Identification of the Enzymatic Active Site of CD38 by Site-directed Mutagenesis*

CD38 is a ubiquitous protein originally identified as a lymphocyte antigen and recently also found to be a multifunctional enzyme participating in the synthesis and metabolism of two Ca\textsuperscript{2+} messengers, cyclic ADP-ribose (cADPR) and nicotinic acid adenine dinucleotide phosphate. It is homologous to Aplysia ADP-ribosyl cyclase, where the crystal structure has been determined. Residues of CD38 corresponding to those at the active site of the Aplysia cyclase were mutated. Changing Glu-226, which corresponded to the catalytic residue of the cyclase, to Asp, Asn, Gln, Leu, or Gly eliminated essentially all enzymatic activities of CD38, indicating it is most likely the catalytic residue. Photoaffinity labeling showed that E226G, nevertheless, retained substantial NAD binding activity. The secondary structures of these inactive mutants as measured by circular dichroism were essentially unperturbed as compared with the wild type. Other nearby residues were also investigated. The mutants D147V and E146L showed 7- and 19-fold reduction in NADase activity, respectively. The cADPR hydrolysis activity of the two mutants was similarly reduced. Asp-155, on the other hand, was crucial for the GDP-ribosyl cyclase activity since its substitution with either Glu, Asn, or Gln stimulated the activity 3-15-fold, whereas other activities remained essentially unchanged. In addition to these acidic residues, two tryptophans were also important, since all enzyme activities of W125F, W125Y, W189G and W189Y were substantially reduced. These six residues correspond to those critical residues of CD38 corresponding to those at the active site of the Aplysia cyclase. The results indicate a strong structural homology between the active sites of CD38 and the Aplysia cyclase.

CD38 was first described as a lymphocyte antigen, the expression of which depends on the stage of differentiation of the lymphocytes (reviewed in Refs. 1 and 2). Mice carrying a null mutation in the CD38 gene exhibit altered humoral immune responses (3). Ligation of CD38 with specific monoclonal anti-bodies triggers a myriad of responses in lymphocytes from proliferation to apoptosis (reviewed in Refs. 2, 4, and 5). Also, increased expression of CD38 on T cells is associated with human immunodeficiency virus infection, and the expression index is shown to be the best predictor of shorter survival of the patients (6, 7).

The biological relevance of CD38, however, is not restricted to lymphocytes. Its expression has since been found to be widespread among non-hematopoietic tissues, including the brain (8, 9). In addition to the antigenic functions, CD38 also possesses a multitude of enzymatic activities. It is homologous to Aplysia ADP-ribosyl cyclase, sharing about 30% sequence identity and a perfect alignment of the cysteine residues (10). Similar to the ADP-ribosyl cyclase, CD38 catalyzes both the cyclization of NAD to cyclic ADP-ribose (cADPR) and a base-exchange reaction using NADP as substrate, producing nicotinic acid adenine dinucleotide phosphate (NAADP) (reviewed in Refs. 11 and 12). In addition, CD38 also catalyzes the hydrolysis of cADPR and NAD. CD38 is thus catalytically novel. Biologically more relevant, however, are its two enzymatic products, NAADP and cADPR. Both are Ca\textsuperscript{2+} messengers mediating the mobilization of intracellular Ca\textsuperscript{2+} stores in a wide variety of cells from protozoan and plant to human (reviewed in Refs. 13 and 14). That a single enzyme is capable of catalyzing the synthesis and metabolism of two structurally and mechanistically distinct Ca\textsuperscript{2+} messengers suggests CD38 may be central to Ca\textsuperscript{2+} signaling in cells.

In this study, we identify the enzymatic active site of CD38 by site-directed mutagenesis. We have recently co-crystallized the Aplysia cyclase with nicotinamide, a substrate of the base-exchange reaction, and we have characterized its active site by x-ray crystallography (15). We have taken advantage of the structural homology between CD38 and the cyclase and have selected four acidic residues and two tryptophans of CD38 for mutagenesis. These six residues correspond to those critical residues at the active site of Aplysia cyclase. The results identified Glu-226 as the most likely candidate for the catalytic residue.

MATERIALS AND METHODS

Expression of the Human CD38 in Yeast—The Pichia expression vector pPICZaA (Invitrogen, Carlsbad, CA) was used, and the construct consisted of cloning the catalytic domain of either the wild type or mutant human CD38 in frame with the yeast mating α-factor signal sequence. The first 44 residues of CD38, containing the transmembrane and cytoplasmic domains (16), were deleted, and the catalytic domain was started at Arg-45. This soluble fragment of the CD38 has enzymatic activities indistinguishable from the full-length protein (17–20). The details of the construct were as we have described previously (19, 21, 22).

1 The abbreviations used are: cADPR, cyclic ADP-ribose; NAADP, nicotinic acid adenine dinucleotide; NGD, nicotinamide guanine dinucleotide; HPLC, high pressure liquid chromatography.
22. The tightly controlled alcohol oxidase promoter AOX1 was used to regulate the expression of the construct. The X-33 strain of yeast was transfected with the construct and the expression induced by methanol. The expressed CD38 was secreted as a soluble protein in the medium (19, 21). Endogeneous proteolytic enzymes that normally are responsible for protein-protein cross-linking also removed the α-factor signal sequence from the expressed CD38.

The advantages of the X-33 yeast strain have been described previously (15). Briefly, the strain contains the native alcohol oxidase gene AOX1, which allows the yeast to metabolize methanol and grow to higher densities. It also contains the native HIS4 gene, enabling them to grow without histidine supplementation. The HIS4 promoter allows for use of positive selection vectors like pPGCzA6. With this vector the transformation efficiency was nearly 100%, and no revertants were detected. After a 5-day induction with methanol in a standard culture tube, the yeast produced and secreted up to 40 μg/ml of the soluble catalytic fragment of CD38 in the culture medium. After dialyzing the culture media to remove salts, the soluble CD38 proteins were purified in a single step using a cation exchange column (SP5 PW, Waters, Milford, MA). A linear gradient of NaCl was used for elution as described previously (19). At least two separate cultures were made from each mutant, and each culture was purified separately.

Enzyme Assays—The wild type or mutant CD38 (0.2–40 μg/ml) was incubated (2 min to 18 h) at room temperature with various concentrations of NAD (0.2–10 mM) and NADP (0.1–100 mM). The reaction mixture is treated with PfuTurbo Klenow enzyme to generate staggered nicks is generated. Following the thermal cycling, the supercoiled plasmid containing the insert. A mutated plasmid containing the enzyme was amplified, together with the crystal coordinates of the cyclase dimer that we have previously determined (15, 26), to generate the homologous structure of the truncated CD38 dimer. This structure was then refined using the Powell relaxation routine in X-PLOR (27). All the cysteines were positioned close enough that the disulfide bond constraint could be imposed during the X-PLOR refinement. In the initial homology model, there were 96 bad contacts and the X-PLOR energy terms for the bonds, angles, dihedrals, and Van der Waals were 27, 172, 16, 484, and 3, respectively. After refinement, there were no bad contacts, and the energies were reduced to 243, 1,958, 3,381, and -3,365, respectively.

Circular Dichroism Measurement—CD38 and its mutants were purified by cation exchange chromatography as described above. The purified fractions were pooled and dialyzed against 10 mM phosphate buffer, pH 7. The dialyzed proteins were concentrated using Centricon-10 (Millipore) concentrators. Circular dichroism measurements were done at a protein concentration of 0.5 mg/ml and a temperature of 25 °C. The samples (125 μl) were added to a 0.05-cm cuvette, and a Jasco 710 Spectropolarimeter was used to record 6 scans at 50 nm/min at 0.1-nm intervals from 195 to 250 nm. The 10 mM phosphate buffer used as a blank was subtracted from each recording. The data were converted to molar ellipticity units and finally to molar circular dichroism units. Protein concentration was determined by the method of Chou and Fasman (28). The data were calculated by using the Contini program (29) and are shown as percent α-helix, β-strand, and unordered structure.

RESULTS

We have recently characterized the active site of the Aplysia ADP-ribosyl cyclase (15). X-ray crystallography shows that nicotinamide, the substrate of the base-exchange reaction, is coordinated by three residues, Glu-98, Asn-107, and Trp-140. Site-directed mutagenesis identifies two additional residues, Trp-77 and Glu-179, at the binding pocket as critical for catalysis. Among these residues, Glu-179 is most likely to be catalytic. The corresponding residues in CD38 are Trp-125, Glu-146, Asp-155, Trp-189, and Glu-226, and they were selected for site-directed mutagenesis. Additionally, Asp-147 was also targeted. The effects of the mutations on four enzymatic activities of CD38 were determined. The cyclization reaction was assayed using NAD as substrate, and the production of cADPR was monitored fluorometrically (20). The exchange of the nicotinamide group of NADP with nicotinic acid was measured as NAADP production using HPLC (18). The two hydrolytic reactions, NADase and cADPR hydrolase were assayed as production of ADP-ribose from NAD and cADPR, respectively.

The effects of mutagenizing Glu-226, which corresponds to the catalytic Glu-179 of the cyclase, are shown in Table I. Substitution of Glu-226 with Leu or even with conservative residues, such as Asp, Asn, and Gln, totally eliminated all enzymatic activities. Only residual activities were detected when substituting with a glycine. This exquisitely specific requirement of a glutamate residue is consistent with it being the catalytic residue. We have previously found a similarly high degree of specificity for a glutamate at the active site of the Aplysia cyclase (15).

That these substitutions did not induce a gross distortion of the structure of the active site is shown by the retention of substantial NAD binding activity of the mutant E226G. The NAD binding activity was assessed by photoaffinity with [3H]P8-azido-NAD in the absence and presence of increasing concentrations of NAD. Fig. 1 shows that the labeling was specific for both the mutant, E226G, and the wild type since it was competitively reduced by NAD. In the case of E226G, the half-maximal effect was between 0.1 and 0.5 μM of NAD. The
Inhibition of enzymatic activities of CD38 by site-directed mutagenesis of Glu-226

Data shown are the \( V_{\text{max}} \) values for the wild type (WT) CD38, whereas the enzymatic activities for the mutants were determined at a saturating substrate concentration of either 0.2 mM for NAD, NADP, and NGD or 1 mM for cADPR. Two different protein preparations were used for the wild type and each of the mutants. The values shown are mean \( \pm \) S.E. for 4–6 determinations. For determining the wild type \( V_{\text{max}} \) of the NADase, [NAD] was varied from 7.5 to 125 \( \mu \)M; for the base exchange, [NADP] was varied from 10 to 160 \( \mu \)M; and for the cADPR hydrolyase activity, [cADPR] was varied from 0.16 to 1.6 mM. The \( V_{\text{max}} \) values for GDPR cyclase activity were determined by a continuous fluorimetric assay using an initial concentration of 25 \( \mu \)M NGD as described under “Materials and Methods.”

|       | NADase | Base exchange | GDPR cyclase | cADPR hydrolyase |
|-------|--------|---------------|---------------|-----------------|
| WT    | 124,200 ± 8,800 | 76,500 ± 2,900 | 15,700 ± 400 | 5,800 ± 500 |
| E226D | 0      | 0             | 0             | 0               |
| E226N | 0      | 0             | 0             | 0               |
| E226L | 0      | 0             | 0             | 0               |
| E226Q | 0      | 0             | 0             | 0               |
| E226G | 1      | 47 ± 7        | 22 ± 1        | 89 ± 17         |

The secondary structures of three of the E226 mutants were evaluated using circular dichroism spectroscopy. The results are listed in Table II. The percentages of the \( \alpha \)-helices, \( \beta \)-sheets, and random coils of the inactive mutants were essentially the same as those of the wild type, giving further support that the point mutation at Glu-226 did not induce a gross perturbation on protein folding.

The catalytic role of the conserved sequence near the middle of the CD38 molecule was next investigated. This sequence (Thr-144–LEDTL) is conserved among three species of CD38 and two species of ADP-ribosyl cyclase (reviewed in Refs. 11, 12, and 14). In the Aplysia cyclase, this sequence constitutes the bottom of the active site pocket (15). Glu-146 and Asp-147 are conserved between CD38 and the Aplysia cyclase. Substituting Asp-147 with valine reduced the NADase activity to about 17,100 ± 1,500 nmol/mg/min (\( n = 4 \), ± S.E.), more than 7-fold reduction as compared with the wild type (cf. Table I). Substituting Glu-146 with leucine depressed the NADase activity even further to 6,600 ± 1,300 (\( n = 4 \), ± S.E.), a 19-fold reduction. That these two conserved residues are indeed important for catalysis is further indicated by the total elimination of all enzymatic activities when both residues were substituted (E146L/D147V).

When using NAD as substrate, CD38 mainly hydrolyzes it to ADP-ribose, and very little cADPR is produced (29–31). The cyclization reaction catalyzed by CD38 is more easily shown by using NGD, instead of NAD, as substrate. The main product in this case is cGDPR (20). Although substitutions of either Asp-147 or Glu-146 decreased the NADase activity by 7–19-fold, the GDP-ribosyl cyclase activity of the mutants remained essentially the same as the wild type. The cADPR hydrolyase activity, however, appears to correlate with the NADase activity since both were correspondingly depressed by the substitutions. This correlation is further shown in Fig. 2, where the cADPR hydrolyase activity of each mutant was plotted against its NADase activity. A roughly linear relationship with an \( r^2 \) value of 0.93 was apparent. The slope of the least square line was 2.22, suggesting that the substitutions more readily affected the cADPR hydrolyase than the NADase activity.

X-ray crystallography of nicotinamide bound to the active site of the Aplysia cyclase shows that Asn-107 is one of the three closest residues (15). This residue is not conserved in

intensity of labeling of the mutant in the absence of NAD was 44% that of the wild type (inset, Fig. 1).

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CD38 but corresponds to Asp-155. Table III shows that the most dramatic effect of substituting Asp-155 with a conservative residue, such as Glu, Asn, or Gln, was the large stimulation of the GDP-ribozyme cyclase activity, by as much as 15-fold in the case of D155E. Since all other enzymatic activities of the mutants were only minimally affected, this suggests that Asp-155 is uniquely important for determining the GDP-ribozyme cyclase activity of CD38.

In addition to the acidic residues described above, two conserved tryptophans were found to be also important for the enzymatic activities of CD38. Substituting either residue depressed all enzymatic activities uniformly as shown in Table IV. Trp-189 corresponds to the Trp-140 in the Aplysia cyclase, which is responsible for coordinating the bound nicotinamide (15). Its substitution with glycine essentially eliminated all enzymatic activities. A similar decrease in activities was seen even when substituting with tyrosine, another aromatic residue. W189Y showed no NADase nor cADPR hydrolyase activities and only residual GDP-ribozyme cyclase, and the exchange activities were detected. Similarly, substituting Trp-125 with similar aromatic residues, phenylalanine and tyrosine, depressed all activities (Table IV).

Fig. 3 shows a homology model of CD38. The crystal coordinates of the dimer of Aplysia cyclase were used first, which resulted in a model having a number of bad contacts. After energy minimization, all bad contacts were removed, and the energy terms showed large reduction, as detailed under "Materials and Methods." The final homology model of CD38 shows that all 12 cysteines are indeed close enough to form disulfide bonds. This is consistent with the crystal structure of the Aplysia cyclase, which shows its 10 cysteines are paired in disulfide bonds (26). The 10 cysteines of the cyclase are in perfect alignment with those in the catalytic domain of CD38. The correct placement of the disulfide bonds lends credence to the homology model. The five conserved disulfide linkages are represented by purple cylinders in Fig. 3A, and the extra one is colored yellow. All the catalytically important residues described in this study are shown to be in a pocket located close to the middle of the CD38 molecule. The stereo image in Fig. 3B shows that Glu-226, which is most likely the catalytic residue, is deep inside the pocket. Glu-146 and Asp-155, which are important in controlling the NADase and GDP-ribozyme cyclase activities, respectively, are close together and positioned opposite to Glu-226. The two critical tryptophans (Trp-189 and Trp-125) line the rim of the pocket, one on each side. The Asp-147, which is less important than Glu-146 in determining the NADase activity, is located farther and slightly away from the pocket. Also indicated in the Fig. 3 is the location of Lys-129, which has been shown by a previous study to be important for the cADPR hydrolyase activity (32).

**DISCUSSION**

The results of site-directed mutagenesis presented in this study identify six residues in the catalytic domain of CD38 as important for the various aspects of catalysis. They span over 100 residues in the CD38 sequence, from Trp-125 to Glu-226, or one-third of the entire domain. There is no a priori reason from the primary sequence to suspect these widely dispersed residues are all important for catalysis. The homology model, however, makes it rather obvious, as all these residues are clustered in a pocket near the middle of the molecule. Two other notable features of the model are as follows: first, the correct placement of Lys-129 at the active site, the mutation of which has previously been shown to inhibit the cADPR hydrolyase of CD38 (32); and second, the appropriate pairing of all the cysteines for disulfide linkage. These results indicate that the model is a good representation of the active site of CD38 and that there is a great structural homology between the active sites of CD38 and the Aplysia cyclase. The homology model should thus be a valuable guide for further dissection of the mechanism involved in the novel multifunctional catalysis of CD38.

We have proposed a unified mechanism for the multifunctionality of CD38 and the Aplysia cyclase (11, 14, 33). For the cyclization reaction to occur, the substrate, either NAD or NADP, must bind to the active site in a folded conformation so that the two ends of the molecule can be linked. The pocket structure of the active site as revealed by the homology model should function well in molding the substrate into such a conformation, which is likely to involve stacking of the nicotinamide and adenine groups of the substrate. Indeed, Trp-189, through hydrophobic interaction with either of the stacked aromatic rings, could stabilize such a conformation. That substitution of Trp-189 greatly inhibited all enzymatic activities (cf. Table IV) is consistent with this notion.

The next step in catalysis could be the release of nicotinamide from NAD, with the remaining ADP-ribose forming an activated intermediate. The catalytic residue, Glu-226, most likely would participate in this step. The activated intermediate could be an oxocarbonium ion, as has been proposed for various related enzymes (34–38). The anionic Glu-226 could serve to stabilize the cationic intermediate, as has been shown in both diphtheria and Clostridium botulinum C2 toxins (36, 37). Indeed, we have shown that replacement of the anionic Glu-226 with either an Asn, Gln, or Leu completely eliminated all enzymatic activities (Table I). Substitution with an anionic Asp, however, also inhibited the activities. This could be because the anionic charge of its smaller side chain is too far away from the intermediate to affect ionic stabilization. In addition to Glu-226, two other nearby anionic residues, Glu-146 and Asp-155, could serve a similar stabilization function if the intermediate is able to orient itself closer to them. Substituting Glu-226 with a glycine, which is essentially equivalent to deletion of its side chain, could provide sufficient room for such reorientation. Indeed, we observed residual activities in E226G.

Intramolecular attack of the intermediate by N-1 of the adenine would result in cyclization and the formation of cADPR.
from NAD. On the other hand, nucleophilic attack of the intermediate by water would instead lead to hydrolysis and the formation of ADP-ribose from NAD. It has previously been reported that mutation of the two non-conserved cysteines in CD38 can stimulate the cyclase activity (39). The homology model shows that these two cysteines are linked by a disulfide bond at the middle of the molecule. Destruction of the linkage by mutation could lead to significant alteration of the conformation of the active site, restricting water access and thus stimulating the cyclase activity of CD38.

The GDP-ribosyl cyclase activity of CD38 appears to be controlled by Asp-155 (Table III). We have previously shown that cyclization of NGD occurred at N-7 instead of N-1 as in the case of cADPR (33). This would require a rotation of the guanine ring around the N-9–ribose bond to an anti-orientation. Substitutions of Asp-155 with either Asn, Glu, or Gln appear to allow easier rotation of the guanine ring, resulting in stimulation of the GDP-ribosyl cyclase activity. The two hydrolase reactions, NADase and cADPR hydrolase, show significant correlation with respect to residue replacement (Fig. 2). Both reactions could result from nucleophilic attack of the same intermediate by water. The only difference is that whether the same activated ADP-ribose intermediate is generated from NAD in one case or from cADPR in the other.

### Table III

|                  | NADase | cADPR hydrolase | GDPR cyclase | Base exchange |
|------------------|--------|-----------------|--------------|--------------|
|                   | V\(_{\text{max}}\) | K\(_{\text{m}}\) | V\(_{\text{max}}\) | K\(_{\text{m}}\) | V\(_{\text{max}}\) | K\(_{\text{m}}\) | V\(_{\text{max}}\) | K\(_{\text{m}}\) |
| WT               | 101,600 ± 8,000 | 4,200 ± 150 | 13,100 ± 800 | 74,500 ± 1,500 |
| D155N            | 67,200 ± 1,800 | 7,400 ± 300 | 40,700 ± 1,100 | 120,000 ± 7,500 |
| D155E            | 66,800 ± 1,800 | 6,800 ± 350 | 190,800 ± 21,000 | 147,000 ± 7,500 |
| D155Q            | 19,300 ± 1,000 | 2,700 ± 100 | 87,400 ± 5,400 | 75,000 ± 1,000 |

### Table IV

|                  | NADase | cADPR hydrolase | GDPR cyclase | Base exchange |
|------------------|--------|-----------------|--------------|--------------|
|                   | V\(_{\text{max}}\) | K\(_{\text{m}}\) | V\(_{\text{max}}\) | K\(_{\text{m}}\) | V\(_{\text{max}}\) | K\(_{\text{m}}\) | V\(_{\text{max}}\) | K\(_{\text{m}}\) |
| W125F            | 13.9 ± 0.5 | 18 ± 2 | 4.8 ± 0.3 | 156 ± 16 | 35.5 ± 2.5 | 5 ± 0.3 | 23.1 ± 0.7 | 21 ± 1 |
| W125Y            | 14.2 ± 0.5 | 6 ± 1 | 6.4 ± 0.4 | 218 ± 30 | 53.4 ± 1.1 | 2 ± 0.2 | 10.3 ± 0.2 | 16 ± 1 |
| W189G            | 2.1 ± 0.1 | – | 0.2 ± 0.5 | – | 0.2 ± 0.03 | – | 1.6 ± 0.7 | – |
| W189Y            | 0 | – | 0 | – | 17.4 ± 1.8 | – | 1.2 ± 0.1 | – |

**Fig. 3.** A homology model of the catalytic domain of CD38. The model is based on the crystallography coordinates of a dimer of *Aplysia* ADP-ribosyl cyclase. A, two identical monomers of CD38 are shown with one rendered with van der Waals contact surface and the other is represented as wire frame. The five conserved disulfide bonds are depicted as purple cylinders, and the non-conserved one is colored yellow. B, a stereo view of the active site pocket. The critical amino acids are shown as space-filling models; carbon atoms are shown in green, oxygen atoms in red, and nitrogen atoms in dark blue. The rendering was done using a program MOLMOL (40).
The base-exchange reaction could result from the nucleophilic attack of the same intermediate by nicotinic acid. The present study presents no clear indication of which residue could control the access of a nicotinic residue to the active site. This issue as well as the verification of the various catalytic features discussed above would have to be resolved by eventual crystallography of CD38 with various substrates bound to the active site.

Acknowledgments—We thank Kevin Mayo for help in the circular dichroism measurements and Malcolm Johns for technical assistance.

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J. Biol. Chem. 2000, 275:21566-21571.
doi: 10.1074/jbc.M909365199 originally published online April 25, 2000

Access the most updated version of this article at doi: 10.1074/jbc.M909365199

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