ADP-ribosyl cyclase synthesizes two Ca\(^{2+}\) messengers by catalyzing NAD to produce cyclic ADP-ribose and exchanging nicotinic acid with the nicotinamide group of NADP to produce nicotinic acid adenine dinucleotide phosphate. Recombinant *Aplysia* cyclase was expressed in yeast and co-crystallized with a substrate, nicotinamide. X-ray crystallography showed that the nicotinamide was bound in a pocket formed in part by a conserved segment and was near the central cleft of the cyclase. Glu\(^{179}\), Asn\(^{107}\), and Trp\(^{140}\) were within 3.5 Å of the bound nicotinamide and appeared to coordinate it. Substituting Glu\(^{179}\) with either Gln, Gly, Leu, or Asn reduced the cyclase activity by 16–222-fold, depending on the substitution. The mutant N107G exhibited only a 2-fold decrease in activity, while the activity of W140G was essentially eliminated. The base exchange activity of all mutants followed a similar pattern of reduction, suggesting that both reactions occur at the same active site. In addition to NAD, the wild-type cyclase also cyclizes nicotinamide guanine dinucleotide to cyclic GDP-ribose. All mutant enzymes had at least half of the GDP-ribosyl cyclase activity of the wild type, some even 2–3-fold higher, indicating that the three coordinating amino acids are responsible for positioning of the substrate but not absolutely critical for catalysis. To search for the catalytic residues, other amino acids in the binding pocket were mutated. E179G was totally devoid of GDP-ribosyl cyclase activity, and both its ADP-ribosyl cyclase and the base exchange activities were reduced by 10,000- and 18,000-fold, respectively. Substituting Glu\(^{179}\) with either Asn, Leu, Asp, or Gln produced similar inactive enzymes, and so was the conversion of Trp\(^{77}\) to Gly. However, both E179G and the double mutant E179G/W77G retained NAD-binding ability as shown by photoaffinity labeling with \(^{32}\)P-8-azido-NAD. These results indicate that both Glu\(^{179}\) and Trp\(^{77}\) are crucial for catalysis and that Glu\(^{179}\) may indeed be the catalytic residue.

Adenylyl cyclase was first discovered in sea urchin egg extracts as an activity converting NAD to a Ca\(^{2+}\)-releasing metabolite, which was later identified as cyclic ADP-ribose (cADPR)\(^{1}\). (1, 2). Accumulating evidence indicates cADPR is a Ca\(^{2+}\) messenger important in regulating various cellular functions in a variety of species from plant to human (reviewed in Refs. 3 and 4). Similarly, the cyclase has since been shown to be ubiquitous and is particularly abundant in *Aplysia* ovotestis (5–9). Sequence comparison reveals that the *Aplysia* cyclase is homologous to antigens CD38 on human lymphocytes and BST1/BP3 on bone marrow cells (10, 11). In addition to sharing 25–30% sequence identity, there is also a perfect alignment of the cysteines of the homologs. Subsequent work shows that all members of the cyclase family are also functionally similar, since they all are enzymes catalyzing the cyclization of NAD to cADPR (Refs. 12 and 13; reviewed in Refs. 14 and 15). More surprising is the discovery that all these enzymes also can use NADP as a substrate and catalyze the exchange of its nicotinamide group with nicotinic acid, producing nicotinic acid adenine dinucleotide phosphate (16), which is capable of activating a novel Ca\(^{2+}\) release mechanism that is totally independent of that activated by cADPR or inositol trisphosphate (17). This novel nicotinic acid adenine dinucleotide phosphate-dependent mechanism was first shown to be present in invertebrate eggs and oocytes (17, 18) and has recently been found to be important in coordinating the hormonal Ca\(^{2+}\) signaling functions in mouse pancreatic acinar cells (19), indicating that it is likely to be also a signaling mechanism of general relevance.

It is uncommon that a single enzyme can use two different substrates and produce two structurally and functionally distinct signaling molecules. A model has been proposed to account for this novel multiplicity in catalysis (15, 20–23). The salient feature of this catalytic model is the proposal that a single intermediate is responsible for cyclization, hydrolysis, and base exchange chemistries of the cyclase family of enzymes. Experimental support for this model has recently been provided (23). X-ray crystallography reveals that the *Aplysia* cyclase is a bean-shaped molecule with a central cleft (24). In solution as well as in the crystalline state, the cyclase forms dimers from two monomers in a head-to-head fashion enveloping a central cavity the size of a molecule of cADPR (24, 25). This structural feature suggests that the active site of the enzyme may be at the central region of the molecule. In this study, the *Aplysia* cyclase was co-crystallized with nicotinamide, a substrate for the base-exchange reaction. The active site of the enzyme was identified and characterized by x-ray crystallography and site-directed mutagenesis.

**MATERIALS AND METHODS**

Expression of the *Aplysia* ADP-ribosyl Cyclase in Yeast—The *Pichia* expression vector pPICZaA (Invitrogen, Carlsbad, CA) was used, and the construct consists of cloning the wild type or mutant cyclase in frame with the yeast mating α-factor signal sequence. The details of the

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‡ The abbreviations used are: cADPR, cyclic ADP-ribose; NGD, nicotinamide guanine dinucleotide; HPLC, high pressure liquid chromatography.
Co-crystallization of the Recombinant ADP-ribose Cyclase and Nicotinamide—Crystals were grown using the hanging drop method. Crystallization conditions for the recombinant cyclase were initially determined and optimized. These conditions were subsequently used to co-crystallize the enzyme with nicotinamide. Co-crystals were obtained using a reservoir solution containing 17% (w/v) polyethylene glycol 8000 (molecular weight of 4,000) and 0.1 M imidazole buffer (pH 7.5). The crystallization drops contained 2.5 μl of the reservoir solution and 2.5 μl of concentrated protein (OD = 12 at 280 nm). Crystallization trays were stored at 18 °C, and crystals displaying thin rod-shaped morphology with a length typically of 0.5 mm generally appeared within 7 days.

X-ray Structure Determination—x-ray diffraction data were collected from a single co-crystal using the A-1 station (λ = 0.919 Å) at the Macromolecular Diffraction Facility at the Cornell High Energy Synchrotron Source (MacCHESS). To reduce radiation damage, the crystal was cooled to liquid nitrogen temperature. Prior to this, the crystal was placed in a cryosolvent containing 22% polyethylene glycol 4000 to prevent ice formation within the crystalline lattice.

The diffraction data were collected using the 82 × 82-mm 2 K × 2 K Princeton CCD-based x-ray detector (29). The data indexed as a triclinic lattice and extended to 2.4 Å, displaying a very high anisotropic mosaicity, which varied from 1.75 to 3.5°. Data reduction and scaling yielded a partially complete data set with a unit cell of 60.5 × 75.3 × 138.1 Å. The overall Rsym for the data set was 6.8%, with the highest resolution shell of 2.6–2.4 Å having a high R_corr of 31%.

The crystal structure of the nicotinamide-cyclase complex was solved by molecular replacement using the program AMORE (30). A search model was constructed from the previously determined dimeric structure of ADP-ribose cyclase. Because the two cyclase monomers of this dimer were built and refined independently, a modified dimer was constructed using only the chain A monomer truncated at residue 247. Side chain atoms were retained in the search model.

Four solutions to the rotation function were found. The translation search using these orientations resulted in the position of four individual dimers in the asymmetric unit of the unit cell. The resulting positions of the four cyclase dimers displayed reasonable crystal packing contacts, suggesting that the solutions were correct. The four molecules were found to be independently positioned within the asymmetric unit of the crystal. This was verified by examining the self-rotation functions using the program GLRF (31). The best solution, according to the AMORE statistics had a correlation coefficient of 42.9 and an R-factor of 41.3.

The presence of four dimers in the asymmetric unit results in a favorable Matthews coefficient of 2.59. The four dimers give a total molecular mass of 240 kDa. The solvent content of the crystals was calculated to be 53%.

Structure Refinement—Residues 127–129 were mutated to alanines in each of the eight monomers for subsequent check of the quality and bias of electron density maps to be calculated. X-ray structure factors were calculated using the position of the atoms resulting from the placement of the four dimers in the unit cell, and these resulting structure factors were refined against the measured diffraction data. 5% of the reflections were set aside for calculating the R-free parameter. Rigid body refinement was conducted using the program X-PLOR (32) and all data between 8 and 3 Å. Because the native monoclinic structure is composed of two discrete domains (the N-terminal domain consisting of residues 1–66 and residues 100–150, the C-terminal domain consisting of residues 74–97 and residues 153–250), the refinement was carried out with each domain defined as a rigid body. In addition, rigid body refinement was conducted with each monomer defined as a rigid body. The approach where each monomer was refined as two discrete domains gave the lower R-factor. With an overall R-factor assigned as 20, the R-factor after rigid body refinement was 36.3%.

Although no proper NCS relations are present in the crystal structure, each domain of each monomer can be related to that of a reference molecule, and this procedure only takes 7 days, starting from the mutation reaction, to transforming E. coli with the mutated DNA and purifying the resulting Saccharomyces cerevisiae ADP-ribose cyclase, and as mentioned above (26, 27). Endogenous proteolytic enzymes that normally are responsible for processing the prepro-a-mating factor also removed the a-factor signal sequence from the expressed cyclase.

Some of the mutant proteins were also expressed in the X-33 strain (Invitrogen) of Pichia pastoris. This strain is isogenic to the GS115 strain, except that it contains the native alcohol oxidase AOX1 and HIS4 genes. The AOX1 gene allows the yeast to metabolize methanol and grow to higher densities, particularly during fermentation. The native histidine gene, HIS4, facilitates production of endogenous histidine and allows rapid growth of the yeast without any external supplementation of histidine. The dominant selectable marker, Zeocin, also allows for use of positive selection vectors pPICZαA. 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 mutant cyclase in the culture medium. A 10-ml culture provides more than enough protein for all of the enzymatic characterizations, and a bioreactor is not needed.

Antibodies against the Aplysia ADP-ribose Cyclase—Polyclonal antibodies were produced in rabbits by Genoys Biotechnologies, Inc. (The Woodlands, TX). The recombinant wild type cyclase produced as described above was used as antigen. Antibodies against the conserved region of the Aplysia cyclase were also raised using a synthetic peptide, TLEDTLTGLY. Western blots were visualized by chemiluminescence using a kit from Amersham Pharmacia Biotech.

Enzyme Assays—The wild type or mutant cyclase (50–3,000 ng/ml) was incubated (2–120 min) at room temperature with various concentrations of NAD (25–1,000 μM) or nicotinamide guanine dinucleotide (NGD) (10–2,000 μM) at pH 7.8 buffered by 40 mM Tris-HCl. The total volume of the reaction mixture was 0.1 ml, and the reaction was stopped by the addition of SDS (0.17% final concentration). The reaction products were analyzed by HPLC (BioCad) using either a Poros 10 HQ column (Roche Molecular Biochemicals) or an AG MP-1 column (6.6 × 150 mm). The resin AG MP-1 was obtained from Bio-Rad. The elution was performed either using a gradient of NaCl (HQ column) or trifluoroacetic acid (AG MP1 column) at a flow rate of 5 ml/min. The base line reaction was similarly assayed at pH 4.5 in the presence of 50 mM nicotinic acid and various concentrations of NADP (20–4,000 μM).

Enzyme activities of the wild type and mutant cyclases were measured, each using two or three different protein preparations. The results shown are mean ± S.E. of 9–12 determinations. The Vmax and Km values were obtained from double-reciprocal plots, and the results shown are mean ± S.E. of at least five determinations.

Site-directed Mutagenesis of ADP-ribose Cyclase—Primers for the 5′- and 3′-ends of the cyclase cDNA were as follows: 1) CYC-F2, hybridizing to the mature soluble cyclase, and also generating an EcoRI restriction site, 5′-CAT GAA TTC ATC GTC CCC ACT CGC-3′ and 2) CYC-R, hybridizing to the 3′ region of the cyclase as well as generating a NotI site, 5′-AAT GCG GCC GCG AAA TTG GTA AAG AGC-3′. Amplification was carried out using 0.5 unit of Vent polymerase (New England Biolabs), 280 μM dNTPs (New England Biolabs), and 0.4 μM primers in a 50.0-μl reaction. The mutations were carried out in two sequential amplification steps. During the first amplification, two fragments were generated. The primer CYC-F2 and an antisense primer for a specific mutation were used to generate one fragment, and the primer CYC-R and a sense primer for the same mutation were used to generate the other. For both of these polymerase chain reactions, the native cyclase sequence served as the template. The two fragments were gel-purified and used for the second amplification, during which the purified fragments served as the templates, and primers CYC-F2 and CYC-R were used to generate the 0.77-kilobase pair cyclase containing the mutation. The mutated cyclase was then gel-purified, digested with EcoRI and NotI, and ligated into a yeast expression vector that we had constructed previously (27).

Some mutations were done using the QuickChange site-directed mutagenesis kit employed by Stratagene. The high fidelity PfuTurbo DNA polymerase and mutant oligonucleotide primers to repress both strands of the supercoiled plasmid containing the insert. A mutated plasmid containing staggered nicks is generated. Following thermal cycling, the reaction mixture is treated with DpnI endonuclease, which digests hemimethylated parent DNA leaving behind the nicked DNA containing the mutation of interest. Plasmid DNA from most Escherichia coli strains is dam-methylated and can be digested away with the DpnI enzyme. This method eliminates the use of various subcloning procedures necessary to generate mutants. The procedure only takes 7 days, starting from the mutation reaction, to transforming E. coli with the mutated DNA and purifying the resulting Saccharomyces cerevisiae ADP-ribose cyclase, and as mentioned above (26, 27).
ment resulted in an R-factor of 28.4% (R-free = 31.6%). Finally, tightly restrained individual B-factor refinement gave an R-factor of 24.6% (R-free = 28.0%).

Synthesis of [32P]8-Azido-NAD and Photoaffinity Labeling—The details of the synthesis were as we have described previously (33, 34). Briefly, 8-azido-3’-AMP was phosphorylated with [γ-32P]ATP using polyribonucleotide kinase (U.S. Biochemical Corp.). The resulting 8-azido-[S-32P]3’-AMP was converted to 8-azido-[S-32P]AMP using nuclease P1 (U.S. Biochemical Corp.) and subsequently coupled to β-nicotinamide mononucleotide using 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide. This procedure produced the radioprobes with high specific activity suitable for photoaffinity labeling (33, 34). Nonradioactive 8-azido-NAD was synthesized by coupling 8-azido-AMP with β-nicotinamide mononucleotide using the same carbodiimide reaction.

Wild type or mutant cyclases (0.3 μg) were incubated on ice in 50-μl droplets containing 50 μM [32P]8-azido-NAD (530,000 cpm), 0–5 mM β-NAD, and 0.5 mM α-NAD. The droplets were exposed to 160 mJ of ultraviolet light energy (45 s) in a Stratalinker chamber (Stratagene, La Jolla, CA). Pilot experiments showed that this amount of UV energy produced optimal labeling without detectable protein-protein cross-linking. α-NAD is not a substrate for the cyclase (5) and was included in all samples to reduce potential nonspecific labeling. Samples were analyzed by SDS-polyacrylamide gel electrophoresis, and the radioactivity associated with the proteins was measured using a phosphor imager (Packard, Meriden, CT). The phosphor images were collected at 16-bit resolution for quantitative analyses but reduced to 8-bit for printout.

RESULTS

X-ray Crystallography of the Aplysia ADP-ribosyl Cyclase—We have previously shown that nicotinamide, similar to nicotinic acid, can serve as a substrate for the base exchange reaction (16). In this case, the product is the same as the substrate (NAD or NADP), and the enzyme appeared inhibited. However, the rapid exchange can be readily demonstrated using radioactive substrate (16). That nicotinamide is a substrate indicates that it should have affinity for the active site of the cyclase. We thus crystallized the recombinant cyclase with nicotinamide. The structure of the complex was refined using various procedures as described under “Materials and Methods,” giving an R-factor of 24.6% (R-free = 28.0%). Electron density maps calculated from these refined coordinates suggest that there are additional atoms present in the crystal structure. None of the maps were biased by substrate assignments, yet in the initial unaveraged 2Fo – Fc and Fc – Fo maps and all subsequent maps, strong peaks appeared where the assignments had been made. Before map averaging, the peaks were around 2.75 σ in the Fc – Fo map. After 8-fold averaging using the RAVE programs (35), the peaks were as large as 6 σ. In particular, between Glu98 and Trp140 there was strong density of the shape of a six-member ring resembling nicotinamide.

Fig. 1A shows the van der Waals contact surface of a cyclase dimer with one monomer colored light blue and the other dark blue. Each monomer was a bean-shaped molecule consisting of two domains separated by a central cleft. The two monomers associated with each other in a head-to-head fashion forming a donut-like molecule with a central cavity (24). We have previously shown by chemical cross-linking and dynamic laser light scattering that the cyclase forms dimers in solution as well (25).

The tentative assignment of the bound nicotinamide is shown in yellow in Fig. 1A. The binding site was in a pocket at the central cleft of the monomer and was very close to the central cavity of the dimer. Nicotinamide was bound to each monomer. The binding pocket of the monomer colored dark blue was not visible, because it was on the other side of the monomer. The cyclase has a stretch of six amino acids (TLEDTL) that are highly conserved with CD38, a homolog that also possesses similar enzymatic activities as the cyclase (10, 12). This conserved region formed part of the binding pocket and is shown in orange in Fig. 1A.

Fig. 1B shows the three amino acids in the binding pocket that were closest to the placement of the bound nicotinamide (yellow) and appeared to complex the bound substrate. These three residues were Trp140 (2.8 Å), Glu98 (3.5 Å), and Asn107 (3.1 Å). The conserved sequence is shown as orange sticks, and Glu98 is part of this sequence. Trp140, although not in the conserved region, is nevertheless conserved among two species of the cyclase and three species of CD38 (24). Asn107, on the other hand, is not a conserved residue. If these amino acids are indeed responsible for substrate binding, their alteration by site-directed mutagenesis should result in reduction or elimination of the enzymatic activities of the cyclase.

Site-directed Mutagenesis of the ADP-ribosyl Cyclase—Various mutations of the three amino acids closest to the bound nicotinamide were produced, and the resulting mutant cyclases were expressed in yeast as secretory proteins. The yeast media containing the mutant proteins were dialyzed and purified by treatment with an anion exchange resin (DEAE), which removed nondialyzable contaminants in the yeast media. SDS-polyacrylamide gel electrophoresis showed that the preparations of the mutant proteins after the DEAE treatment contained essentially a single protein with molecular weight of about 29,000, similar to that of the wild type protein (Fig. 2). Examples of Western analysis of some of the mutant proteins are shown in Fig. 2. All mutant proteins are recognized by the polyclonal antibody against the wild type cyclase. On the other hand, the antibody raised against the conserved peptide (anti-Con Pep) recognized the wild type, N107G, and W140G proteins but not the mutant proteins E98L and E98G. Since Glu98 is one of the amino acids in the conserved sequence, its change to leucine or glycine is expected to render the mutant proteins unrecognizable by the antipeptide antibody. The DNA from the regions containing the cyclase insert in all four mutants was amplified by the polymerase chain reaction using appropriate primers. The sequence results confirmed that they harbored the correct mutations (Fig. 2, lower panel).

Fig. 3 shows that substitution of Glu98 with either Gln, Gly, Leu, or Asn resulted in a 16–222-fold decrease in the rate of cADPR synthesis compared with the wild type cyclase, indicating that Glu98 is important for catalysis. The large decrease in activity is likely to be due to the elimination of the negative charge on Glu98 by all these mutations. The size of the substituting amino acids appears to have a secondary effect on the activity. The mutant with the conservative substitution by Gln, which is similar in size to Glu98, had only about 6% of the activity of the wild type enzyme. Smaller side chains such as Leu produced a slightly larger decrease in activity. The conversion to glycine (E98G), which is equivalent to deleting the side chain of the glutamate, produced similar inhibition. Much more destructive is substituting with a residue only slightly smaller than Gln, such as Asn, which resulted in more than 200-fold decrease in activity. It is unlikely that the inhibitory effect of the mutation was due to nonspecific alterations of the structure of the enzyme, since similar decreases were seen with such conservative substitutions as Asn and Gln, which are generally considered to have minimal effect on protein structures. More likely is that the enzyme activity has an exquisitely specific requirement for both the charge and size of the side chain at the 98-position, and any alteration would result in a decrease in activity.

Table I summarizes and compares the enzymatic parameters of several mutant cyclases with the wild type. Of the three amino acids that complex the nicotinamide, Trp140 is the most critical to the cyclase activity. It is closest to the bound nicotinamide, and its conversion to glycine resulted in a 4,648-fold decrease in activity. Asn107 is the least critical; only a 2-fold increase in activity.
decrease in activity was measured for the mutant N107G. Asp99 is next to Glu98 and is similarly charged. It is also in the highly conserved sequence, TLEDTL. Nevertheless, its conversion to glycine produced much less pronounced effect on the cyclase activity, only a 10-fold decrease. These results indicate that the effect of the site-directed mutagenesis is residue-specific and that Trp140 is the most critical residue for the cyclase activity.

In addition to comparing the maximal cyclase activity at high substrate concentration ($V_{\text{max}}$ values), Table I also lists $V_{\text{max}}/K_m$ ratios of the wild type and mutants. The Michaelis-Menten model predicts that at low substrate concentrations the enzyme activity is proportional to the substrate concentration and the proportionality constant is the $V_{\text{max}}/K_m$ ratio. The pattern of changes of the ratios follows that of the $V_{\text{max}}$ values, indicating that the mutant cyclases had lower activity at both limits of high and low substrate concentration. The kinetic parameters for W140G were not determined because its activity was too low. The value shown in Table I was measured at 1 mM NAD, which should be approximately the maximal activity.

Fig. 4 compares the cyclase activity with the base exchange activity of five of the mutants. The pattern of inhibition of both activities was similar. The mutant W140G had the least cyclase and exchange activity, while N107G had the most. The mutation thus affected both enzymatic activities similarly, suggesting that the same active site is responsible for catalyzing both reactions.

We have previously shown that the wild type cyclase can also use NGD, an analog of NAD, as substrate and cyclize it to cyclic GDP-ribose (cGDPR), a fluorescent analog of cADPR (21, 36). Table II compares the GDP-ribosyl cyclase activities of the wild type and the mutants. It can be seen that, contrary to the large decrease in the ADP-ribosyl cyclase activity (cf. Table I), all mutant cyclases retained substantial GDP-ribosyl cyclase activity. Indeed, both Glu98 mutants had activity 2–3-fold higher than the wild type. Even the W140G mutant, which only had residual ADP-ribosyl cyclase activity (Table I), had substantial GDP-ribosyl cyclase activity. Its $K_m$ value, however, was close to 40-fold higher. This disparity of the effect of the mutations on the two enzyme activities suggests that Glu98 and Trp140 may be important mainly in the positioning of the substrate and that the binding of NGD and NAD may involve different
was measured at 960 μmol/mg/min. The rate of cADPR production of the mutant cyclases was 0.79 and Methods " and compared with that of the wild type. The cyclase domains of the mutants suggest that the affinity of the mutant active sites for NAD is less than the wild type. As shown in the inset of Fig. 5 (lane 1), in the absence of NAD, the labeling intensity of E179G and the double mutant was actually higher than the wild type although the same amounts of protein were loaded onto the gel and the labeling was performed under identical conditions. In fact, quantification of the phosphor image shows that the labeling intensity of E179G and the double mutant was 2- and 1.5-fold higher, respectively, than that of the wild type. These results indicate that the inactivating effect of the mutations is not due to nonspecific alteration of the protein structure, which would expectedly destroy the substrate binding ability of the enzyme.

**The Structure of the Active Site of ADP-ribosyl Cyclase—** Fig. 1C shows a stereo view of the active site. The van der Waals contact surface of the site is shown with the three most critical amino acids, Trp77 and Trp77, and Glu179, rendered as space-filling models. The active site is in a pocket with Glu179 deep inside the pocket, while Trp77 and Trp77 line the rim of the pocket, one on each side. The carboxyl oxygen atoms of Glu179 and the carbonyl oxygen atom of Trp77, which are likely to participate directly in catalysis, are shown in red. The bound nicotinamide is shown in yellow. The aromatic ring of Trp77 is close to, and appears to position, the bound nicotinamide, presumably through hydrophobic interaction.

**DISCUSSION**

In this study, the active site of the cyclase was first identified by co-crystallizing the cyclase with nicotinamide, a substrate of the base exchange reaction. X-ray crystallography allowed the unambiguous identification of the three amino acids in the cyclase that were closest to the bound nicotinamide. To determine if they are the catalytic residues, site-directed mutagenesis was used to alter these amino acids. Although large de-
The recombinant wild type and mutant ADP-ribosyl cyclases were incubated with various concentrations of NAD. The rates of cADPR produced were measured by HPLC. The kinetic parameters, $K_m$ and $V_{max}$, were determined by double-reciprocal plots of the data. The distances shown are the shortest distances between the indicated amino acid residues to the bound nicotinamide and were determined from x-ray crystallography data.

| Amino acid residue mutagenized | Distance to the bound nicotinamide | $K_m$ (μM) | $V_{max}$ (nmol/mg/min) | $V_{max}/K_m$ (nmol/mg/min) |
|-------------------------------|-----------------------------------|------------|--------------------------|-----------------------------|
| Wild type                     |                                   | 119 ± 59   | 36,922 ± 6,503           | 310                         |
| Glu$^{90}$ → Leu              |                                   | 887 ± 73   | 78,431 ± 11,507          | 88                          |
| Glu$^{90}$ → Gly              |                                   | 283 ± 35   | 81,428 ± 12,455          | 287                         |
| Trp$^{140}$ → Gly             |                                   | 4,083 ± 469| 16,460 ± 4,069           | 4                           |
| Asn$^{107}$ → Gly             |                                   | 81 ± 17    | 52,848 ± 9,378           | 652                         |
| Asp$^{99}$ → Val              |                                   | 225 ± 32   | 30,783 ± 5,658           | 137                         |

TABLE III
Identification of the catalytic amino acid residues of the ADP-ribosyl cyclase by site-directed mutagenesis

The rates of the ADP-ribosyl cyclase and the GDP-ribosyl cyclase activity were determined using 5 mM NAD and 5 mM NGD, respectively. The base exchange activity was determined in the presence of 2 mM NADP and 50 mM nicotinic acid. The rates listed for the wild type are the $V_{max}$ values.

| Enzymatic activity | ADP-ribosyl cyclase | GDP-ribosyl cyclase | Base exchange |
|--------------------|---------------------|---------------------|---------------|
|                    | $nmol/mg/min$       | $nmol/mg/min$       | $nmol/mg/min$ |
| Wild type ($V_{max}$) | 952,785 ± 193,220 | 36,922 ± 6,503 | 351,277 ± 62,813 |
| Trp$^{140}$ → Gly | 32 ± 5             | 150 ± 21           | 112 ± 14 |
| Glu$^{90}$ → Gly | 92 ± 6             | 0                  | 19 ± 13 |
| W77G/E179G       | 0                  | 0                  | 0             |

TABLE IV
Inactivation of ADP-ribosyl cyclase activity by substituting Glu$^{97}$ with other amino acid residues

The rates of the ADP-ribosyl cyclase were determined using 960 μM NAD.

| ADP-ribosyl cyclase activity | $nmol/mg/min$ |
|-----------------------------|---------------|
| Wild Type                   | 791,000 ± 54,000 |
| Glu179 → Gly                | 92 ± 6        |
| Glu179 → Asn                | 4 ± 1         |
| Glu179 → Leu                | 5 ± 1         |
| Glu179 → Asp                | 50 ± 2        |
| Glu179 → Gln                | 40 ± 10       |

bind to the active site differently. Since the site of cyclization in cADPR is at the N1-position of the adenine, but is at the N7-position of the guanine in cGDP (36, 37), the purine ring must thus be in the anti-configuration during cyclization in the case of NGD but is in the syn-configuration in the case of NAD. These structural differences of the products are consistent with the idea that different amino acid residues may be involved in binding of the two substrates.

It is reasonable to expect that the catalytic residues should be closer to the substrate, NAD, instead of nicotinamide. It is technically difficult to co-crystallize the cyclase with NAD because it can quickly convert NAD to its product, cADPR, which may not have much affinity for the active site of the cyclase. Nevertheless, it is logical to assume that the NAD-binding site must be close to the nicotinamide-binding site. Asp$^{99}$ was a good candidate, since it was next to Glu$^{90}$, one of the coordinating residues for the bound nicotinamide, and it was also in the highly conserved sequence. Conversion of Asp$^{99}$ to glycine produced, however, less inhibition of the enzymatic activities than the mutant, E98G. To search for the catalytic residues, other residues were explored. X-ray crystallography showed that the nicotinamide-binding site is in a pocket formed in part.
that the nucleophilic attack of the oxocarbonium intermediate by the 1-nitrogen of the adenine is the dominant reaction, leading to the cyclization of NAD to produce cADPR. Access of water to the active site, on the other hand, would lead to the formation of ADP-ribose through hydrolysis, which is the dominant reaction catalyzed by CD38 (12, 41).

Another notable structural feature of the active site shown in this study is its close proximity to the central cavity formed by the two monomer cyclase molecules (Fig. 1A). The size of the central cavity is similar to the dimensions of a molecule of cADPR. This suggests the possibility that the central cavity may serve as a channel, through which the product of catalysis, cADPR, can pass (4). A recent report has now provided strong experimental support that a homolog of the cyclase, CD38, may indeed function as such a novel transporter of cADPR (42). It is shown that when resealed right-side-out red cell ghosts that contain ecto-CD38 are incubated with NAD (or NGD), active transport of cADPR (or cGDPR) occurs, generating a concentration gradient of the product 10–80-fold higher inside than outside (42). Inhibition of the enzymatic activity of CD38 by mercaptoethanol blocks the process. Similar active transport of the enzymatic products is seen with purified CD38 reconstituted into liposomes (42). That CD38 is a transporter capable of active translocation of its enzymatic products across membranes provides a novel explanation of how a surface antigen with its catalytic domain on the outside of the cells can participate in intracellular Ca²⁺ signaling (43). The structural features of the active site of the cyclase shown in this study are consistent with such a proposal.

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**FIG. 5. Photoaffinity labeling of the wild type and mutant cyclases using [32P]8-azido-NAD.** The labeled proteins (~6 μg/ lane) were analyzed by SDS-polyacrylamide gel electrophoresis, and the radioactivity associated with the proteins was visualized by a phosphor imager. The phosphor images were quantitatively analyzed at 16-bit resolution. The inset shows images of the proteins labeled with 50 μM [32P]8-azido-NAD alone (lane 1) and in the presence of 0.1 mM (lane 2), 0.5 mM (lane 2), 1 mM (lane 4), and 5 mM (lane 5) NAD. The resolution of the images shown in the inset was reduced to 8-bit.
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