The 1.8 Å Cholix Toxin Crystal Structure in Complex with NAD\(^+\) and Evidence for a New Kinetic Model

**Background:** Cholix toxin from *Vibrio cholerae* inactivates eukaryotic elongation factor 2 by transferring ADP-ribose from NAD\(^+\).

**Results:** This crystal structure of cholix toxin with NAD\(^+\) reveals new insights into the reaction mechanism of this bacterial enzyme.

**Conclusion:** This mechanism may be generally applicable to other members of this bacterial virulence factor family.

**Significance:** This new insight provides impetus for therapeutic development for treating bacterial diseases.

Protein toxins may hold the key to new drugs, vaccines, and cancer therapies. For example, antibiotic resistance is on the rise, and we need new strategies to combat bacterial pathogens that secrete exotoxins when they cause illnesses such as diphtheria, cholera, intestinal diarrhea, and whooping cough (1). One strategy is to directly inhibit such toxins using small-molecule drugs, designed with knowledge of the protein structure active site (2). This strategy reduces selection pressure by disarming, rather than killing, pathogens (3). Protein toxins also have a long history as the basis for vaccines (created by inactivating toxins) (4) and are even being used to kill cancer cells (5). In fact, a diphtheria toxin (DT)-based\(^{2}\) cancer therapy is United States Food and Drug Administration (FDA)-approved, *Pseudomonas aeruginosa* exotoxin A (ExoA)-based therapies are in phase II clinical trials (6), and there is reason to believe that cholix toxin, characterized here, holds the same potential. For example, recently, domains II and III of cholix toxin from *Vibrio cholerae* have been joined to an Fv antibody (Fv refers to the variable domain on antibodies that binds to antigens) that directs this toxin to the human transferrin receptor. This fusion protein, dubbed HB21-CET40, is potently toxic against several human cancer cell lines (7). Our interest, however, is in using cholix as a representative of an important class of toxins involved in infectious diseases.

Normally, cholera disease is associated with secreted toxins specifically from the O1 and O139 *V. cholerae* strains. However, other strains of *V. cholerae* also use secreted toxins, likely to promote survival in aquatic environments. Such toxins may allow colonization by nonpathogenic bacteria involved in symbioses. Several non-O1 non-O139 *V. cholerae* strains have also displayed virulence, indicating that other virulence factors play a role in this pathogenesis (8).

Cholix was first isolated from the *V. cholerae* TP strain from an aquatic setting in southern California (9). A screen of 83 *V. cholerae* strains from both environmental and clinical origin revealed that the *chxA* gene encoding cholix is present in one-third of cholerae strains and that it is more common in non-O1, non-O139 strains than in the O1 and O139 strains (10).

APD-ribosyltransferase toxins fall into two groups: CT (after cholera toxin) and DT (after diphtheria toxin). Cholix is the third member of the DT group. It is most similar to ExoA with respect to structure, enzyme activity, and inhibitor specificity (2, 11). Structurally, it is a three-domain A/B toxin. It consists of a receptor binding domain (R) for receptor-mediated endocytosis, a membrane translocation domain (T) for translocation to the host cytoplasm, and the catalytic domain (C) (11).

Cholix is active against eukaryotes, including crustaceans and mammals (11); it also kills yeast cells when expressed intracellularly (2, 12). Cholix enters eukaryotic cells by receptor-mediated endocytosis (11). Structural similarity to ExoA suggests activation by both reduction of a disulfide bond and cleavage by furin (or a similar protease) in the endosome, releasing the catalytic domain, which enters the cytoplasm (11).
Cholix covalently transfers ADP-ribose from NAD\(^+\) to diphthamide on eukaryotic elongation factor 2 (eEF2) to inhibit protein synthesis and cause cell death. The DT group reaction has been extensively studied (13, 14) and involves scission of the glycosidic bond (C–N) between nicotinamide and its conjugated ribose as well as transfer of the ADP-ribose group to a nucleophilic residue, diphthamide, on eEF2. Specifically, loop 1 flexibility allows this loop to form a solvent cover to exclude water and help stabilize the transition state during the reaction.

Cholix serves as a good working model for ExoA and DT because it is more amenable to crystallization (data not shown); cholix inhibitors may work against ExoA as well. Recently, our group characterized the structure and activity of several cholix inhibitors that fully protect human lung cells (2). Several cholix structures have now been reported, including the 2.1 Å full-length cholix toxin structure and several high-resolution structures of the catalytic C-terminal domain (cholix\(_C\)) (2, 11, 12). So far, there is no report of cholix in complex with its natural substrate, NAD\(^+\). However, DT and ExoA (14, 15) have previously been crystallized with NAD\(^+\) as well as CT group toxins C3b0t1 (Protein Data Bank (PDB) 2A9K) (16), iota toxin (PDB 1GIQ) (17), SpvB (PDB 2GWL) (18), EDIN-B (PDB 1OJZ) (19), CdtA (PDB 2WN7) (20), Art2.2 (PDB 1OG3) (21), Vip2 (PDB 1QS2) (22), and cholera toxin (PDB 2A5F) (23). Key to understanding the toxin reaction mechanism, and how toxin inhibitors work, is to view toxins in the context of NAD\(^+\), especially given that the NAD\(^+\) conformation is different in the toxins when compared with most other NAD\(^+\) binding proteins (24).

In this study, we present a x-ray crystal structure of cholix in complex with NAD\(^+\) coupled with a mutational and kinetic analysis of key residues and a discussion of the implications of these findings on small molecule therapies.

**EXPERIMENTAL PROCEDURES**

**Cloning chxA Gene and Site-directed Mutagenesis**—Cloning was described previously (11). Site-directed mutagenesis was performed using the Stratagene QuikChange protocol and primers ordered from Invitrogen. Sequences were confirmed by DNA cycle sequencing using an Applied Biosystems 3730 DNA analyzer.

**Overexpression and Purification of Cholix Toxin and Purification of eEF2**—We purified full-length cholix toxin, cholix catalytic fragment (cholix\(_C\)), various cholix mutants, and eEF2 as reported previously (11). Correct production of cholix mutants was confirmed using a Bruker Reflex II MALDI-TOF DNA analyzer.

**Crystallization**—Full-length cholix toxin was crystallized by vapor diffusion against reservoirs containing 23% polyethylene glycol 10,000, 7.5% ethylene glycol, and 0.1 M HEPES, pH 7.5, at 19°C. We added ~40 µl of reservoir solution containing 1.25 mM NAD\(^+\) solution to a ~2-µl crystal-containing drop and allowed the NAD\(^+\) to soak into the crystals for ~2–3 min. The crystals were transferred to Paratone-N (Hampton Research) for cryoprotection and then flash-frozen in liquid N\(_2\). Data were collected at the Canadian Light Source (Saskatoon, SK, Canada). Data were indexed, integrated, and scaled using HKL2000 (25). The full-length cholix-NAD\(^+\) structure was solved by molecular replacement using the refined structure of full-length cholix as a search model (PDB 2Q5T) (11). After transferring the R\(_{free}\) set from the 2Q5T structure and extending the resolution from 2.1 to 1.8 Å, the model was rebuilt in Coot (26) and refined first in Refmac5 (27) and finally in Phenix (28) using TLS with the molecule divided into 20 groups. The structure was validated by PROCHECK (29), and figures were prepared using PyMOL (30). The two-dimensional visualization was done using PoseView (31), LigPlot (32), and Molecular Operating Environment (MOE) 2011 (Chemical Computing Group).

**Fluorescence Measurements**—Steady-state fluorescence measurements were obtained using a Cary Eclipse fluorescence spectrophotometer (Varian Inc.) according to previously reported methods (33–37). Buffer conditions were 20 mM Tris, 50 mM NaCl, pH 7.9, at 25°C.

**Folding Integrity by Intrinsic Trp Fluorescence**—The cholix (4 µM) Trp emission spectra were obtained with excitation wavelength 295 nm and the emission collection from 305 to 450 nm (2.5 nm band-pass for both).

**Substrate Binding by NAD\(^+\)-dependent Quenching of Intrinsic Trp Fluorescence**—Cholix (1 µM, 600 µl, triplicate) was titrated using small aliquots of NAD\(^+\) (3–63 mM, ~6.5 µl). Fluorescence quantum yield of each titration point was measured with an excitation of 295 nm and emission of 340 nm (5 nm band-pass for both). Fluorescence intensity of N-acetyltryptophanamide was used to correct for the inner filter effect.

**Fluorescence-based ADPRT and NAD\(^+\) Glycohydrolase Assay**—Cholix proteins (5 nM to 59 µM) were mixed with eEF2 (~40 µM) and e-NAD\(^+\) (0–500 µM). Measurements were made in disposable ultra-microcuvettes (BrandTech Scientific) with a 1-cm path length. Settings were as follows: 305 nm excitation, 405 nm emission wavelengths, 5 nm band-pass, 300 s. The NAD\(^+\)-glycohydrolase assay was similar, except that eEF2 was replaced with buffer, the buffer contained no salt, and the assay was run for 30 min with 2 µM cholix toxin. For inhibitor characterization, a FLUOstar Omega microplate reader (BMG Labtech) was used. The excitation and emission wavelengths were set at 320 and 405 nm, respectively, with a band-pass slit width of 10 nm.

**N1-Acetyl-AMP Standard Curve and Assay Calibration**—Fluorescence intensity measurements of N1-AMP standards (1–10 µM, in water) were used to prepare standard curve with slope µM\(^{-1}\).

**Kinetic Model**—For the binding system and catalytic activity, we developed a general kinetic model for cholix. E represents cholix enzyme, S represents NAD\(^+\), and B represents eEF2. We propose sequential binding for S. The dissociation constant for S at the first substrate binding site is \(K_{D1}\); the dissociation constant at the second substrate binding site is \(K_{D2}\). We assume random binding for B, with dissociation constant \(K_{DB}\). The rate constant of the catalytic step was defined as \(k\), and the interaction factors of the binding among various intermediates were represented as \(\alpha\) and \(\beta\). See “Discussion” for further details.

We developed three kinetic scenarios based on the presence of \(E_S\) and/or \(E_{BS}\). Thus, assuming a rapid equilibrium approach, the variable \(den\), the partition function, was defined.

\[
den = 1 + \frac{[S]}{K_{D1}} + \frac{[B]}{K_{DB}} + \frac{[B][S]}{\alpha \times K_{DB} \times K_{D1}} \quad (\text{Eq. 1})
\]
Cholix-NAD\textsuperscript{+} Structure and Kinetic Analysis

Equation 1 is for the single model that considers one binding site for S \((K_{D1} \rightarrow \infty)\) in the presence or absence of B.

\[
den = 1 + \frac{[S]}{K_{D1}} + \frac{[B]}{K_{DB}} + \frac{[B][S]}{\alpha \times K_{DB} \times K_{D1}} + \frac{[B][S]^2}{\alpha \times \beta \times K_{DB} \times K_{D1} \times K_{D2}} \quad (Eq. 2)
\]

Equation 2 is for one-site binding of S in the absence of B but two-site sequential binding of S in the presence of B \((K_{D2} \rightarrow \infty, \beta \rightarrow 0)\).

\[
den = 1 + \frac{[S]}{K_{D1}} + \frac{[S]^2}{K_{D1} \times K_{D2}} + \frac{[B]}{K_{DB}} + \frac{[B][S]}{\alpha \times K_{DB} \times K_{D1}} + \frac{[B][S]^2}{\alpha \times \beta \times K_{DB} \times K_{D1} \times K_{D2}} \quad (Eq. 3)
\]

Equation 3 is for full two-site sequential binding of S in the presence or absence of B.

Now, we write explicit expressions for the observables. First, considering the full kinetic scheme, the Trp emission of the enzyme observed by fluorometric titration with substrate S is written as Equation 4

\[
\frac{F}{F_0} |_{[\theta] = 0} = 1 + (\gamma_1 - 1) \frac{[ES]}{[E]} + (\gamma_2 - 1) \frac{[ES_2]}{[E]} \quad (Eq. 4)
\]

with

\[
\frac{[ES]}{[E]} = \frac{[S]}{K_{D1}} \times \frac{1}{den} \quad (Eq. 5)
\]

\[
\frac{[ES_2]}{[E]} = \frac{[S]^2}{K_{D1} \times K_{D2}} \times \frac{1}{den} \quad (Eq. 6)
\]

where \(den\) is defined under Equation 3, \([B] = 0\), and \(\gamma_i\) is the fractional quenching or enhancement of molar fluorescence \(Q_i\) for each bound species with respect to the apo enzyme \(Q_E\)

\[
\gamma_1 = \frac{Q_{ES}}{Q_E} \quad (Eq. 7)
\]

\[
\gamma_2 = \frac{Q_{ES_2}}{Q_E} \quad (Eq. 8)
\]

and

\[
F_0 = Q_E [E] \quad (Eq. 9)
\]

using \(F\), as defined above, corrected for both background signal and the inner filter effect using N-acetyltryptophanamide as a reference.

Second, the fractional concentration of the catalytic intermediate, in terms of free substrate concentration of [S] and [B], would be

\[
\frac{[BES]}{[E]} = \frac{[B][S]}{\alpha \times K_{DB} \times K_{D1}} \times \frac{1}{den} \quad (Eq. 10)
\]

So the concentration dependence of the initial catalytic rate is Equation 11

\[
v_o = k \times [BES] = V_{max} \times \frac{[BES]}{[E]} \quad (Eq. 11)
\]

where \(v_o\) is the initial slope (in s\(^{-1}\)) of the time course of the fluorescent product formed. Independent fitting of binding data for each mutant with Equation 4 involved four parameters applying to two phases of the binding curve: \(K_{D1}\) and \(K_{D2}\) and the fluorescence descriptors \(\gamma_1\) and \(\gamma_2\). Independent fitting of activity data with Equation 11 involved six parameters applying to two phases of the activity curve: \(K_{ET}\) and \(K_{D2}\) (initially fixed to values from the binding data), \(K_{DB}\) (fixed to a literature value), and the free-floating parameters \(\alpha\), \(\beta\), and \(k\). To refine our initial fit, we then fixed \(\alpha\), \(\beta\), and \(k\) and allowed \(K_{ET}\) and \(K_{D2}\) to float freely. We minimized \(\chi^2\) by adjusting fixed and free parameter combinations until they converged with errors lower than 10% of the nominal value. Furthermore, we confirmed our results with global iterative fitting with shared parameters. We used Origin 6.1 for fitting and Maple 15 simulations to rule out alternative hypotheses and possible artifacts.

Structural Comparison with Known Inhibitors—We scored putative cholix inhibitors with the OpenEye software suite using the library of experimentally characterized poly (ADP-ribose) polymerase (PARP) inhibitors assembled by Novikov et al. (38). The library was filtered for drug-likeness (OpenEye/Filter), and the molecules were prepared considering various tautomeric states (OpenEye/Tautomers), various protonation states (OpenEye/pKatyp) using AM1-BCC charges, and various conformations (OpenEye/omega2). Fred Receptor (version 2.2.5) was used to prepare the cholix-NAD\textsuperscript{+} receptor. A 8010 Å\(^3\) box surrounding NAD\textsuperscript{+} was considered using molecular site detection. We used a high quality site shape potential, with a 49 Å\(^3\) inner contour and a 1665 Å\(^3\) outer contour. We constrained the docking by forcing Gly-461 to be an H-bond donor and acceptor. We docked the ligands (OpenEye/FRED) 10,000 times into the cholix active site and 12 reference active sites to enable multiple active site correlation. Consensus poses were selected based on scoring them with reference to the active site (Shapegauss, Piecewise Linear Potential, Chemgauss3, OEChemScore, and ScreenScore scoring functions) and with reference to the bound NAD\textsuperscript{+} (Chemical Gaussian Overlay and Chemical Gaussian Tanimoto scoring functions). The scoring was also based on how much better the molecule fit into cholix when compared with the reference active sites. The top four results were diagrammed and compared with NAD\textsuperscript{+} using PoseView (31).

RESULTS AND DISCUSSION

Cholix-NAD\textsuperscript{+} Structure—The NAD\textsuperscript{+}-bound structure of full-length cholix is similar, although not identical, to the apo form previously solved in our laboratory (11). Supplemental Table 1 shows crystallography summary statistics. The resolution is 1.8 Å, \(R_{free}\) is 20.35%, and no forbidden conformations were observed in the Ramachandran plot.

Domain I (1a–264, lb 387–423) (Structural Classification of Proteins (SCOP) b.29.1.7, CATH protein structure classifica-
tion 2.60.120.200) is an all-β domain. This domain resembles concanavalin A-like lectins and glucanases. It has a complex topology, forming a sandwich with ~12–14 strands in two sheets (Fig. 1a, supplemental Fig. 1). Domain II (265–386) (SCOP f.1.5.1, CATH 3.90.1350.10) is characteristic of membrane and cell surface proteins. It is a multihelical domain that unfolds in the membrane. The catalytic domain III (424–634) (SCOP d.166.1.1, CATH 3.90.175.10) consists of antiparallel β sheets and a separate helical region.

There are four disulfide bonds: C11–C15, C208–C225, C278–C300, C394–C401 (Fig. 1a). Apparently, none of these block the active site. It is generally thought that the flexible loop L1 is closed in full-length toxin such that it sterically blocks NAD⁺ binding and that it is only open in the enzymatically active catalytic domain (39). In full-length ExoA, the backbone of residues 458–462 and side chains of Arg-458, Gln-460, and Leu-462 sterically interfere with binding of the adenosine moiety and the diphosphates. However, in cholix, the NAD⁺ was able to bind without any structural activation or perturbation of the protein. The L1 swivel points in ExoA are Ala-457 (replaced by Pro-476 in cholix) and Ala-464 (replaced by Glu-487 in cholix). Perhaps Pro-476 influences the L1 swivel to lessen its ability to cap the binding pocket.

The cholix active site is shown in Fig. 1b (and in two-dimen- sional format in supplemental Fig. 2). Hydrogen bonds include: Gly-461 to nicotinamide, Arg-479 and Glu-581 to nicotinamide ribose, Gly-480 to adenine phosphate, His-460 and Thr-462 to adenine ribose, and Ala-475 and Gln-356 to adenine. Lys-508 may form an ionic interaction with the nicotinamide phosphate. Aromatic interactions include stacking the nicotinamide ring between Tyr-493 and Tyr-504. We highlight Trp-586 because it is conserved in other toxins and may be important to the mechanism. Hydrophobic interactions with NAD⁺ may include Val-360, Thr-469, Ile-470, Val-477, Leu-492, and Val-500. Destabilizing hydrophobic-hydrophilic contacts with NAD⁺ may include Thr-359, Ala-466, Thr-469, Ile-474, Val-477, Pro-478, and Leu-492 (supplemental Table 2). Considering all interaction categories, the most energetically favorable interactions with NAD⁺, predicted with MOE and in order of decreasing importance are: Lys-508, Glu-581, His-460, Gly-480, Gly-461, Gln-356, Thr-462, Arg-479, Ile-470, and Tyr-504. NAD⁺ is shown in the nicotinamide binding pocket, indicating that as expected, it is well matched to the active site both sterically and electrostatically (Fig. 1c).

Interestingly, the NAD⁺ adenine ring is rotated in this structure when compared with DT and ExoA (Fig. 1d; supplemental Fig. 3). Variation in the adenine ring has been previously observed for CT group toxins, but not for the DT group. The adenine orientation may not be crucial to the enzyme function, giving rise to such variation. In fact, comparing all the mART-A complexes (Fig. 1e), we see that adenine orientation varies much more than the rest of the molecule. Also, NAD⁺ binding failed to trigger loop movements (Fig. 2a) and resulted in minimal changes in side chain orientation overall (Fig. 2b), except that Arg-479 and Lys-508 were somewhat rotated in the NAD⁺-bound structure when compared with the apo structure. A proposed overall cholix-NAD⁺-eEF2 Michaelis complex is shown in Fig. 2c based on our previous x-ray structure of ExoA with eEF2 and NAD⁺ (13, 14). A close-up of the cholix-NAD⁺-eEF2 interface within the proposed complex is shown in Fig. 2d. Cholix-NAD⁺ is shown in the context of its many relat- es, ExoA and DT, as well as the PARPs to illustrate sequence-structure-function relationships (supplemental Fig. 4). We also show cholix-NAD⁺ alongside three states of its closest relative ExoA, illustrating the range of variation in loops L1 and L4 (supplemental Fig. 5).

**Toxin-NAD⁺ Architecture Comparison—**NAD⁺ conformation within toxin active sites has been extensively examined recently (24). Generally, the NAD⁺ conformation is a nontrivial consideration because it can access a large conformational space and it can potentially form many interactions, including electrostatic interactions with the negative pyrophosphate and the positive nicotinamide; hydrogen bonding between the carboxamide and N-ribose hydroxyl; and σ-stacking with the nicotinamide and adenine rings. NAD⁺ usually adopts a compact conformation in solution and a more extended conformation when bound to protein. Enzymes that cleave the N-glycosidic bond, such as the ADP-ribosyltransferases, bind NAD⁺ with various χ₅ angles and adenine mononucleotide conformations (the χ₅ is defined as the dihedral O⁴D–C¹D–N¹N–C²N; see supplemental Fig. 6). Previously, it was thought that χ₅ ≈ 0 and that short P⁵N₁–N¹ distance in many toxin structures strained the N-glycosidic bond to promote cleavage of the nicotinamide. For cholix-NAD⁺, χ₅ is 5.7°, and the P⁵N₁–N¹ distance is 5.06 Å. These values are typical and may not be the basis for straining the N-glycosidic bond because they are observed in other NAD⁺ complexes where cleavage does not occur including enoyl reductases and dehydrogenases (24).

**NAD⁺ Binding and ADP-Ribosyltransferase Activity—**Our NAD⁺ binding and ADP-ribosyltransferase data are summarized in Fig. 3 and Table 1. The cholix-NAD⁺ structure shows a single bound NAD⁺, and the NAD⁺ binding data can be fit using a single-site model for some mutants (e.g. E574A, E581A, and the E574A/E581A double mutant). For some cases, we observed biphasic quenching (γ₁ ≠ γ₂). (See supplemental Fig. 7 for an example.) In the Y493A, Y504A, and Y504F cases, we observed some recovery of Trp fluorescence (γ₂ > γ₁), which is only possible with more than one NAD⁺ substrate bound; in the cholix-wild type, case we observed further quenching (γ₂ < γ₁) pertaining to the doubly bound species. The binding experiments were done with the same conditions (|E|ₜₐ₉₉₉ range of NAD⁺, temperature, buffer, cuvette, and geometry) and corrected for the inner filter effect using the same procedure (empirical correction factors for quenching of N-acetyltrypto-phanamide as a reference signal); the biphasic behavior can only be explained by substrate interaction.

Further, our ADPRT activity assays often showed lower activity at high ε-NAD⁺ concentration (e.g. for wild type cholix, Y493A, Y504A, and E574A/E581A), also suggesting substrate inhibition, explained only by a second bound substrate; other mutants did not present this behavior (e.g. E581A) even under the same range of substrate explored (ε-NAD⁺) and concentration of co-substrate (eEF2). Thus, the possibility of an artifact in the assays or the analysis is small. One possible consideration is the difference in |E|ₜₐ₉₉₉ and the absolute value of the activity; however, the phenomenon was observed for low
**FIGURE 1. Cholix-NAD⁺** structure.  
*a* Full-length cholix-NAD⁺ structure. Blue, domain Ia; orange, domain Ib; red, domain II; green, domain III; black, NAD⁺ (colored by atom). Interestingly, this structure contains NAD⁺ with intact disulfide bonds (C11–C15, C208–C225, C278–C300, C394–C401) in cholix, which are shown in yellow. 
*b* cholix active site with bound NAD⁺. Key residues discussed in this work are highlighted. Hydrogen bonds include: Gly-461 to nicotinamide, Arg-479 and Glu-581 to N-ribose, His-460 and Thr-462 to adenine ribose, and Ala-475 to adenine. Aromatic interactions include stacking the nicotinamide ring between Tyr-493 and Tyr-504. We highlight Trp-586 because it is conserved in other toxins and may be important to the mechanism.  
*c* active site electrostatics for cholix-NAD⁺, generated using the AMBER force field and using the APBS PyMOL plugin (57). NAD⁺ is shown in ball-and-stick format and is colored by element.  
*d* NAD⁺ conformation. Orientation of the adenine ring is flipped for cholix (gold) when compared with ExoA (388H, purple, r.m.s.d. 1.623 Å) and DT (1TOX, red, r.m.s.d. 1.502 Å). There is much more variation in the position of the adenine than the nicotinamide in an overlay of toxin-NAD⁺ structures for both the CT and the DT groups. NAD⁺ from cholix (black), DT (1TOX, red, r.m.s.d. 1.502 Å), ExoA (2ZIT, green, r.m.s.d. 1.672 Å), CT (2ASF, blue, r.m.s.d. 1.669 Å), iota (1GIQ, gold, r.m.s.d. 1.421 Å), SpvB (2GW, purple, 2.04 Å), Vip2 (1IQ2, cyan, 2.06 Å), C3bot (2A9K, orange, r.m.s.d. 1.532 Å), EDIN-B (1OJZ, wheat, 1.603 Å), CdtA (2WN7, brown, 1.442 Å), and Art2.2 (1OG3, pale blue, r.m.s.d. 2.074 Å) is shown. Alignments were constructed using LigAlign (58) and considered all 44 NAD⁺ atoms encoded in the PDB files.
(e.g. wild type cholix), medium (e.g. Y504A), and high (e.g. Y493A, E574A/E581A) toxin concentrations, indicating that the model applies for different $[E]_{\text{total}}$.

Furthermore, we ruled out numerous alternative explanations (product inhibition, limiting effects of eEF2, equilibrium among diverse $e$-NAD$^+$ intermediates with different hydrolysis rates, fluorescence inner filter effect, and other fluorescence measurement artifacts) and ensured that the measured slopes pertained to the initial rate (linear portion of the progress curves). With these considerations, we present a model of cholix function that reconciles NAD$^+$ binding and ADPRT data.

As described under “Experimental Procedures,” we established the simplest model (Fig. 3a) that accounts for our observations in binding and activity of cholix ($E$) with NAD$^+$ ($S$) and eEF2 ($B$). We consider the affinity of $E$ for $S$ for one or two binding sites ($K_{D1}$ for the first $S$ and $K_{D2}$ for the second $S$) and $B$ ($K_{Dh}$), the rate constant $k$ of the catalytic step; and the interaction factors between various species ($\alpha$ and $\beta$, as in Fig. 3a). We consider all steps bidirectional (described by dissociation con-
Cholix-NAD⁺ Structure and Kinetic Analysis

FIGURE 3. Kinetic model to reconcile NAD⁺ binding and ADP-ribosyltransferase activity data. a, a model for cholix toxin (E) and its interaction with NAD⁺ (S) and eEF2 (B). The model is random with respect to S and B and sequential with respect to the first and second S molecules. $K_{D1}$ applies to the first S occupancy of the active site, and $K_{D2}$ applies to the second S occupancy; $K_{D0}$ applies to B. The rate constant of the catalytic step $k$ and the interaction factors between various species are $\alpha$ and $\beta$ as shown. All steps are bidirectional except the catalytic step, which is unidirectional because only initial velocities were measured. B is needed for catalysis, and BES is the only active ternary species. The quaternary complex BES₂ is considered inactive. b, a graphic showing hypothetical NAD⁺ binding and two possible models of substrate inhibition. Model 1 involves two physical sites for NAD⁺; the first NAD⁺ is in black, and the second NAD⁺ is in gray. Model 2 is based on greater dissociation of nicotinamide when compared with the phosphates and ribose of NAD⁺. A = adenine, R = ribose, P = phosphate, and N = nicotinamide. c, summary of NAD⁺ binding based on quenching of intrinsic Trp fluorescence for full-length cholix, cholixc, and various cholixc mutants. Cholix cat, cholix catalytic fragment. d, a sample graph showing quenching of intrinsic fluorescence (FL, fluorescence intensity) of wild type cholix by fluorometric titration. e, relative ADPRT activity for full-length cholix, cholixc, and various cholixc mutants. f, sample graph showing concentration dependence on initial velocity for wild type cholixc. For comparison with the observed data, we show both a theoretical uninhibited curve (dashed line) as well as the theoretical pure inhibition component (dotted line). See “Experimental Procedures” and Table 1 for experimental details. Error bars in panels C, D, E, and F indicate S.D.

stants) except the catalytic step, which was assumed unidirectional (described by a unimolecular rate constant) because only initial velocities were measured and product was assumed to be absent at initial time.

The model is a random binding system for both substrates; that is, either S or B can bind first. It is sequential with respect to the first and second S molecule; that is, S must bind to the active site before another S molecule binds. B is required for catalysis, but the quaternary complex BES₂ was considered inactive ($k_1 > 0$, $k_2 = 0$), leaving the ternary BES as the only active species. (A model involving an active BES₂ quaternary complex where $k_1 > k_2 > 0$ may be possible, but was not chosen because it is more complex without specific supporting evidence.) The equations for the model are under “Experimental Procedures.”
Biphasic quenching during the binding assay and substrate inhibition during the activity assay indicate two binding sites, which could be either in a distinct physical location (Fig. 3b, Model 1) or in the same physical location (Fig. 3b, Model 2). The location of the second binding site is not clear. The cholixc-NAD\textsuperscript{+} structure presented here and the various ExoA-NAD\textsuperscript{+}-eEF2 complexes (PDBs 2ZIT, 3B78, 3B82, and 3B8H) show no structural evidence of a second bound NAD\textsuperscript{+}. Furthermore, a second physical site does not explain the sequential nature of our kinetic model or the dramatic differences in $K_{d2}$. However, several crystal structures of the wild type cholixc catalytic domain show small molecule inhibitors consistently binding to the same secondary site (Supplemental Fig. 8; PDBs 2Q6M, 3K1O, 3K1I, 3K1Z, 3K1S) (2, 11, 12). This secondary site is occluded in the full-length structure by domain Ib, perhaps blocking NAD\textsuperscript{+} from binding there in the full-length crystal structure (but not the catalytic fragments used during the fluorescence-based assays).

Alternatively, the complex nature of the active site might allow more than one NAD\textsuperscript{+} molecule to bind to the same physical site (model 2). Interestingly, the tankyrase (relatives of the mART toxins with a similar structure) can sometimes accommodate multiple, small molecule inhibitors in a single physical active site (40).

The sequential nature of our model may arise because the first NAD\textsuperscript{+} molecule occupies the whole binding site, minimizing the energy of the complex. We believe that the second NAD\textsuperscript{+} molecule only binds because of the dynamic nature of the interaction with the first NAD\textsuperscript{+} molecule. That is, physically, there is only one binding site visualized by the crystal structure, but functionally, there are two sites in some cases, allowing substrate inhibition to occur. Furthermore, a single physical site is consistent with dramatically different $K_{d2}$ values among the cholixc mutants. Notably, physiological concentration of NAD\textsuperscript{+} has been reported as ~370 $\mu$M in animal cells (41, 42) and 1–2 mM in yeast cells (43). Thus, the range of concentrations used in our experiments is physiologically relevant. Of course, not all of the solution interactions are captured in the crystal structure. The sequential character of NAD\textsuperscript{+} binding proposed here includes the possibility that only one kinetic (or conformational) intermediate of the complex, the one where the nicotinamide moiety is detached, is able to bind a second molecule of NAD\textsuperscript{+}. Crystallization and crystal freezing conditions may abrogate that interaction.

The NAD\textsuperscript{+} molecule is complex, and its corresponding binding site involves multiple parts (Fig. 3b). Previous work by our laboratory (44) has shown that for ExoA, nicotinamide dissociates more readily than NAD\textsuperscript{+}, AMP, ADP, or ADP-ribose. Thus, nicotinamide dissociation might be the basis for allowing a second molecule of NAD\textsuperscript{+} to bind to the active site, producing a quaternary complex that hinders activity.

The marginal interaction of the second NAD\textsuperscript{+} molecule is consistent with the high $K_{d2}$ found here. Considering ExoA, our laboratory (44) previously reported that two molecules of AMP and ADP bind the active site, which is consistent with our idea that more than one molecule can share the active site, with affinities dependent on the individual interaction components. However, the binding of only one ADP-ribose molecule reported by our laboratory (44) may be because the terminal ribose may stabilize the complex with ADP-ribose and occupy the site more fully; lacking nicotinamide, a second ADP-ribose molecule cannot bind. Further, our laboratory (44) also showed that the nicotinamide failed to have a substantial difference in binding affinity ($K_{d2}$ for NAD\textsuperscript{+} and ADP-ribose were 47 and 58 $\mu$M, respectively). Further evidence for the simultaneous binding of two NAD\textsuperscript{+} molecules is that the quantum yield of e-NAD\textsuperscript{+} is enhanced only after cleavage and release of the nicotinamide moiety, rather than upon binding to the toxin, combined with the lower fluorescence quantum yield of the free e-NAD\textsuperscript{+} because of intramolecular self-quenching by the col-

### TABLE 1

**Kinetic parameters for cholixc wild type and mutants**

$K_{d1}, \gamma_1$, and $\gamma_2$ were determined for NAD\textsuperscript{+} by quenching intrinsic Trp fluorescence as described under “Experimental Procedures.” Briefly, 600-$\mu$L solutions of 1 $\mu$M cholixc were prepared in triplicate in 20 mM Tris and 30 mM NaCl, pH 7.9, at 25 °C and titrated using small aliquots of NAD\textsuperscript{+} (~6.5 $\mu$M NAD\textsuperscript{+}; concentration ranging from 3 to 62.76 mM). The fluorescence quantum yield was monitored in triplicate, and values were corrected to account for background signal using a blank sample and inner-filter effect using N-acetyltryptophanamide titration. ADPR activity was assessed in triplicate using various concentration of cholixc and cholixc mutant (ranging from 5 nM to 59 $\mu$M final) with a saturating amount of eEF2 (~40 $\mu$M final) and various concentrations of e-NAD\textsuperscript{+} (ranging from 0 to 500 $\mu$M final) in 20 mM Tris-HCl and 50 mM NaCl, pH 7.9, at 25 °C in a total reaction volume of 70 $\mu$L. For all cases, $\chi^2 < 5 \times 10^{-4}$ and $r^2 > 0.92$.

| Full-length cholixc | Cholixc\textsubscript{E574A} | Cholixc\textsubscript{E581A} | Cholixc\textsubscript{E574A}\textsubscript{E581A} | Cholixc\textsuperscript{+} | Cholixc\textsubscript{Y493A} | Cholixc\textsubscript{Y504A} | Cholixc\textsubscript{Y504F} | Cholixc\textsubscript{E579R} |
|---------------------|---------------------|---------------------|---------------------|---------------------|---------------------|---------------------|---------------------|---------------------|
| $K_{d1}$ ($\mu$M)   | 513 ± 21             | 79 ± 6              | 333 ± 10             | 531 ± 21             | 67 ± 8              | 87 ± 2              | 87 ± 2              | 216 ± 9              |
| $\gamma_1$          | 0.83 ± 0.004         | 0.326 ± 0.012       | 0.442 ± 0.006        | 0.396 ± 0.009        | 0.659 ± 0.030       | 0.440 ± 0.005       | 0.075 ± 0.005       | ND                  |
| $K_{d2}$ (s\textsuperscript{-1}) | 2.22 ± 0.14\textsuperscript{a} | 9.6 ± 0.3\textsuperscript{b} | 0.0012 ± 0.0001      | 0.0028 ± 0.0002      | 35.2 ± 1.7          | 0.078 ± 0.004       | 0.22 ± 0.02         | 28.3 ± 0.3\textsuperscript{c} |
| $\gamma_2$          | ND                   | ND                   | ND                   | N/A                 | 105 ± 6             | 1333 ± 460          | 1103 ± 88\textsuperscript{d} | 5129 ± 5552         |
| $K_c$ (s\textsuperscript{-1}) | ND                   | ND                   | ND                   | ND                  | 0.380 ± 0.008       | 0.643 ± 0.026       | ND                  | 1.000 ± 0.317       |
| $E_{\text{bound}}$ (s\textsuperscript{-1}) | 0.010                | 0.010               | 26                   | 5.32 ± 0.57         | 1.47 ± 0.17         | 4.19 ± 0.57         | ND                  |
| $E_{\text{bound}}$ (s\textsuperscript{-1}) | 0.006–0.020          | 1.0                 | 1.0                  | 0.010               | ND                  |

\textsuperscript{a} Measurement based on two independent experiments.

\textsuperscript{b} These binding values were obtained from activity fitting because quenching was minimal against background during quenching experiments and accurate fitting of those data was not possible.

\textsuperscript{c} ND implies not determined.

\textsuperscript{d} Detailed activity measurements were made only in cases where a substantial (>15-fold) reduction of activity occurred when compared with wild type cholixc, during preliminary assessment at saturating conditions where [e-NAD\textsuperscript{+}] = 300 $\mu$M. This particular value is based on a ratio against wild type cholixc, from our initial assessment.

\textsuperscript{e} N/A implies value not needed for fitting.

\textsuperscript{f} The value of $K_{d1}$ was not directly measured. It was assumed to be 1.4 $\mu$M during fitting, the value for ExoA and a typical value for this family (1.36 ± 0.090 for ExoA and 1.204 ± 0.117 for DT, unpublished data by X. Liu (59)).

\textsuperscript{g} Interaction factors may differ from the true values due to high [E] required to observe activity for these extremely inactive mutants.

\textsuperscript{h} Concentrations refer to activity measurements.
Cholix-NAD⁺ Structure and Kinetic Analysis

lision of the nicotinamide and e-adenine moieties (45). It appears that the nicotinamide and -adenine moiety continue interacting even in the bound state, which is consistent with our idea that nicotinamide forms dynamic interactions (Fig. 3b).

The first characterization of cholix, based on a Michaelis-Menten expression, produced a \( K_m \) of 45 \( \mu \text{m} \) (11). However, because this substrate inhibition prevents direct observation of the true \( K_m \), we can mathematically estimate it as \( \approx 417 \) \( \mu \text{m} \) (Fig. 3, “no inhibition” curve). The former value corresponds approximately to \( K_D \) in our expanded substrate inhibition model, that is, the concentration at 5 half of the apparent maximum velocity, \( V_{\text{max app}} \). Also, it is possible to set \( K_D \) artificially low during fitting to simulate the “pure” inhibition component (Fig. 3, “inhibition component”). In this case, \( K_D \) for inhibition corresponds to \( K_D^{22} \). During fitting to the various mutants, we established only the minimal necessary binding and activity scheme to explain the data. As part of adopting a minimal model, not all parts of the reaction scheme were always used; sometimes, binding or activity required only a single site (i.e., using \( K_D^{21} \) only) to achieve an appropriate fit. For example, for fitting the wild type and E574A/E581A data, we used two sites for both binding and activity, whereas for E581A, we used only one site for both binding and activity (making this case Michaelis-Menten). For Y493A and Y504A, we found the best results using one site for binding, but two for activity.

The relative binding and activity results are shown in Fig. 3, and the absolute values are presented in Table 1. Considering binding first (Fig. 3, c and d), the catalytic domain of cholix, cholix⁺, had a much greater affinity for NAD⁺ (\( K_D^{21} = 67 \pm 8 \) \( \mu \text{m} \)) than the full-length toxin (\( K_D^{21} = 513 \pm 21 \) \( \mu \text{m} \)). Low affinity for NAD⁺ in the full-length toxin is consistent with DT group toxins requiring activation by furin cleavage at an arginine-rich loop and/or reduction at disulfide bonds (46, 47); however, it is clear both structurally and biochemically that full-length cholix is still capable of binding NAD⁺, although relatively poorly. Interestingly, some recent studies claim that the toxicity of ExoA results from translocation of the full-length toxin directly across the endosome and into the cytoplasm (rather than via the retrograde pathway to the endoplasmic reticulum) and that the C-terminal fragment is unstable in the cytoplasm (48), casting some doubt on the conventional model. Notably, the \( K_D^{21} \) for cholix⁺ is in the same range as the \( K_D^{21} \) for ExoA (\( K_D^{21} = 35 \pm 3 \) \( \mu \text{m} \)) (11).

Within the region 3 ARTT loop (L3) that is implicated in both NAD⁺ binding and interaction with the eEF2 target, both the E581A mutant (corresponding to the primary catalytic Glu, Glu-553, in ExoA) and the double mutant E574A/E581A (corresponding to the Glu-546/Glu-581 double mutant in ExoA, which involves both the primary and the secondary catalytic Glu residues) had lower affinity for NAD⁺ (\( K_D^{21} = 333 \pm 10 \) and 531 \( \pm 21 \) \( \mu \text{m} \), respectively). Recall from the crystal structure that E581A H-bonds to the N-ribose, so substitutions involving this residue are expected to abrogate binding. Interestingly, E574A (E546A in ExoA) did not have a large effect on NAD⁺ binding on its own (\( K_D^{21} = 79 \pm 6 \) \( \mu \text{m} \)), but appeared to contribute in the case of the double mutant. Interestingly, cholix Glu-579 corresponds to Arg-551 in ExoA, resulting in a reversal of charge at that position. To probe this difference, we assessed E579R and found that it did not change binding significantly (\( K_D^{21} = 98 \pm 2 \) \( \mu \text{m} \)).

The Tyr residues that flank the nicotinamide, Tyr-493 and Tyr-504 (Tyr-470 and Tyr-481 in ExoA), had little effect on NAD⁺ binding (\( K_D^{21} \) and \( K_D^{22} \) = 87 \( \pm 2 \) and 87 \( \pm 7 \) \( \mu \text{m} \), respectively), not differing substantially from wild type cholix⁺. Interestingly, the Y504F mutant apparently had lower affinity for NAD⁺ than Y504A, despite Y504F generally being regarded as a more conservative mutation. In both cases, the H-bond to the nicotinamide ribose is lost, but perhaps the bulky nonpolar Phe disrupted NAD⁺ binding more than the much less bulky Ala. As well, because

\[
\Delta G = \Delta H - T \Delta S \tag{Eq. 12}
\]

and

\[
\Delta G = -RT \ln(K_D) \tag{Eq. 13}
\]

there can be entropic (\( \Delta S \)) effects that play a role in changing \( K_D \) as well as enthalpic effects. For example, mutation to Ala might make \( \Delta G \) more negative by making \( \Delta S \) larger and overcome a more positive \( \Delta H \). Also, Y493A and Y504A had a significantly lower preference for binding a second NAD⁺, providing some support that the binding of a second NAD⁺ may occur in the same cleft as the first.

As mentioned, \( K_D^{22} \) tended to be high, indicating clear preference for the primary NAD⁺ binding site and, in some cases, \( K_D^{22} \) was assumed infinite (no second site, Equation 1). However, the wild type catalytic domain had a relatively low \( K_D^{22} \) (\( 105 \pm 6 \) \( \mu \text{m} \)), and we were able to estimate \( K_D^{22} \) for Y493A, Y504A, and E579R.

In terms of ADP-ribose transferase activity (Fig. 3, e and f), wild type full-length toxin apparently has some activity, although at levels more than 15-fold lower than the corresponding catalytic fragment, cholix⁺. Activity was virtually abolished for the E581A and E574A/E581A mutants (29,300- and 12,600-fold, respectively), in part due to weaker NAD⁺ substrate binding, impaired productive substrate binding, and an inability to stabilize the transition state complex (11, 14). Activity in these mutants is so low that accurate measurements are challenging. In particular, the high enzyme concentration required to observe activity for these extremely inactive mutants may affect the [S] \( \gg \) [E] Michaelis-Menten kinetic assumption in these cases; the interaction factors \( \alpha \) and \( \beta \) may differ from their true values and could also explain why \( k \) was higher for E574A/E581A when compared with E581A. In region 3, the E574A substitution reduced activity \( \approx 3.7\)-fold, a mild effect, but still indicating possible involvement of this residue during the course of the reaction. E579R was very similar to wild type, with only a 1.6-fold reduction in activity.

Marked reduction in activity was observed for both region 2 mutants, Y493A and Y504A (450- and 160-fold, respectively), indicating that these residues play an important role during the reaction. This is also consistent with Tyr residues playing a key role during the reaction for CT group toxins (e.g., Tyr-251 in iota toxin). Activity was about 8-fold lower for Y504F when compared with wild type cholix⁺, representing a modest reduction.
FIGURE 4. Cholix-NAD<sup>+</sup> as context for cholix inhibitors. a–d, 4-amino-1,8-naphthalimide (a), 1-(4-(2,4-dioxo-3,4-dihydroquinazolin-2H-yl)butyl)-4-phenyl-1,2,3,6-tetrahydropyridinium (b), 10-(aminomethyl)-4,5,6,7-tetrahydro-1H-cyclopenta[a]pyrrolo[3,4-c]carbazole-1,3(2H)-dione (c), 4-(4-hydroxyphenyl)-1-(3-(4-oxo-1,4-dihydroquinazolin-2-yl)propyl)-1,2,3,6-tetrahydropyridinium (d). Structures were depicted with PoseView (31). Further details are in supplemental Table 4.
Cholix-NAD$^+$ Structure and Kinetic Analysis

in activity likely due to loss of the H-bond between Tyr-504 and the N-ribose.

As stated, our favored model explains the phenomenological evidence of substrate inhibition and biphasic binding curve by means of a direct interaction between two NAD$^+$ molecules in the same binding pocket, although other explanations might be possible. Similar experimental evidence would come from allosteric interaction between two distant NAD$^+$ binding sites; both models are mathematically the same. For further comparison of activity and binding data between cholix, ExoA, and diphtheria toxin, see supplemental Table 3.

Glycohydrolase Activity and Folded Integrity—Many ADPRTs show glycohydrolase activity corresponding to the hydrolysis of NAD$^+$ in the absence of target protein. Glycohydrolase activity is considered background activity, representing enzyme “leakiness” and having unknown physiological relevance. However, it can also be viewed as a measure of the integrity of the active site. Both full-length cholix and the catalytic domain, cholixc, showed glycohydrolase activity, although the full-length cholix was at levels more than 15-fold lower than wild type cholix (supplemental Fig. 9a). That full-length cholix retains some glycohydrolase activity is consistent with the enzyme binding NAD$^+$. The E581A mutants, both full-length cholix E581A and catalytic cholix, E581A, were tested for glycohydrolase activity, and marked differences when compared with wild type were observed in both cases. The integrity assay confirmed that the cholix, mutants were correctly folded (supplemental Fig. 9b).

Implications for Cholix Reaction Mechanism—Interestingly, NAD$^+$ binding in the absence of the eEF2 substrate fails to trigger loop movements. However, flexible loop 1 (L1, Fig. 2 and supplemental Fig. 5) is expected to cap the binding pocket upon eEF2 binding, forming a cover over the active site and restricting solvent entry (11). Loop closure generally excludes water from the active site and decreases the dielectric constant to allow enhanced interaction with polar amino acids and stabilization of the transition state within the active site (49). (A low dielectric is also favorable to the extended NAD$^+$ conformation.) Also, the basicity of the carboxylate groups of the glutamic acid residues may increase due to this same effect, which would increase their catalytic power (49). The current structure and kinetic information is consistent with the existing model of a random, third order SN1 mechanism. During the reaction, the trimethyl ammonium group of the diphthamide reaches toward the nicotinamide phosphate. L1 flips down and hydrogen-bonds to NAD$^+$ and diphthamide. The ADP-ribose is transferred from NAD$^+$ to N3 of the diphthamide imidazole. Refer to Refs. 13 and 14 for exhaustive studies on the proposed mechanism.

Cholix-NAD$^+$ Provides Context for mART and PARP Inhibitors—The DT, ExoA, cholix, and PARP active sites show remarkable similarity. (For PARPs active sites, see PDBs: 1WFX, 1GS0, 1PAX, 2PQF, 2RF5, 2X5Y, 3BLJ, 3C4H, 3GWJ, 3G0Y, 3HKV, 3KJD, and 3MHJ). Furthermore, some inhibitors work across these similar active sites; for example, PJ34 works for both toxin and PARPs (PDBs 1XK9, 2Q6M, 3CE0, and 3GEY). One use of the cholix-NAD$^+$ structure is to score and rank sets of inhibitors. We conducted a focused, small-scale test in which we scored known PARP inhibitors against the cholix active site, the bound NAD$^+$ ligand, and reference active sites. There are no reported PARP-NAD$^+$ structures, the current cholix-NAD$^+$ structure is slightly better resolved than the existing DT-NAD$^+$ (PDB 1ITOX, resolution 2.30 Å), and the existing ExoA-NAD$^+$ structure (PDB 3882, resolution 2.35 Å) is in the presence of eEF2, which may influence the position or conformation of NAD$^+$. Thus, the current structure is the highest resolution DT/PARP group structure in complex with NAD$^+$ and presents a unique opportunity to create a context for mART/PARP inhibitors without any confounding factors. Importantly, computational algorithms have allowed us to reproduce crystallographic poses from previous work (2) to good approximation for our purposes and provide insight to other representative, drug-like inhibitor types. The top-ranked, drug-like inhibitors based on this approach are shown in Fig. 4 and detailed in supplemental Table 4. For example, 4-amino-1,8-naphthalimide in Fig. 4a was experimentally characterized as a PARP inhibitor (IC$_{50}$ in the nanomolar range) (50) and crystallized in the PARP active site (51). As expected, 4-amino-1,8-naphthalimide showed strong inhibition. We measured the IC$_{50}$ against cholix, as $42 \pm 10$ nM at 45 μM e-NAD$^+$. The other three compounds in Fig. 4 are not commercially available. However, the quinazolinone in Fig. 4b was characterized previously (52) as a potent PARP-1 inhibitor, and a similar structure was crystallized in the PARP-1 active site (53). The pyrrolocarbazole in Fig. 4c was described as part of a class of potent PARP-1 inhibitors (54). These compounds also inhibit kinases (see PDB 1WVX) and are in accordance with growing interest in the crossover between ADPRT and kinase inhibitors (55). The quinazolinedione in Fig. 4d was characterized as a potent PARP-1 inhibitor (53) and crystallized in the PARP active site (56). Although the focus of the present work is not on inhibitor characterization, the new cholix-NAD$^+$ structure provides valuable context and points to several possibilities for expanding and refining our menu of options for inhibiting these toxins.

Acknowledgments—We are especially grateful to Dawn White for molecular biology support and to Gerry Prentice, Nirja Patel, and Peter Spangioannopoulos for protein purification support. We also thank our laboratory colleagues for useful discussions, in particular Danielle Visschedyk, Ravi Ravulapalli, Toni Forde, XiaoBo Liu, Yolanda Wang, and Anthony Petrie. We thank Prof. Matt Kimber, Jose A. Cuesta-Seijo, and Valerie Campagna-Slater for helpful advice. We also thank the Shared Hierarchical Academic Research Computing Network (SHARCNET) technical staff, especially Terry McKay.

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