Structural Basis for Formation and Hydrolysis of Calcium Messenger Cyclic ADP-ribose by Human CD38

Qun Liu‡, Irina A. Kriksunov‡, Richard Graeff§, Hon Cheung Lee§¶, Quan Hao‡
‡MacCHESS, Cornell High Energy Synchrotron Source, Cornell University, Ithaca, NY 14853, USA
§Department of Pharmacology, University of Minnesota, Minneapolis, MN 55455, USA
¶Department of Physiology, University of Hong Kong, Hong Kong, China

Corresponding authors: Q.H.: Email: qh22@cornell.edu, Tel.: 607-254-8983, Fax: 607-255-9001
and H.C.L.: Email: leehc@hku.hk, Tel.: 852-2819-9163, Fax: 852-2819-9230

Running title: Mechanism of cADPR formation and hydrolysis by human CD38

Human CD38 is a multifunctional ectoenzyme responsible for catalyzing the conversions from nicotinamide adenine dinucleotide (NAD) to cyclic ADP-ribose (cADPR) and from cADPR to ADP-ribose (ADPR). Both cADPR and ADPR are calcium messengers that can mobilize intracellular stores and activate influx as well. In this study, we determined three crystal structures of human CD38's enzymatic domain complexed with cADPR at 1.5 Å resolution, with its analog, cyclic GDP-ribose (cGDPR) (1.68 Å), and with NGD (2.1 Å), a substrate analog of NAD. The results indicate that the binding of cADPR or cGDPR to the active site induces structural rearrangements in the dipeptide Glu146-Asp147 by as much as 2.7 Å, providing the first direct evidence of a conformational change at the active site during catalysis. In addition, Glu226 is shown to be critical not only in catalysis but also in positioning of cADPR at the catalytic site through strong hydrogen bonding interactions. Structural details obtained from these complexes provide a step-by-step description of the catalytic processes in the synthesis and hydrolysis of cADPR.

INTRODUCTION
Calcium is a universal intracellular signal in regulating cellular functions (1). The ability of cells to use this single metal ion for distinct functions is mainly relied on modulating the generation, localization, and termination of spatial, temporal, and amplitudal characteristics of calcium waves by cell-type-dependent toolkits (2). Intracellular calcium signals are regulated through three structural divergent messengers inositol 1,4,5-trisphosphate (InsP3) (3), cyclic ADP-ribose (cADPR), and nicotinic acid adenine dinucleotide phosphate (NAADP) (4), which mainly elicit mobilization of intracellular calcium stores. Two of these messengers, cADPR and NAADP, are synthesized by CD38, a transmembrane enzyme and a member of ADP-ribosyl cyclase superfamily (5-8). Surprisingly, the same enzyme can also hydrolyze both cADPR and NAADP to their final products ADP-ribose (ADPR) and 2'-ADPR-phosphate (ADPRP), respectively (6, 9). It is intriguing that ADPRP itself, the breakdown product of cADPR, has been shown to be able to gate the influx of calcium as well, and with cADPR synergizing such an action (10, 11). That the same enzyme is involved in the synthesis and hydrolysis of distinct calcium messengers indicates its central role in maintaining the homeostasis of these signals. To understand the multi-functionality of this novel signaling enzyme, it is imperative to obtain structural details at the atomic level.

CD38 cyclizes NAD, a long linear molecule, to a compact cyclic nucleotide, cADPR. The active site of human CD38 has been biochemically and structurally characterized (12, 13). Glu226 is identified as the catalytic residue since its mutation to other residues essentially eliminates all its catalytic activities (12). Ser193 is also important for catalysis as its mutation to alanine also greatly reduces the enzyme's activities (14). Conversion of NAD to cADPR, however, is not the dominant reaction catalyzed by wild-type human CD38. In fact, the large majority of the substrate NAD is hydrolyzed to ADPR (6).
Completely the opposite is observed when NGD, an analog of NAD, is used as substrate. The dominant reaction is now cyclization instead of hydrolysis, producing cyclic GDP-ribose (cGDPR) as the major product (15). Considering the similarity of NGD and NAD, which differ only in the purine rings, it is puzzling why the reactions are so different. Scheme 1 shows two different cyclization reactions catalyzed by human CD38 with NAD and NGD as substrates. It is interesting that, by mutating a conserved active site residue Glu\textsuperscript{146} to alanine, the mutant acquires greatly increased ADP-ribosyl cyclase activity. With NAD as substrate, CD38 E146A mutant can produce three times more cADPR than ADPR, indicative of Glu\textsuperscript{146} role in controlling the cyclization and hydrolysis reactions.

In this study, we employed X-ray crystallography to determine the structures of enzymatic domain of human CD38 complexed with three relevant ligands, cADPR, cGDPR, and NGD. These complexes, together with previously solved NAD and ADPR complexes (14), allow us to provide a step-by-step description of the catalytic processes involved in the synthesis and hydrolysis of cADPR. The results also provide the first evidence of a conformational change at the active site of CD38 during catalysis.

**EXPERIMENTAL PROCEDURES**

*Proteins Expression, Site-directed Mutagenesis, and Crystallization*

E226Q, E226G, and E226D mutants were obtained by site-directed mutagenesis and expressed in a yeast expression system. The *Pichia pastoris* vector pPICzα (purchased from Invitrogen) was used for generating all the mutants. The soluble domain of hCD38, starting with Arginine-45, was cloned into the vector between the EcoRI and NotI restriction sites in the multiple cloning site of the vector. This subcloning method allows for the ligation of the soluble CD38 in-frame with the α-factor secretion signal. Expression is driven by the tightly controlled alcohol oxidase promoter AOX1 which is induced by methanol. The protein is initially expressed as a fusion of the secretion signal peptide which is cleaved off by endogenous proteolytic enzymes, and the CD38 secreted into the expression medium.

Mutations were performed using the QuickChange Site-directed Mutagenesis kit obtained from Stratagene. Complementary primers containing the mutations were used to amplify the entire plasmid using the high-fidelity *PfuTurbo* DNA polymerase. The amplification is linear, and only 20 cycles were used in order to minimize the chances of generating errors during the process. At the end of the amplification process the reaction mixture contained the newly synthesized vector with nicks at the priming site, and the parent (template) vector which is hemi-methylated due to being amplified in an E. coli strain. Using the endonuclease *DpnI*, the methylated vector is digested away leaving behind the newly-synthesized vector containing the mutation of interest which was used to transform super-competent XL1-Blue *E. coli* cells purchased from Stratagene. The positive clones were checked for the presence of the insert, sequenced and used for the transformation of the X-33 yeast cells.

Primers for the mutations are listed below. Changes in the base-pairs are indicated by bold and underlined bases.

**CD38-E226G**

**Top strand primer**

5’

CAGCACTTTTGGGAGTGGGAGTCCATA

ATTTGCAACCAGAGAA 3’

**Bottom strand primer**

5’

TTCTCTGGTTGCAAATTAATGGACTGCCACA

CTCCCAAAAGTGCTG 3’

**CD38-E226Q**

**Top strand primer**

5’

CAGCACTTTTGGGAGTGGCAGTCCATA

ATTTGCAACCAGAGAA 3’

**Bottom strand primer**

5’

TTCTCTGGTTGCAAATTATGGACTGCCACA

CTCCCAAAAGTGCTG 3’

**CD38-E226D**

**Top strand primer**

5’

CAGCACTTTTGGGAGTGGACGTCCATA

ATTTGCAACCAGAGAA 3’

**Bottom strand primer**

5’

TTCTCTGGTTGCAAATTATGGACGTCCACA

CTCCCAAAAGTGCTG 3’
The positive clones containing the mutation were linearized by digesting the vector with SacI, and using the linearized vector to transform electrocompetent X-33 yeast cells. The transformed cells were selected under 100 g/ml zeocin. Colonies were formed between 3 – 4 days posttransfection, and 6 clones from each transfection selected for expression screening by activity assays and SDS PAGE. Previously established purification strategies were followed to get pure proteins (12, 16).

Before crystallization, the samples were concentrated to 8 mg/ml and stored at -80°C. The E226Q mutant was crystallized based on a previously reported recipe (14). E226G and E226D crystals were formed by hanging drop vapor-diffusion technique. For E226G crystals, 1 µl mg/ml sample was mixed with 1 µl reservoir that contains 100 mM MES, 13% PEG400, pH 6.0. For E226D crystals, the optimized crystallization condition was determined by increasing the concentration of PEG 4000 from 13% to 15%, while kept other conditions the same as that for E226G crystals. At room temperature, crystals usable for data collection appeared within one week.

**Preparation of cADPR, cGDPR, and NGD complexes**

NGD and cADPR were purchased from Sigma (Sigma-Aldrich, St. Louis). Cyclic cGDPR sample were synthesized by incubating NGD with Aplysia cyclase followed by HPLC column purification as described before (15). The formation of complexes by co-crystallization was not possible, as the residual hydrolysis activities of the mutants were sufficient to breakdown the ligands during the process. Instead, all complexes were obtained by soaking preformed crystals with solutions containing cADPR, cGDPR, or NGD. The soaking process was performed at 4°C to prevent damage to the crystals, which could happen in minutes at room temperature. Specifically, the cADPR complexes were obtained by incubating E226Q, E226G, or E226D crystals with 48 mM cADPR solution buffered with the crystallization mother liquor for 3-8 min at 4°C; the cGDPR complex was obtained by incubating E226Q crystals with 40 mM cGDPR for 5 min; and the NGD complex was obtained by incubating E226Q crystals with 20 mM NGD for 2 min. These soaked crystals were then quickly transferred and preserved in liquid nitrogen until data acquisition.

**Data Collection, Reduction and Structures Refinements**

Crystallographic data were collected at the Cornell High-Energy Synchrotron Source A1 station with a fixed wavelength of 0.976 Å. Under the cryo-stream protection at 100K, each crystal was rotated 360 degrees with 1° oscillation. Data sets were integrated and scaled by using the program package HKL2000 (17). The apo structure of shCD38 (PDB id 1YH3) was used as the initial model for structure solution by the molecular replacement method implemented in MOLREP (18). The initial model of cADPR was derived from its crystal structure (19). The NGD and cGDPR models were manually built in O (20) based on the electron densities and optimized with the program PRODRG (21). Subsequent crystallographic refinements and solvent addition were done with the program REFMAC (18) and ARP/WARP (22). The crystallographic statistics for data reduction and structure refinements are listed in Table 1.

**RESULTS**

**Overview of Complex Structures**

The structures of the soluble extra-membrane domain of human CD38 (shCD38) complexed with various nucleotides were obtained by soaking substrates cADPR, cGDPR, or NGD with crystals of E226 mutants (E226Q, E226D, or E226G). All these mutants show greatly reduced catalytic abilities compared with that of the wild-type shCD38 (12). However, the residual activities prevented us from obtaining these complexes by co-crystallization, wherein only products were observed. Among these mutants, E226Q is the least inactive one. By incubating pre-formed E226Q crystals with cADPR, cGDPR, or NGD for minutes in a cold room, stable complexes can be obtained. The low temperature reduces the catalytic activity and slows down the diffusion of substrates into the active site, alleviating damages to the crystals.

Like wild-type shCD38, the shCD38 mutants were crystallized in space group P1 with two shCD38 molecules in the crystallographic asymmetric unit in a head-to-tail fashion (14). The structures of the complexes were determined by molecular replacement with the wild-type structure as the search model. In all three complex structures, cADPR, cGDPR, and NGD were bound...
tightly in the catalytic sites defined by the residues Trp125, Trp189, Glu146, Glu155, and Glu226 as proposed previously (12, 23). No secondary binding sites were seen for any of the substrates/products (cADPR, cGDPR, and NGD) either within the protein or between the interfaces of the two shCD38 molecules in the unit cell. This is consistent with our previous findings that NAD and ADPR bind to the same site of the enzyme (14). Therefore, the multiple catalytic reactions of shCD38 occur within the same active site.

The cyclic molecules, cADPR and cGDPR, possess common ribose and phosphate groups, but with a different purine ring. The sites of cyclization are also different. In cADPR, the adenine ring is cyclized with the terminal ribose at its N1 of the adenine, while in cGDPR, it is at the N7 of the guanine. It is the same N1/N7-glycosidic bond that is cleaved, respectively, during the CD38 catalyzed hydrolysis. Consistently, as seen in the complexes (Fig. 1A and 1B), the bound cADPR and cGDPR are oriented with the portions containing the N1/N7-glycosyl bonds embedded deep toward the bottom of the active site pocket where catalysis occurs.

Fig. 1C shows shCD38 complexes with NGD, the substrate for the synthesis of cGDPR. It can be seen that the part of NGD, a long linear molecule, containing the nicotinamide group penetrates deep into the active site pocket. This is again consistent with catalysis occurring at the bottom of the pocket and indicates that the nicotinamide group of NGD is the first to be removed during catalysis, before cyclization.

cADPR Complexes Reveals the Role of Glu226 in Substrate cADPR Activation

Human CD38 is the only human enzyme known to hydrolyze cADPR to ADPR. The refined E226Q-cADPR complex structure at 1.98 Å resolution is the first reported structure of cADPR complexed with a protein (Fig. 1A and Fig. 2). The high resolution diffraction data allowed unambiguous identification of the position and orientation of cADPR in the active site from the Fo-Fc omit map (Fig. 2).

The cADPR binding site in the E226Q mutant is formed by residues Arg127, Thr221, Glu146, Asp155, and Trp189. All these residues have direct polar or no-polar interactions with cADPR (Fig. 2). The adenine ring of cADPR adopts an orientation such that it has both hydrophobic interactions with the side chain of Trp189 and hydrophilic interactions with Glu146 and Glu155.

Although the N-1-glycosidic bond of the bound cADPR is facing and is close to the bottom of the active site pocket, it is striking that it is 7.9 Å away from the catalytic residue Glu226 (Glu226 in wild-type), too far to be attacked by it. That Glu226 is the catalytic residue has previously been established by both structural and mutagenesis studies (12, 14). The gap between cADPR and the catalytic residue is filled with five water molecules which form stable hydrogen bonds network to prevent the further entry of cADPR (Fig. 2). It is thus clear that this form represents an inactive complex between shCD38 and cADPR, and is consistent with E226Q being catalytically impaired. In fact, this inactive form of complex is characteristic of all inactive mutants we have examined. The superposition of the cADPR complex structures obtained from three inactive mutants of shCD38, E226Q, E226G and E226D reveals essentially the same cADPR structure. It appears, for these E226 mutants, that cADPR cannot be correctly positioned in their active sites in close enough distance to Glu226 for catalysis. Repeating the soaking experiments at room temperature gave similar results, indicating this form of cADPR binding is temperature independent. With wild-type shCD38 crystals, however, even short soaking below 0ºC resulted in its hydrolysis to ADPR. The ability of wild-type shCD38 to hydrolyze cADPR demonstrates that Glu226 is not only catalytic but, surprisingly, is also essential for the binding of cADPR to an active position.

It should be noted that all inactive mutants have some residual catalytic activities (12), indicating that cADPR can transit from the inactive position in the complex to an active one, albeit at a very low rate. Once in the active position, it can then be readily hydrolyzed. This has been verified structurally, as ADPR product is found in the active site during long term co-crystallization of E226Q with cADPR (14). The complexes of cADPR with the CD38 mutants thus reveal an unexpectedly active role of Glu226 in promoting the binding of cADPR to an active position in the catalytic pocket.

Binding of cGDPR and cADPR Requires Structural Movement of Dippeptide Glu146-Asp147

What this active position for the hydrolysis of cADPR is, in fact, can be inferred from the cGDPR complex obtained by incubating cGDPR...
with E226Q crystals at 4ºC, as shown in Fig. 3A. The structure was refined at 1.68 Å resolution and the electron density map clearly defines positions for all the cGDPR atoms (Fig. 3A). In contrast to cADPR, cGDPR enters deeply into the active site with its N7-glycosyl bond positioned only 3.96 Å from Gln226. Its guanine ring group is recognized and positioned by residues Glu146, Asp155, and Trp189 through hydrophilic and hydrophobic interactions. Water molecules, Wat 1 and Wat 2, forms two additional hydrogen bonds to cGDPR O6 and 2'-OH, respectively. The nicotinamide ribose has direct polar interactions with Gln226 carboxyl group and the main-chain nitrogen of Trp125. Two phosphate groups on cGDPR form extensive polar interactions with dipeptide Ser126Arp127 and the main-chain nitrogen of Phe222. Even the guanine ribose, which is located at the entrance of the active site, is stabilized by a hydrogen bond with Thr221 (Fig. 3A).

There are two shCD38 E226Q molecules in the crystallographic asymmetric unit. We observed that cGDPR can only bind to the active site of molecule A. In the active site of molecule B, the electron density can only be modeled as solvent. The inability of molecule B to bind with cGDPR suggests its reduced affinity for cGDPR. It is thus interesting to see the structural differences between the active sites of the two E226Q molecules in the same crystallographic asymmetric unit (molecule A and B). This can be done by structurally aligning the two molecules. The alignment reveals that Glu146 in the apo structure (dark-green) is required to move away from the active site to make sufficient space for the binding of the guanine ring of cGDPR (Fig. 3B). This backward movement of Glu146 (from its dark-green position to gray position) can be as far as 2.66 Å, a distance corresponding to the length of a hydrogen bond (Fig. 3B). As a consequence of the backward shift of Glu146, its neighboring residue Asp155 retreats accordingly (from its dark-green position to gray position). Hence the comparison of the apo and the cGDPR bound complex indicates the overall effect induced by cGDPR binding is the backward movement of the dipeptide Glu146-Asp155. This movement can be illustrated more dramatically by adding the cADPR complex to the alignment as shown in Fig. 3C. Residues Glu146 and Asp155 in the cADPR complex (magenta) adopt a position between the apo and cGDPR bound position. It can be concluded that the entry of either cADPR or cGDPR to the active site requires the consistent movements of Glu146 and Asp155, more extensive with cGDPR than with cADPR.

Comparing the cADPR and the cGDPR complexes, it can be seen that cGDPR bound much tighter than cADPR, with the ribosyl C1’ atoms of cGDPR approximately 3.7 Å deeper into the active site. The tight association is due mainly to the fact that cGDPR is larger than cADPR, such that the guanine ring forms stable interactions with Trp189, Asp155, and Glu146 through both hydrophobic and hydrophilic interactions (Fig. 3A). Particularly is the extensive overlap between the tryptophen ring and the guanine ring with face-to-face π-π interactions between their five-membered rings. This tight binding likewise induces the much larger movement of the dipeptide Glu146-Asp155 than cADPR.

NGD Complex together with cGDPR/NAD Complexes Defines a Site Responsible for Substrate Specificity

When NGD is used as substrate, human CD38 mainly cyclizes it to cGDPR (15). This is in contrast to when NAD is used as substrate, which is predominately hydrolyzed to ADPR. To find the structural basis for this disparity, we determined E226Q mutant complexed with its substrate NGD at 2.1 Å resolution.

Fig. 4A shows that the nicotinamide group of the bound NGD points toward Glu146 and Glu155 and forms two hydrogen bonds with them (Fig. 4A). The nicotinamide ribose and the diphosphate of NGD interact with protein residues in the same way as their corresponding parts in cGDPR (Fig. 3A and Fig. 4A). The Fo-Fc omit electron densities are clear for the nicotinamide terminus and the diphosphate, but not good for its guanine terminus (Fig. 4A). The disordered density reflects the flexibility of the guanine ring of the bound NGD, analogous to the poor density observed for the adenine terminus of bound NAD (14). Structural alignment of the NGD and NAD complexes indicates that NGD and NAD share a common interaction pattern with the enzyme (Fig. 4B). NGD overlaps quite well with NAD except for its guanine terminus (Fig. 4A). The disordered density reflects the flexibility of the guanine ring of the bound NGD, analogous to the poor density observed for the adenine terminus of bound NAD (14). Structural alignment of the NGD and NAD complexes indicates that NGD and NAD share a common interaction pattern with the enzyme (Fig. 4B). NGD overlaps quite well with NAD except for its guanine terminus. The similarity indicates the structural determinants responsible for the differences in the reactions between NGD and NAD are not this part of the molecules, but the purine rings instead. As described above, the guanine ring can form much tighter association with the active site (i.e. Trp189, Asp155, and Glu146).
than adenine. Once folded back, the higher affinity should increase the probability of the guanine ring for coupling with the terminal ribose and thus facilitate the cyclization.

Another feature revealed by the structure comparison is that the nicotinamide ribose and the diphosphate in NGD, cGDPR, and NAD interact with a set of common residues at the active site, namely, Thr221, Phe222, Trp125, Ser126, Arg127 and Gln226. These residues thus form a docking site capable of accepting NGD, cGDPR, or NAD, and likely any other nucleotides possessing a ribose-diphosphate motif as well. The purine rings of the bound NAD and NGD extend outward from the active site and found to be disordered in the complexes, indicating they are not specifically recognized. This is consistent with the promiscuous nature of CD38, capable of accepting a variety of purine nucleotides as substrate (24).

Tightly Controlled cADPR Recognition and Catalysis

All the Glu226 mutants characterized so far are catalytically inactive (12) and the structures of the complex indeed show that cADPR is bound too far from the catalytic Glu226 to be activated. In order for the N-1 glycosidic bond to be cleavable, cADPR must bind closer, at least as close as seen in the cGDPR complex. This can be modeled by aligning the two complexes. All the protein residues of the complexes were fixed and only cADPR was mapped on cGDPR after proper translation and distortion. The nicotinamide ribose and diphosphate of cADPR were aligned with that of cGDPR because they are the structural determinants recognized by the active site as described above. The resultant cADPR model has correct geometry without obstructing contacts with protein residues (Fig. 5A). In this active cADPR model, Gln226 is close enough to form a hydrogen bond to cADPR’s 3’-OH group. Ser193 is about 3 Å to cADPR’s C-1’ carbon, which is consistent with our previous studies showing that Ser193 is critical for catalysis and in the stabilization of the non-covalent intermediate (14).

From Fig. 5A and Fig. 3C, it can be seen that in order to be catalyzed, cADPR needs to bind at least 3.7 Å deeper into active site. In this new position, the N-6 atom of cADPR forms two hydrogen bonds to Glu146 with distances of 2.81 Å and 2.69 Å. This close interaction with Glu146 is consistent with our previous finding that the residue is a critical determinant of the bifurcation between the cyclization and hydrolysis processes (23). Additionally, the 2’-, 3’-OH groups of cADPR is now close enough to form two hydrogen bonds with Glu226. This interaction with the ribose positions cADPR properly such that the carboxyl group of Glu226 is ~3.3 Å to the C-1’ of the ribose (Fig. 6A), making it suitable as a nucleophile. It can thus be inferred that the strong interactions between Glu226 and cADPR are not only critical for catalysis but also important for positioning cADPR to the catalytic position. Any change of the residue, as in the inactive mutants, renders cADPR incapable of binding deep enough into the active site for catalysis.

It has been established above that the binding of cADPR to inactive E226 mutants induces backward movement of Glu146. This is also true for the binding of cADPR to its catalytic position. From the structural alignments of the apo E226Q mutant (molecule B of cGDPR complex) and the catalytic cADPR model, Glu146 (green stick) in apo structure is only 1.55 Å to N-6 of cADPR, strongly suggesting the backward movement of Glu146 would necessarily occur during the entry of cADPR to its catalytic position (Fig. 5B). The movement of Glu146 is always accompanied with the movement of neighboring residue Asp147, as indicated in Fig. 5B. Residue Asp147 is located on the surface of the protein (red surface). Therefore, the external force exerted on Asp147 can potentially be transduced to residue Glu146. Such a connection between the active site residue Glu146 and the outside environment through Asp147 can well be the first clue to how the multiple catalysis of CD38 can be regulated by external factors.

DISCUSSION

In this study, we present detailed characterizations of the CD38 complexes. The observations allow us to put together a step-by-step scenario for the production of cADPR from substrate NAD and the hydrolysis of cADPR to produce final product ADPR.

NAD enters the active site as a linear molecule with the nicotinamide end positioned and fixed by polar interactions with Asp155 and Glu146 (14). The nicotinamide ring is enforced parallel to Trp189 ring by the ring-ring stacking interactions between them (Fig. 4A and Fig. 6A). Furthermore, the substrate recognition site, composed by residues Arg127, Ser126, Thr221, Phe222, Trp125, and Gln226, binds to the ribose-diphosphate portion of NAD. Meanwhile, the catalytic Glu226 forms two
hydrogen bonds with 2'-and 3'-OH groups of the nicotinamide ribose, positioning its C-1' for nucleophilic attack by the OE2 of the carboxyl group of Glu226. The OE2 atom has two lone pairs. One is involved in the formation of H bond with 2'-OH; the other is used for attacking of C-1' carbon from the alpha face of the ring. This results in the nicotinamide group disassociating from the substrate through an SN1 mechanism. The electron pair forming the C-1'-N bond will migrate to the nitrogen side, making C-1' positively charged and forming the oxocarbanium ion intermediate, which is stabilized by joint contributions from Glu226 and Ser193 (both are ~3.3 Å away from C-1') (14). The formation of H bond between Glu226 OE2 and 2'-OH prevents a direct covalent linkage between C-1' and the OE2 of Glu226, and promotes the formation of an ionic intermediate instead. This could be the major structural differences between NAD utilizing enzymes and other glycosidases/glycohydrolases.

The release of nicotinamide partially vacates the active site, allowing the folding back of adenine ring at the other end of the molecule toward the C-1' of the intermediate. This folding induces a concerted retreat of Glu146–Asp147. The backward shift of the dipeptide accumulates strain, making it behaves as a harmonic oscillator. The folding process is not random but is guided by residues Asp155, Glu146, and Trp189, which together control the positioning and orientation of the adenine ring, such that the N-1 atom is directed toward the positively charged C-1' of the intermediate, resulting in coupling intra-molecularly of the two ends and producing cADPR (Fig. 6A and 6B). The compaction of the intermediate to a smaller cyclic product releases the strain of the harmonic dipeptide and facilitates the release of cADPR from the active site (reaction 2, Fig. 6B).

Because of the affinity of the docking site formed by the Trp125, Ser126, Arg127, and Phe222 (Fig. 4A) for the ribose-diphosphate motif, cADPR has finite possibility of re-entering the active site, resulting in its hydrolysis. The compact size of cADPR, however, makes its binding to the extended docking site not as optimal as for NAD, NGD, or cGDPR. The hydrogen bonding with the Glu226 becomes critical for cADPR to bind deep enough into the active site for catalysis (Fig. 5A). Otherwise, cADPR would be bound peripherally as observed in the complexes with the inactive mutants (Fig. 2). Similar to the process of cADPR formation, the binding of cADPR to its catalytic position also induces the retreat of the same dipeptide (Fig. 6A). Likewise, the cleavage of the N-glycosidic bond of cADPR by Glu226 proceeds also in much the same way as described above.

After the bond cleavage, the forward movement of the dipeptide helps the release and unfolding of the adenine ring from the active site (Fig. 6A). Once again, the same ionic intermediate is formed. The unfolding of the adenine ring allows the entry of a water molecule nearby, whose lone pair electron serves as a nucleophile for attacking the intermediate, resulting in the formation of product ADPR (reaction 3). In contrast to cADPR, the linear substrate NAD binds favorably to the docking site and thus is readily hydrolyzed to ADPR (reaction 1, Fig. 6B), which is consistent with hydrolysis being the dominant reaction when NAD is used as a substrate.

As described, both the cyclization and hydrolysis reactions occur via the same intermediate and involve similar catalysis. They are not mutually exclusive, however, nor do they occur sequentially. Which one of the two reactions is dominant would depend on how accessible water is to the intermediate relative to the probability of intra-molecular attack by the adenine.

In fact, the dependence on the accessibility of water of the catalysis outcome can explain why CD38 mainly cyclizes NGD instead of hydrolyzing it. As shown in the complexes with NGD and cGDPR, the guanine ring interacts strongly with Trp189, much more so than the adenine ring of cADPR. This higher affinity of the guanine ring is further enhanced by formation of three hydrogen bonds with Glu146, Asp155, and Wat2 (Fig. 3C). Once the nicotinamide ring of the substrate is released, the extensive overlap and stacking of the guanine ring with the tryptophen ring not only increase greatly the probability that the guanine ring is in position for reacting with the intermediate, but also decrease the water access at the same time. Consequently, cyclization is predominant over hydrolysis.

In addition to Glu226, Glu146 is also critically important. Its mutation does not eliminate enzymatic activities but alter the dominance of the reactions CD38 catalyzes. In addition to catalyzing the synthesis and hydrolysis of cADPR, CD38 also catalyzes the synthesis and hydrolysis of another calcium messenger, NAADP (7, 9). The latter occurs only at acidic pH. Changing Glu146 to a
neutral residue allows the reactions to occur at neutral or alkaline pH (9). Structural characterizations indicate that the electrostatic repulsion between the Glu\textsuperscript{146} and the substrates that are negatively charged at neutral pH prohibits their binding and thus inhibits the reactions (9).

Mutation of Glu\textsuperscript{146} to many other residues, such as alanine, also greatly increases cyclization of NAD and inhibits its hydrolysis (23). This may well be related to the present finding that the dipeptide Glu\textsuperscript{146}-Asp\textsuperscript{147} undergoes structural rearrangement upon the binding of cADPR or cGDPR (Fig. 3C). The change to alanine is likely to alleviate the strain on the dipeptide during the entry of adenine ring to the active site, resulting in enhancement of the cyclization process. In any case, the movement of the dipeptide described here represents the first direct evidence for a conformational change at the active site of CD38 during catalysis.

Perhaps the most intriguing possibility raised by this study is that structural changes of the dipeptide may provide a mean for regulating CD38 catalysis. It is entirely possible that protein factors or membrane lipids interacting with Asp\textsuperscript{147} of the dipeptide that is exposing to the surface of CD38 can induce conformational changes of the dipeptide and thus alter the catalysis.
Footnotes
Atomic coordinates and structure factors have been deposited at the Protein Data Bank (http://www.pdb.org) with the accession codes of 2O3Q (E226Q cADPR complex), 2O3R (E226D-cADPR), 2O3S (E226G-cADPR complex), 2O3T (E226Q-cGDPR complex) and 2O3U (E226Q-NGD complex).

The abbreviations used are cADPR, cyclic ADP-ribose; ADPR, ADP-ribose; NADP, nicotinamide adenine dinucleotide phosphate; NAADP, nicotinic acid adenine dinucleotide phosphate; NGD, nicotinamide guanine dinucleotide; cGDPR, cyclic GDP-ribose.

Acknowledgements
This work was supported by grants from the NIH to MacCHESS (RR01646) and H.C.L./Q.H. (GM061568). The crystallographic data were collected at the Cornell High Energy Synchrotron Source (CHESS), which is supported by the NSF and NIH National Institute of General Medical Sciences under award DMR-0225180.
REFERENCES

1. Berridge, M. J., Lipp, P., and Bootman, M. D. (2000) *Nat. Rev. Mol. Cell. Biol.* 1, 11-21
2. Berridge, M. J., Bootman, M. D., and Roderick, H. L. (2003) *Nat. Rev. Mol. Cell Biol.* 4, 517-529
3. Berridge, M. J. (2005) *Annu. Rev. Physiol.* 67, 1-21
4. Lee, H. C. (2001) *Annu. Rev. Pharmacol. Toxicol.* 41, 317-345
5. Lee, H. C., Zocchi, E., Guida, L., Franco, L., Benatti, U., and De Flora, A. (1993) *Biochem. Biophys. Res. Commun.* 191, 639-645
6. Howard, M., Grimaldi, J. C., Bazan, J. F., Lund, F. E., Santos-Argumedo, L., Parkhouse, R. M. E., Walseth, T. F., and Lee, H.C. (1993) *Science* 262, 1056-1059
7. Aarhus, R., Graeff, R. M., Dickey, D. M., Walseth, T. F., and Lee, H. C. (1995) *J. Biol. Chem.* 270, 30327-30334
8. Lee, H. C. (2005) *J. Biol. Chem.* 280, 33693-33696
9. Graeff, R., Liu, Q., Kriksunov, I. A., Hao, Q., and Lee, H. C. (2006) *J. Biol. Chem.* 281, 28951-28957.
10. Perraud, A. L., Fleig, A., Dunn, C. A., Bagley, L. A., Launay, P., Schmitz, C., Stokes, A. J., Zhu, Q., Bessman, M. J., Penner, R., Kinet, J. P., and Scharenberg, A. M. (2001) *Nature* 411, 595-599
11. Kolisek, M., Beck, A., Fleig, A., and Penner, R. (2005) *Molecular Cell* 18, 61-69
12. Munshi, C., Aarhus, R., Graeff, R., Walseth, T. F., Levitt, D., and Lee, H. C. (2000) *J. Biol. Chem.* 275, 21566-21571
13. Liu, Q., Kriksunov, I. A., Graeff, R., Munshi, C., Lee, H. C., and Hao, Q. (2005) *Structure* 13, 1331-1339
14. Liu, Q., Kriksunov, I. A., Graeff, R., Lee, H. C., and Hao, Q. (2006) *J. Biol. Chem.* 281, 32861-32869
15. Graeff, R., Walseth, T. F., Fryxell, K., Branton, W. D., and Lee, H. C. (1994) *J. Biol. Chem.* 269, 30260-30267
16. Munshi, C., Fryxell, K. B., Lee, H. C., and Branton, W. D. (1997). *Methods Enzymol.* 280, 318-330
17. Otwinowski, Z., Minor, W. (1997) *Methods Enzymol.* 276, 307-326
18. Collaborative Computational Project, Number 4 (1994) *Acta Crystallogr. D Biol. Crystallogr.* 50, 760-763
19. Lee, H. C., Aarhus, R. and Levitt, D. (1994) *Nat. Struct. Biol.* 1, 143-144
20. Jones, T. A., Zou, J. Y., Cowan, S. W., and kajeldgaard, M. (1991) Acta. Crystallogr. A47, 110-119
21. Schuettelkopf, A. W. and van Aalten, D. M. F. (2004) *Acta Crystallogr. D Biol. Crystallogr.* 60, 1355-1363
22. Zwart, P. H., Langer, G. G., and Lamzin, V. S. (2004) Modelling bound ligands in protein crystal structures. *Acta Crystallogr. D Biol. Crystallogr.* 60, 2230-2239
23. Graeff, R., Munshi, C., Aarhus, R., Johns, M., and Lee, H.C. (2001) *J. Biol. Chem.* 276, 12169-12173
24. Graeff, R. M., Walseth, T. F., Hill, H. K., and Lee, H. C. (1996) *Biochemistry* 35, 379-386
Table 1. Crystallographic data and refinement statistics.

| Data collection | E226Q-cADPR | E226D-cADPR | E226G-cADPR | E226Q-cGDPR | E226Q-NGD |
|-----------------|-------------|-------------|-------------|-------------|-----------|
| Cell dimensions | 41.994      | 41.724      | 42.350      | 41.71       | 41.976    |
| a, b, c (Å) /  | 53.289      | 52.999      | 53.768      | 52.812      | 53.091    |
| α, β, γ (°)     | 65.729/     | 65.973/     | 66.826/     | 65.424/     | 65.678/   |
|                | 105.900     | 104.960     | 104.966     | 105.93      | 106.086   |
|                | 91.760      | 91.217      | 91.705      | 91.80       | 91.914    |
|                | 95.160      | 94.570      | 95.042      | 95.22       | 95.183    |
| Space group     | P1          | P1          | P1          | P1          | P1        |
| Resolution (Å)  | 30-1.98 (2.05-) | 30-1.75 (1.81-) | 50-1.50 (1.55-) | 50-1.68 (1.74-) | 30-2.10 (2.18-) |
| | 1.98 | 1.75 | 1.50 | 1.68 | 2.10 |
| Unique reflections | 34368     | 51453       | 78040       | 59536       | 29497     |
| Multiplicity    | 3.8 (3.8)  | 2.9 (2.6)   | 3.4 (2.0)   | 3.8 (2.8)   | 3.7 (2.9) |
| I/sigma         | 13.98 (3.68)| 30.83 (2.41)| 25.1 (1.85) | 27.3 (2.69) | 16.07 (2.41) |
| Rmerge (%)      | 7.8 (40.3) | 3.8 (33.7)  | 4.0 (38.9)  | 5.7 (34.7)  | 7.1 (40.2) |
| Completeness (%)| 91.9 (96.7)| 95.1 (81.0) | 87.0 (41.2) | 96.6 (89.5) | 95.6 (78.9) |
| Refinement      |             |             |             |             |           |
| R factor (%)    | 20.69       | 18.30       | 18.89       | 17.17       | 17.79     |
| Rfree factor (%)| 27.82       | 22.91       | 23.82       | 20.84       | 22.27     |
| Protein atoms   | 4016        | 4014        | 4076        | 4100        | 4100      |
| Water molecules | 293         | 399         | 697         | 479         | 183       |
| Ligands (atoms) | 35+35       | 35+35       | 35+35       | 36          | 45+45     |
| Mean B (Å²)     | 44.97       | 46.12       | 40.07       | 32.78       | 40.62     |
| R.M.S. deviations |             |             |             |             |           |
| Bond lengths (Å) | 0.035      | 0.031       | 0.021       | 0.012       | 0.013     |
| Bond angles (°) | 2.520       | 2.376       | 1.654       | 1.346       | 1.398     |

Values in parentheses are from the highest resolution shell.

Rmerge = \[\sum |I - \langle I\rangle|/\sum I\], where I is the integrated intensity of a given reflection.

R = \[\sum ||F_{obs}| - |F_{calc}||/\sum |F_{obs}|\]. Rfree was calculated using 5% of data excluded from refinement.
FIGURE CAPTIONS

SCHEME 1. Cyclization reactions catalyzed by human CD38.

FIGURE 1. Structural overview of shCD38 complexed with various substrates and products.
The structure of shCD38 is shown as gray transparent surface. Cyclic ADPR (A), cGDPR (B), and NGD (C) are drawn in sticks and colored with the scheme: C, white; N, blue; O, red; P, orange. Figures were prepared with Pymol (http://pymol.sourceforge.org).

FIGURE 2. Stereo representation of cyclic ADP-Ribose complex.
The interactions of the bound cADPR to various residues at the active site pocket of the mutant E226Q are shown. The catalytic Glu$^{226}$ was mutated to a glutamine, shown as yellow sticks, and the mutant protein has greatly reduced catalytic activities. Other residues involved in cADPR binding are shown as sticks and colored in violet for polar interactions and marine for hydrophobic interactions, respectively. Fo-Fc omit difference density for cADPR is drawn as isomesh at 2.5σ.

FIGURE 3. Cyclic GDP-Ribose complex.
(A) Interactions between cGDPR and the enzyme are shown. Polar interactions are shown as dashed cyan lines. Protein residues involved in the polar interactions were drawn as sticks and colored as C, gray; N, blue; O, red; P, orange. Trp$^{189}$ has hydrophobic interactions with cGDPR and is shown as marine sticks. Ser$^{193}$, shown as yellow sticks, is 3.1 Å to the C-1’ atom of cGDPR. (B) The structural comparison of two CD38 molecules in the crystallographic asymmetric unit reveals the structural changes induced by cGDPR binding. Only one of the molecules (A, colored gray) contains a bound cGDPR in the active site and the other (B) does not. The two molecules were aligned based on the minimization of all the Ca atoms. Comparing the active site structures of molecule A and B, the binding of cGDPR to molecule A induces the consistent movement of residues Glu$^{146}$ and Asp$^{147}$ from dark green position (apo form) to gray position (cGDPR bound form). (C) The superimposition of the apo-structure with the structures of the cADPR and cGDPR complexes. The residues Glu$^{146}$-Asp$^{147}$ in the cADPR complex (magenta) are located in between the free form (green) and cGDPR bound form (gray).

FIGURE 4. NGD complex and its comparison with the NAD complex.
(A) NGD complex. Protein residues involved in polar interactions are drawn as magenta sticks. Polar interactions are shown as dashed lines colored in cyan. Residue Trp$^{189}$ has hydrophobic interactions with the nicotinamide portion of the substrate is also shown in dark blue. Residue Ser$^{193}$, whose OH group is only 3.21 Å away from the substrate’s C1’ atom, is shown as yellow sticks. A structural water molecule in the active site is shown as a red sphere. The Fo-Fc omit difference density for NGD is drawn as gray isomesh and contoured at 2.5σ. (B) The NGD complex superimposed with the NAD complex. NAD and its nearby protein residues are shown as green sticks. Both complex structures are alike except for the parts of the purine rings that are flexible and outside of the active site.

FIGURE 5. cADPR recognition and catalysis.
A) A stereo model of the cADPR complex with the bound cADPR transformed to the catalytic position seen in the cGDPR complex. The positions of the protein residues were unchanged and only cADPR was modeled. The bound cGDPR is shown as yellow sticks as reference. The adenine ring on cADPR and guanine ring on cGDPR are coplanar and parallel to the ring of Trp$^{189}$. Residue Glu$^{146}$ in the position that it adopts in the cADPR complex (Figure 2) is shown as magenta sticks; two dashed blue lines and associated values indicate the distances between adenine and Glu$^{146}$. B) A model showing the dynamics of Glu$^{146}$ during cADPR catalysis. Glu$^{146}$ is shown in green when it is in the position as seen in the apo E226Q structure, while it is colored yellow when it is in the position as seen in the cGDPR-E226Q complex. The active site and its nearest surface to Glu$^{146}$ are shown as gray.
surface. Asp<sup>147</sup> as seen in the cGDPR-E226Q structure is located on the surface of CD38 and colored magenta. Red and dark arrows show the reciprocal movement of Glu<sup>146</sup> and Asp<sup>147</sup> during the cADPR binding and release, respectively. The conformational changes of Glu<sup>146</sup>-Asp<sup>147</sup> are crucial in regulating cADPR catalysis.

**FIGURE 6. A catalytic model of cADPR formation and hydrolysis.**

A) A stereo representation of the structural alignment of the active cADPR (yellow) model with NAD (green) complexed by wild-type CD38 (magenta sticks). The active cADPR model is derived by transforming cADPR from its inactive conformation, seen in the complexes with the inactive CD38 mutants (Fig. 2), to the catalytic conformation seen in the cGDPR complex (Fig. 5A).

B) Reaction schemes showing the proposed mechanism for the cADPR production and hydrolysis. Pathways 1, 2, and 3 represent respectively the NAD hydrolase, the ADP-ribosyl cyclase, and the cADPR hydrolase activities catalyzed by CD38.
Scheme 1.
Figure 3.
Figure 4.
Figure 6.
Structural basis for formation and hydrolysis of calcium messenger cyclic ADP-ribose by human CD38
Qun Liu, Irina A. Kriksunov, Richard Graeff, Hon Cheung Lee and Quan Hao

J. Biol. Chem. published online December 20, 2006

Access the most updated version of this article at doi: 10.1074/jbc.M609093200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts