FAFDCVENPR
ratBST1 PK.GAYPTKG FFADFEIPYL QKDKITRIEI WVMHEVGGPH VESCGEGSVK ILEDRLEALG FQHSCINDYP
mouBST1 PK.GAYPTRG FFADFEIPYL QKDKVTRIEI WVMHDVGGPN VESCGEGSVK ILEDRLEALG FQHSCINDYR
humBST1 PT.GAYPIKG FFADYEIPNL QKEKITRIEI WVMHEIGGPN VESCGEGSMK VLEKRLKDMG FQYSCINDYR
mouCD38 RE..PFYKNS TFGSLEVFSL DPNKVHKLQA WVMHDIEGAS SNACSSSSLN ELKMIVQKRN MIFACVDNYR
ratCD38 SE..PFYRNS TFGSVEVFNL DPNKVHKLQA WVMHDIKGTS SNACSSPSIN ELKSIVNKRN MIFACQDNYR
humCD38 SK..IFDKNS TFGSVEVHNL QPEKVQTLEA WVIHGGREDS RDLCQDPTIK ELESIISKRN IQFSCKNIYR
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Figure 6. Homology plot of ADP-ribosyl-cyclases and NAD^+ glycohydrolases. The sequences are akADPRC, *Aplysia kurodai* ADPribosyl-cyclase; acADPRC, *Aplysia californica* ADPribosyl-cyclase; ratBST1, rat bone stromal cell antigen; mouBST1, mouse bone stromal cell antigen; humBST1, human bone stromal cell antigen; mouCD38, mouse CD38; ratCD38, rat CD38; humCD38, human CD38. The residues marked by an asterisk correspond to the conserved glutamate covalently modified by *ara*-F-NMN^+ in the human CD38 protein. The numbers above the sequences correspond to the residue numbers for the full length human CD38 sequence. Recombinant human CD38 has substitutions for N at sites 100, 164, 209 and 219.

20% completion based upon the consumption of total NGD^+. From the reaction mixture 150 μL aliquots were removed and GDPR and cGDPR were separated by HPLC,[28] and fractions were collected and separately counted after addition of scintillation fluid in a 9:1 ratio to aqueous volume. 100% reactions were obtained by allowing CD38 to react with the remaining substrate overnight and then purified cGDPR and GDPR fractions were combined and counted. KIE observed was determined using the equation $KIE_{obs} = (^3H/^{14}C$ at 100% conversion)/($^3H/^{14}C$ at 20% conversion). The reported KIE values are observed values and are not corrected for isotopic depletion.

Exchange of [carbonyl-^{14}C]Nicotinamide into *ara*-F-NMN^+. The compound *ara*-F-NMN^+ was purified as reported,[39] dissolved in 0.5 mL of potassium phosphate, and determined by UV/vis to be at a concentration of 200 μM using 4600 M^{-1} cm^{-1} for the extinction coefficient at 266 nm. CD38 was added to reach a concentration of 1 μM and the total sample was added to 5 μmol of [carbonyl-^{14}C]-nicotinamide possessinga specific activity of 50 μCi/μmol. After 24 h at room temperature the reaction mixture was separated by HPLC using 50 mM ammonium acetate, pH 5.0, as eluant on a Waters C-18 μBondapak column. The peaks for *ara*-F-NMN^+ and for nicotinamide were collected. Specific activity was determined by taking an aliquot of *ara*-F-NMN^+ and counting radioactivity after addition of a scintillation cocktail. Concentration was determined by UV/vis measurement of 266 nm absorbance. Similar measurements were done for eluted nicotinamide. Specific activity incorporated into *ara*-F-NMN^+ was found to be at least 95% of theoretical based upon complete incorporation of radiolabel.

Radiotitration of CD38 with [carbonyl-^{14}C]*ara*-F-NMN^+ and Enzyme Activity Measurements. Samples of 250 μL containing 1.8 μM [carbonyl-^{14}C]*ara*-F-NMN^+ and 228 nM CD38 (1.5 μL CD38 at 38 μM determined by UV/vis using 280 nm measurement and an extinction coefficient of 1.70 mg^{-1} mL) in 50 mM potassium phosphate, pH 7.0, were incubated for times 5, 10, 15, 20, 30, 45, 60, 90, and 120 min at 37 °C. After these times 2 μL was removed and added to a cuvette containing 200 μM NGD^+ in 0.5 mL of 50 mM potassium phosphate, pH 7.0. The residual catalytic activity was determined by monitoring 295 nm absorbance over 5 min. A control was also prepared that did not contain *ara*-F-NMN^+ and it was assayed to determine the 100% catalytic activity. A volume of 10 μL of 5 mM nicotinamide was added to the residual 248 μL and the total sample separated as above by HPLC. The peak containing nicotinamide was collected and radioactivity counted in separate vials each containing 1 mL of eluant and 9 mL of cocktail. Plots of activity were fit to a single-exponential curve versus time using the program Kaleidagraph and the equation $A(t) = Ae^{(-k_{obs}t)}$ for decay of activity, where A is 100% activity, $A(t)$ is activity measured at a given time t, and k_{obs} is the rate constant. Counts of nicotinamide were fit according to $N(1 - e^{(-k_{obs}t)}) + C = N(t)$, where $N(t)$ is counts measured in cpm at a given time, C is counts at time = 0, and k_{obs} is the rate constant.

Rescue of CD38 Activity by Nicotinamide. A solution of CD38 (10 mL of 0.050 μM) inhibited by *ara*-F-NMN^+ was prepared by incubation of the enzyme with 1 μM inhibitor for 6 h at 37 °C in 50 mM potassium phosphate, pH 7.0. Solutions of 200 μM NGD^+ and 0, 2, 10, 20, 30, 40, 60, and 200 mM nicotinamide were prepared in the identical buffer. For each nicotinamide solution pH was adjusted to 7.0 using concentrated NaOH or HCl. In a stopped flow apparatus fitted with two syringes the solutions containing inhibited enzymes and NGD^+/nicotinamide solutions were mixed and fluorescence (360 nm filter) measured as a function of time. The resulting curves were fit using the equation $E(t) = vt + (v - b)(1 - e^{(-kt)})/k + d$, where $E(t)$ is emission at time t, v is the initial rate of emission change, b is the final rate of emission change, k is the observed rate constant for rescue, and d is the initial emission intensity. A plot of k versus concentration of nicotinamide was fit to the Michaelis equation to derive values for V_{max} and K_m.

Mass Spectrum of CD38. A solution of CD38 (50 μM in 10 mM Tris buffer, pH 7.0) was incubated for several hours with 200 μM *ara*-F-NMN^+ and then submitted for MS analysis in either MALDI or electrospray modes. A control lacking inhibitor was similarly incubated and analyzed.

Mass Spectrum of CD38 Peptides. A solution was prepared as above and after 5 h 50 μL was removed and 50 mg of solid urea was added to denature protein. After 15 min 50 μL of trypsin (60 μg) was added in 10 mM Tris/acetic acid buffer. The final pH was adjusted to 6 using pH paper and microliter additions of 100 mM Tris, pH 7.0. After 3 h of room-temperature incubation 10 μL aliquots were injected onto a C-8 column equilibrated with 17 mM ammonium acetate, pH 6.0, in 5% acetonitrile. A gradient of 1 mL/min was run that changed acetonitrile composition to 70% over 60 min. Peaks containing derivatized peptide eluted at 25−32 min. Two peptides were found, one of mass 2720, a 22mer + sugar, the other of mass 2505, a 20mer + sugar, representing different extents of tryptic digestion. The mixture of 2505 and 2720 peaks was digested with alkaline phosphatase overnight at pH 6.0 (1 μL of enzyme, 0.5 units), then repurified by HPLC as above. The 25−32 min fractions were concentrated and analyzed by MALDI-PSD to identify the site of modification. A control similarly treated in all respects but not inhibited did not show evidence for sugar modification.

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