Crystal Structure of ADP-ribosylated Ribosomal Translocase from Saccharomyces cerevisiae*

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The crystal structure of ADP-ribosylated yeast elongation factor 2 in the presence of sordarin and GDP has been determined at 2.6 Å resolution. The diphthamide at the tip of domain IV, which is the target for diphtheria toxin and Pseudomonas aeruginosa exotoxin A, contains a covalently attached ADP-ribose that functions as a very potent inhibitor of the factor. We have obtained an electron density map of ADP-ribosylated translation factor 2 revealing both the ADP-ribosylation and the diphthamide. This is the first structure showing the conformation of an ADP-ribosylated residue and confirms the inversion of configuration at the glycosidic linkage. Binding experiments show that the ADP-ribosylation has limited effect on nucleotide binding affinity, on ribosome binding, and on association with exotoxin A. These results provide insight into the inhibitory mechanism and suggest that inhibition may be caused by erroneous interaction of the translation factor with the codon-anticodon area in the P-site of the ribosome.

Eukaryotic elongation factor 2 (eEF2)1 catalyzes the step in the elongation cycle of protein synthesis where peptidyl-tRNA in the ribosome is translocated from the A-site to the P-site and the deacylated tRNA is translocated from the P-site to the E-site, preparing the ribosome for another cycle. Recently, we solved the crystal structure of eEF2 from yeast in the presence and absence of the fungal inhibitor, sordarin (1). This structure revealed large conformational changes upon binding of the inhibitor demonstrating the highly dynamic nature of the translocase. This flexibility is supported by cryo-EM studies of both eEF2 and the prokaryotic eEF2 homolog EF-G bound to the ribosome showing that the C-terminal domains III, IV, and V can reorient relative to domains I, G’, and II (2–4). In addition, previous results have shown that GTP hydrolysis in EF-G occurs prior to translocation and that both substituting GTP with a non-hydrolyzable analogue and deletion of domain IV results in a dramatic decrease in translocation (5). These results indicate that EF-G and eEF2 act as GTP-driven motor proteins with domain IV being important for coupling hydrolysis and translocation. This is in contrast to the classical switch mechanism in G-proteins where GTP hydrolysis allows release of the G-protein from its substrate (reviewed in Ref. 6).

eEF2 contains a post-translationally modified histidine residue, which from previous NMR spectral analysis was suggested to be a 2-[3-carboxyamido-3-(trimethylammonio)propyl]histidine, referred to as diphthamide (7, 8). This residue is located at position 699 in domain IV of yeast eEF2 (9). Cryo-EM studies of eEF2 in complex with the 80 S ribosome have shown that the area from residue 694 to 698 is close enough to interact with the codon-anticodon duplex between P-site bound tRNA and mRNA (4). This has led to the suggestion that the tip of domain IV including the diphthamide might have a role in stabilizing the codon-anticodon pairing during the translocation, thereby preventing frameshifts. Diphthamide is found only in eEF2 and is completely conserved throughout eukaryotic and archaeobacterial evolution (10, 11). Although, post-translation modification of histidine to diphthamide requires at least six enzymes, several mutagenesis experiments with eEF2 lacking the ability to form diphthamide have shown that the diphthamide residue is not necessary for eEF2 function (12–16).

The diphthamide of eEF2 is the exclusive cellular substrate for irreversible inactivation by diphtheria toxin and Pseudomonas aeruginosa exotoxin A (ETA) (17, 18), which both catalyze the transfer of ADP-ribose from NAD+ to N-3 (equivalent to Nε2) at the diphthamide in eEF2 (7). A single molecule of ETA can be sufficient to arrest the protein synthesis in a cell and eventually lead to cell death, and therefore this places it among some of the most lethal toxins to humans and other susceptible animals (19). Both crystallographic and kinetic data suggest that the reaction mechanism most likely follows a S1 nucleophilic substitution involving an oxacarbenium cation (20–24). This is despite the observed inversion of configuration for the glycosidic bond formed between the ribose and diphthamide (23). The inversion is probably caused by the anomic carbon of the nicotinamide ribose being susceptible to a backside nucleophilic attack from the N-3 atom in diphthamide (20). Several studies have shown that mutations in eEF2 preventing the biosynthesis of diphthamide confer resistance to both diphth-

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1 The abbreviations used are: eEF2, eukaryotic elongation factor 2; ETA, exotoxin A; ADPR, ADP-ribose; ADPR-eEF2, ADP-ribosylated eukaryotic elongation factor 2; EF-G, elongation factor-G; PE24H, P. aeruginosa exotoxin A 24-kDa C-terminal fragment containing a His6 tag; GDPNP, guanosine 5'-[β,γ-imido]triphosphate; GDP-β-S, guanosine 5'-O-(2-thiodiphosphate); GTP-γ-S, guanosine 5'-O-(3-thiodiphosphate); 5-AF, 5-acetamide fluorescein; AEDANS, 5-(2-aminoethylamino)-1-naphthalenesulfonic acid; EM, electron microscopy.

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nia toxin and ETA (12–16). Previous work using photoaffinity labeling and mutagenesis on ETA has recognized Glu-553 as an active site residue (25, 26). In addition, the crystal structure of ETA shows that it is located on a short β-strand with its side chain protruding into the active site cleft of domain III (27). It was proposed that its side chain is closely associated with the nicotinamide-ribose bond of bound NAD⁺ that is broken during the ADP-ribosylation of eEF2 and thus constitutes part of the NAD⁺ binding site (25, 26). In confirmation of this, the structure of domain III of exotoxin A in complex with the non-hydrolyzable NAD⁺ may account for the apparent irreversibility observed for the ADP-ribosylation activity of these toxins is extremely high and differs from the toxin in that it only ADP-ribosylates a fraction of the eEF2 pool within the cell and that the removal of the ADP-ribo moiety from eEF2 possibly results in the reactivation of the eEF2 pool within eEF2 and Mg2⁺. In addition, the crystal structure of the ADP-ribosylated eEF2 differs from the toxin in that it only ADP-ribosylates a fraction of the eEF2 pool within the cell and that the removal of the ADP-ribo moiety from eEF2 possibly results in the reactivation of the eEF2 pool within eEF2 and Mg2⁺.

A cytoplasmic ADP-ribosyltransferase, found in hamster kidney cells and possessing a similar mechanism of action to ETA and diphtheria toxin, has been identified (28). The cellular enzyme also transfers the ADP-ribo moiety of NAD⁺ to the diphthamide residue of eEF2 leading to its inactivation and thereby inhibiting protein synthesis. This suggests that the highly conserved diphthamide residue is a site of regulation within eEF2 and is not simply a target for bacterial attack (29, 30). It has been shown that the cellular ADP-ribosyltransferases differ from the toxin in that they not only ADP-ribosylates a fraction of the eEF2 pool within the cell and that the removal of the ADP-ribo moiety from eEF2 possibly results in the reactivation of the eEF2 pool, further suggesting its importance in regulation (30). Diphertheria toxin and ETA appear to be mimicking the action of these cellular ADP-ribosyltransferases; however, the ADP-ribosylation activity of these toxins is extremely high and may account for the apparent irreversibility observed in vivo.

We have solved the structure of ADP-ribosylated diphthamide in eEF2 (ADPR-eEF2) purified from yeast and crystallized in complex with GDP and the fungal inhibitor sordarin. The crystal structure supports the previously described inversion of configuration of the N-glycosidic linkage formed between C1’ of the nicotinamide-ribose of NAD⁺ and N-3 of the diphthamide residue (23). In addition, the structure shows that the interaction between Glu-553 in ETA and the nicotinamide-ribose in NAD⁺ is replaced by the highly conserved Asp-696 in eEF2 after the reaction. Furthermore, it appears that the trimethylammonium group of the diphthamide is close enough to interact with the β-phosphate at the ADP-ribose. The structure of the ADPR-eEF2 reaction product also reveals nearly the entire electron density for the diphthamide moiety. In addition, this structure of eEF2 contains well defined electron density for GDP and Mg2⁺, which results in a peptide flip of the conserved Val-28 in the P-loop compared with the previous structures of eEF2 without GDP (1). We also present binding data showing that ADPR-eEF2 has comparable binding affinity to the catalytic domain of ETA as does native eEF2. Furthermore, it is shown that both ADPR-eEF2 and eEF2 bind GTP and GDP with similar affinity. In agreement with earlier results (31) and in disagreement with others (32–35), we show that yeast ADPR-eEF2 does form a stable complex with empty yeast ribosomes, and finally we determine the dissociation constant for sordarin binding to eEF2. The structure of the ADP-ribosylated diphthamide is the first high-resolution structure showing the conformation of an ADP-ribose covalently attached to a target protein residue and provides further insight to the reaction mechanism of ADP-ribosylation and the inhibitory effect on the elongation cycle of protein translation. Finally, the binding data indicate that this inhibition is caused by erroneous interactions of the protein with the ribosome during a late phase of translocation.

**EXPERIMENTAL PROCEDURES**

**Purification—Purification of eEF2 from Saccharomyces cerevisiae** was performed as described (36). The catalytic fragment of ETA with a C-terminal His₆ tag (PE24H) was overexpressed and purified as described (37).

**Riboylation of eEF2**—One microgram of PE24H was added per 1 mg of eEF2 with 500 µM NAD⁺ in buffer Q (20 mM Tris-HCl (pH 7.6), 5 mM MgCl₂, 0.5 mM EDTA, 10% glycerol and 0.5 mM dithiothreitol) with 125 mM KCl (Q-125). The reaction was mixed on a nutator at room temperature for 80 min. Subsequently, the reaction volume was diluted 3-fold with 0 (0 mM KCl) and loaded onto a 9-mL Source-Q column (Amersham Biosciences) equilibrated in buffer Q-50 (50 mM KCl). The concentration of ADPR-eEF2 was eluted with a salt gradient from Q-50 to Q-250. The eluted protein was then loaded onto a 1-mL Uno-Q column (Bio-Rad) equilibrated in buffer Q-30 and eluted with a 20-mL linear gradient from Q-30 to Q-500. Protein-containing fractions were identified by SDS-PAGE, and labeling efficiency was assessed by non-denaturing PAGE (6%).

**Fluorescence Labeling of PE24H and eEF2**—The PE24H protein containing a single cysteine residue was labeled with the 5,6-dioctanoylaminohexyl-1-naphthalenesulfonic acid fluorophore, and both eEF2 and ADPR-eEF2 proteins were labeled with 5-lodoacetamide fluorescein (5-IAF) as described previously (38) with some modifications.

**Fluorescence-based eEF2 Binding Assay**—The fluorescence resonance energy transfer-based assay was used as previously described (38). The dissociation constant for eEF2 or ADPR-eEF2 binding with PE24H was determined using the following equation as part of the non-linear fitting function Origin 6.0 (OriginLab, Northhampton, MA): $\Delta F/\Delta F_{\text{max}} = (|\text{eEF2 or ADPR-eEF2} \times B_{\text{total}}|)/(K_D + |\text{eEF2 or ADPR-eEF2}|)$, where $\Delta F$ is the change in fluorescence intensity for each ligand (eEF2 or ADPR-eEF2) concentration upon macromolecular association, $B_{\text{total}}$ is the maximum change in fluorescence intensity at saturation of the ligand-binding site within PE24H, $K_D$ is the dissociation constant for the binding of eEF2 with PE24H, and $B_{\text{max}}$ is the total PE24H concentration (number of binding sites).

**Measurement of Ligand Binding to eEF2**—The binding of GDP, GTP, GDP-β-S, GTP-γ-S (Sigma), and sordarin (Merck) to eEF2 was measured in 0.5 cm × 0.5 cm quartz cuvettes by the quenching of intrinsic protein (Trp) fluorescence of the protein as a function of ligand concentration. Triplicate measurements were performed over a concentration range of 0–70 µM (guanine nucleotides) or 0–90 µM (sordarin) in the presence of 0.5 µM eEF2 at 25 °C in an initial volume of 600 µl of buffer (20 mM Tris-HCl (pH 7.6), 100 mM KCl, 1 mM dithiothreitol, 10% glycerol, 8 mM MgCl₂). Samples were excited at 295 nm (5-nm band pass), and the fluorescence emission intensity was measured at 340 nm (5-nm band pass) in a Varian Cary Eclipse spectrophotometer (Varian Canada, Inc., Mississauga, ON). The fluorescence signal was collected and averaged for 15 s for each measurement. A cuvette containing buffer only was also titrated with the corresponding ligand, and the signal from this sample was subtracted from the protein-containing samples. The fluorescence intensity measurements were also corrected for the dilution factor resulting from the titration (total dilution factor was less than 5%). $K_D$ for sordarin or guanine nucleotides binding to eEF2 was determined using the same equation as described above for the fluorescence-based eEF2 binding assay where sordarin or GDP/GTP/GDP-β-S/GTP-γ-S represent the ligands binding to eEF2.

**ADPR-eEF2 Binding to the Ribosome**—Ten pmol of 80 S ribosomes from S. cerevisiae purified on a 10–40% sucrose gradient were incubated with 100 pmol of either eEF2 or ADPR-eEF2 in 100 µl of buffer A (100 mM NH₄Cl, 10 mM MgCl₂, 20 mM Hepes-Ne, (pH 7.2), 5 mM β-mercaptoethanol) with or without 100 µM GDPNP (Sigma). The reaction mixture was incubated for 20 min on ice and placed on a 50-µl volume of 30% sucrose cushion in buffer A and centrifuged at 204,000× g for 20 min. The pellets were resuspended in Laemmli sample buffer and analyzed by SDS-PAGE.

**Structure Determination of ADPR-eEF2—ADPR-eEF2 in complex with sordarin was crystallized as described (1), but the cryoscreening was done very rapidly in a single step where a slow gradual soak was used for our structures of both apo-eEF2 and eEF2-sordarin. A 2.6 Å dataset was collected at 7171 (MAC3-7) in Sweden and processed and reduced with MOSFLM and SCALAM (39), and refinement was performed in CNS (40). A model of the ADP-ribosylated diphthamide was created using the PRODRG server (41), which calculated coordinates, CNS parameter and topology files, and stereochemistry file for “O” (42). The ADP-ribosylated diphthamide was then placed instead of His-699 in “O” and fitted into the difference Fourier density map. The refined structure was validated using PROCHECK (43) and MOLPRO-
The addition of the negatively charged phosphates for 5-AF-eEF2 proteins were then run on a non-denaturing PAGE gel. The fluorescently labeled ADPR-eEF2 resulted in a higher mobility through the gel and accommodated the bulky ADPR group. The fluorescently labeled ADPR-eEF2 was pelleted. The SDS-PAGE gel clearly demonstrates binding of ADPR-eEF2 to empty 80 S ribosomes and that ADPR-eEF2 binds at a comparable but probably slightly lower level than unmodified eEF2.

**RESULTS**

**Interaction of ADPR-eEF2 with Toxin—**Both native eEF2 and ADPR-eEF2 were labeled with the fluorescein fluorophore (5-AF). These proteins were labeled under the same conditions and resulted in the identical number of labels per protein. This suggests that the addition of the ADPR moiety does not affect the accessibility of any of the cysteines targeted for conjugation with the fluorescent probe. The affinity of ADPR-eEF2 for PE24H was evaluated using a fluorescence resonance energy transfer-based binding assay. This assay measures the fluorescence resonance energy transfer between the AEDANS moiety in PE24H (donor) and the fluorescein on eEF2 or ADPR-eEF2 (acceptor) (Fig. 1A). From the binding isotherms (Fig. 1A), the eEF2 binding constants were determined to be 1.4 (± 0.1) and 2.5 (± 0.4) μM, respectively, for the absence and presence of the ADPR moiety on eEF2. ADPR-eEF2 maintained the ability to bind to the toxin (although more weakly than for native eEF2) suggesting that the active site cleft of PE24H can still accommodate the bulky ADPR group. The fluorescently labeled eEF2 proteins were then run on a non-denaturing PAGE gel.

The addition of the negatively charged phosphates for 5-AF-ADPR-eEF2 resulted in a higher mobility through the gel and also showed only one population of eEF2 for either the native or the ADP-ribosylated forms (Fig. 1B).

**Ribosome Binding—**Ribosome binding of ADPR-eEF2 and eEF2 was illustrated by a simple co-sedimentation assay (Fig. 1C). When adding the non-hydrolyzable GTP derivative, GDPNP, to the reaction mixture, eEF2 and ADPR-eEF2 were trapped by the ribosome molecules and can therefore be pelleted. Without GDPNP only a small portion of the translocation factors was pelleted. The SDS-PAGE gel clearly demonstrates binding of ADPR-eEF2 to empty 80 S ribosomes and that ADPR-eEF2 binds at a comparable but probably slightly lower level than unmodified eEF2.

**GDP Binding—**Using fluorescence spectroscopy, we have determined the dissociation constants for GDP and GDP binding to yeast eEF2 (Table II). Titration of eEF2 with either GTP or GDP resulted in the quenching of the intrinsic Trp fluorescence of the protein to 20–25% of the original signal at saturating levels of bound nucleotide (data not shown). Comparable levels of fluorescence quenching were also observed when eEF2 was titrated with the guanine nucleotide analogues (GDP-β-S and GTP-γ-S). To investigate the effect of ADP-ribosylation, dissociation constants for GDP and GDP binding to either ADPR-eEF2 or eEF2 were determined. These results show that ADP-ribosylation of eEF2 has very little (if any) effect on binding affinity for the two nucleotides and that there are no differences in affinity for GDP and GTP. The lack of difference in binding affinity for GTP and GDP to eEF2 was also shown for the two analogues GDP-β-S and GTP-γ-S, which showed dissociation constants slightly higher (lower affinity) but comparable with the authentic nucleotides (Table II).

**Sordarin Binding—**The structure of ADPR-eEF2 was solved in complex with the antifungal inhibitor sordarin, and as for the previous eEF2-sordarin structure sordarin binds in a pocket between domains III, IV, and V (results not shown). The binding of sordarin to yeast eEF2 in the absence of ribosomes was determined by fluorescence spectroscopy (sordarin binding resulted in quenching of the eEF2 intrinsic Trp fluorescence to 30% of the original signal at saturating levels of bound sordarin; data not shown) and is comparable with the authentic nucleotides (GDP-β-S and GTP-γ-S), which showed dissociation constants slightly higher (lower affinity) but comparable with the authentic nucleotides (Table II).

**Structure of ADPR-eEF2—**Diffraction data to a maximum resolution of 2.6 Å were collected from ADPR-eEF2 crystals grown in vapor diffusion drops containing GDP, Mg2⁺, and sordarin (Table I). The dataset collected from the crystals of ADPR-eEF2 in complex with yeast eEF2 was also shown for the two analogues GDP-β-S and GTP-γ-S, which showed dissociation constants slightly higher (lower affinity) but comparable with the authentic nucleotides (Table II). The six structural domains: residues 2–218 and 329–345 (domain I or G-domain), 219–328 (G-domain), 346–481 (domain II), 482–558 (domain III), 559–726 and 801–842 (domain IV), and 727–800 (domain V) of the two structures can be superimposed directly with a root mean square deviation of only 0.35 Å (data not shown). The ADP-ribosylation at the diphthamide, however, is rather clear in the unbiased FE₉₀ electron density map (Fig. 2A) where the two ribose rings and the two phosphates are well defined. As expected for such a long side chain, the density becomes weaker with increasing distance to the main chain (compare the density for the first and second ribose in Fig. 2A), and the map shows no density for the bulky adenine base, which most likely has freedom of rotation. Subsequently, the ADP-ribose was built into this initial electron density map, and the model was refined in CNS. A 2FE₀ − FE₁ electron density map also revealed clear density for the diphthamide attached to the C-2 (equivalent to Cₛ) at the

| Data collection* |
|------------------|
| Unit cell parameters: |
| Space group: P2₁, 2, 2 |
| Cell dimensions (Å): a = 119.73, b = 151.00, c = 65.17 |
| λ (Å): 1.089 |
| Resolution (Å): 35–2.6 (2.74–2.6) |
| Rsym (°): 5.6 (25.7) |
| Completeness (°): 99.7 (100.0) |
| Average I/σ (I): 15.4 (4.3) |
| Redundancy: 3.4 (3.4) |
| Refinement |
| Resolution (Å): 35–2.6 |
| Reflections: Work set 35,839, Test set 1126 |
| Number of atoms |
| Protein: 6421 |
| Water molecules: 185 |
| Sordarin/GDP/Mg²⁺: 35/28/1 |
| Root mean square deviation |
| Bond lengths (Å): 0.008 |
| Bond angles (°): 1.344 |
| R-factor (°): 22.73 |
| Rfree-factor (°): 26.18 |
| Ramachandran plot (°): 99.7 |
| Favorable and additionally allowed regions: 0.3 |
| Generously allowed regions: 0.0 |
| Disallowed regions: 0.0 |

* Data were collected at the b1711 MAX-lab synchrotron at Lund University in Sweden.
* Values in brackets are for outer shell.
* Rsym = (ΣhΣi |Ih| - ΣhΣi |Ih|)/|ΣhΣi |Ih| |
* R-factor = ΣhΣi |Ih| Fcal (h) - kFobs (h) |ΣhΣi |Fobs (h)|, where Fobs and Fcal are the observed and calculated structure factor respectively, and k is a scaling factor.
* Rfree-factor is identical to R-factor on a subset of test reflections not used in refinement. The same test set was used as for the previously determined eEF2-sordarin structure at 2.1 Å (1).
ADP-ribosylated eEF2

ribose and Glu-304 and Val-306 residues of a symmetry related eEF2 molecule in the crystal 3.5 to 4.0 Å from the diphthamide; however, no specific hydrogen bonds or salt bridges can be established. The positively charged trimethylammonio group of the diphthamide is positioned 3.8 Å from O2 at the β-phosphate of the ADP-ribose, whereas the diphthamide amide group is positioned 4.1 Å from Asn-581. These atoms may form weak electrostatic interactions or hydrogen bonds possibly mediated by water molecules not detectable at 2.6-Å resolution.

Structure of GDP Binding to eEF2—The 2F0 - Fc electron density omit map calculated using the refined model of ADPR-eEF2 without GDP and Mg2+ shows very clear density for GDP (Fig. 3A). As for all other G-proteins the GDP is placed in a pocket formed by the P-loop (G1) and G4 and G5 loops (6). It interacts extensively with residues found in these three loops. Residues Lys-159 and Asp-161 in G4 together with residues Ser-213, Gly-214, and Leu-215 in G5 form hydrogen bonds to the guanine base, whereas Asp-29, Gly-31, Ser-33, and Thr-34 in the P-loop form hydrogen bonds to oxygen atoms from the α- and β-phosphates (Fig. 3B). Three water molecules can also be seen to mediate contact between eEF2 and GDP. Electron density with short coordination distances at the classical Mg2+ position is interpreted as an Mg2+ ion and is coordinated by two β-phosphate oxygen atoms and the hydroxyl group of Ser-33 (Fig. 3B). Furthermore, the ligand position suggests an octahedral coordination with water molecules as ligands in the three remaining positions; however, density for these water molecules is missing. Compared with the previous structures of eEF2 without GDP and Mg2+ this new eEF2 structure contains a peptide flip of Val-28 in the P-loop leaving space for the β-phosphate in the binding pocket (Fig. 2B) but has virtually no other conformational changes. A comparable flip is also seen in the EF-G structures with and without binding of GDP and Mg2+ (46–48).

**DISCUSSION**

The ADP-ribosylation of a variety of cellular proteins by both toxins and endogenous enzymes represents a very important protein modification process involved in pathogenic mechanisms, cell division, DNA repair, and intracellular signaling systems. Many of the toxins performing these reactions are
**FIG. 2. Structure determination of ADP-ribosylated eEF2.** A, stereo view of the 2.6 Å $F_o - F_c$ electron density map around residue His-699 of the initial model before the diphthamide and ADP-ribose were modeled (orange, carbon atoms). The map was contoured at 1.7 $\sigma$. B, stereo view of a $2F_o - F_c$ electron density map around the ADP-ribosylated diphthamide residue based on the model of ADPR-eEF2 without the diphthamide and contoured at 0.9 $\sigma$. C, stereo view of the final $2F_o - F_c$ electron density map around the fully modeled ADP-ribosylated diphthamide based on the refined model of ADPR-eEF2 contoured at 0.9 $\sigma$. The maps are shown with a mask made around residue 699 atoms with a 1-Å solvent radius. D, interactions between ADP-ribose and eEF2. The diphthamide is placed at the tip of domain IV protruding into the solvent together with the ADP-ribose.
among the most lethal known and are principal components of major epidemic diseases like cholera and diphtheria. Although these diseases are now controlled with vaccines and antibiotics, Pseudomonas infections, in which ETA plays an important function as a virulence factor, still represents a major problem for immunocompromised patients (49, 50).

Many structures of both the ADP-ribosylating toxins and of their target proteins have been determined. Surprisingly, however, no one has ever determined the structure of the product of the ADP ribosylation reaction despite its obvious importance as the underlying cause for such major diseases. Our ADPR-eEF2 structure is therefore the first of any ADP-ribosylated protein. The modification is not simply attached to the diphthamide but is specifically recognized and cradled by strictly conserved residues within the protein.

We chose the sordarin state of eEF2 because crystallization of this state is quite straightforward, and crystals of eEF2-sordarin also diffract better than crystals of apo-eEF2. Because the His-699 at the far end of domain IV is quite distant from the guanine-nucleotide binding site within domain I, but it may be explained by a much more rapid cryosoaking procedure used in the work presented here. Alternatively, the GDP preparation used in our early study (1) may have been partially hydrolyzed.

Our measurements of the affinities of eEF2 for GDP or GTP show that the nucleotide binding properties of yeast eEF2 in solution are not significantly influenced by ADP-ribosylation. In agreement with this, we also show that binding of eEF2 in the presence of GDPNP to empty 80 S ribosomes is only slightly influenced by the presence of the ADP modification. This is also supported by the recent cryo-EM reconstruction of eEF2-sordarin in complex with posttranslational 80 S ribosome. These results suggest that the diphthamide on the tip of domain IV is located close to the codon-anticodon duplex of P-site-bound tRNA with mRNA, and that the interaction of the diphthamide with this duplex may function to stabilize this complex during translocation (4). In addition, it has been shown that ADP-ribosylation of rabbit eEF2 leads to a loss of its nonspecific affinity for RNA, and it was suggested that this could be a reason for the inactivation (33). If this is indeed the function of the diphthamide, it is likely that its modification with the bulky ADP-ribose moiety may impair the proposed interaction with the codon-anticodon and thereby prevent complete translocation followed by release of eEF2. These observations argue in favor of inhibition by ADP-ribosylation to occur during the late phase of translocation by preventing eEF2 turnover. The similar complex is biologically relevant.

The mechanism of inhibition of translation caused by ADP-ribosylation of eEF2 is still not clear. Previous experiments have shown that complex formation between eEF2 and ribosomes from rabbit was not influenced by ADP-ribosylation (31). This indicates that inhibition takes place in the late phase of translocation by inhibiting the cycling of eEF2 probably by locking eEF2 on the ribosome. This could allow initial binding and GTP hydrolysis, which has been shown for EF-G to be a very early event in translocation (5). Other reports have indicated a marked decrease in the ability of ADPR-eEF2 to associate with the 80 S ribosome (32–35) as well as a significant reduction in binding and hydrolysis of GTP upon ADP-ribosylation (32, 35). Furthermore, ADPR-eEF2 binds weaker than eEF2 to a synthetic oligonucleotide (SRD RNA) mimicking the sarcin/ricin loop of 28 S ribosomal RNA (51). Together, these observations indicate that ADP-ribosylation of eEF2 causes the inhibition of translocation prior to or in the early phase of translocation thus preventing even early events within a single cycle of translocation, possibly by inducing conformational changes within ADPR-eEF2.

Our structures of apo-eEF2, the eEF2-sordarin complex, and now ADPR-eEF2-sordarin-GDP do not support a direct coupling between the site of ADP-ribosylation (tip of domain IV) and the GTP/GDP binding pocket (domain I), which is also very close to the sarcin-ricin loop in cryo-EM reconstructions of EF-G/eEF2 in complex with 70 S/80 S ribosomes (4, 52). The tip of domain IV with the diphthamide and the nucleotide-binding site in domain I are separated by 60–70 Å depending on the conformation of eEF2. Furthermore, ADP-ribosylation does not induce any significant change in domain IV, which might be transmitted to domain I through domains III or V. This agrees with previous data that suggested that the binding of guanyl nucleotides does not affect the binding of eEF2 to ETA (37).

The structure presented herein contains bound GDP, which could not be observed in our earlier structures of apo-eEF2 or eEF2-sordarin despite inclusion of GDP in buffers used for crystallization and soaking (1). We have no clear explanation for this observation. It appears unlikely that this is caused by the ADP-ribosylation in domain IV at a site distant from the guanine-nucleotide binding site within domain I, but it may be explained by a much more rapid cryosoaking procedure used in the work presented here. Alternatively, the GDP preparation used in our early study (1) may have been partially hydrolyzed.
$K_D$ values for GTP and GDP binding to yeast eEF2 are in contrast to previous results for both prokaryotic EF-G and eukaryotic eEF2 in the absence of ribosomes where the $K_D$ for GDP binding is significantly lower than for GTP (53, 54). In addition, our observation that the binding affinity of eEF2 for GTP and GDP is unaffected by ADP-ribosylation disagrees with previous results showing the affinity for GTP to wheat germ ADP-eEF2 was significantly reduced (32).

A key feature of our structure is that the ADP-ribosylated product is specifically recognized by eEF2. It appears that the surface area around the diphthamide modification itself. Based on the cryo-EM reconstruction alluded to earlier (4), the interaction of domain IV in eEF2 with the ADP-ribose moiety may mimic the recognition of RNA by eEF2 in the late phase of translocation, and ADP-ribosylation of eEF2 might prevent this. Interestingly, the adenine base could participate in an A-minor tertiary interaction, as previously described for ribosomal RNA (55).

The completely conserved Asp-696 in eEF2 replaces Glu-553 in ETA by interacting with 2'-OH of the nicotinamide-ribose. This conservation indicates an important function for this residue, and therefore this replacement may be important for stabilizing the transition state of the ADP-ribosylation reaction where the C1' anomic carbon of the nicotinamide ribose is made susceptible for a nucleophilic attack by the N-3 of the imidazole on the diphthamide residue. Further evidence for the role of conserved residues within eEF2 (substrate for the ADP ribosylation reaction) participating in its own catalytic process arises from the interaction of Hs-894 with the nicotinamide-ribose of the ADP-ribose moiety. As previously suggested, this new ADP-eEF2 structure confirms that the nucleophilic attack on the nicotinamide-ribose results in an inversion of configuration at C1' of the ribose (23). Other ADP-ribosylating toxins such as cholera toxin and Escherichia coli heat-labile enterotoxin have also been shown to lead to inversion of configuration for the ADP-ribose:nucleophile C-N bond linkage, which indicates that these transfersases may share a common mechanism (56).

The binding affinities between eEF2 and ETA are comparable before and after ADP-ribosylation of eEF2, which indicates that there are no major conformational changes invoked within eEF2 during the ADP ribosylation reaction. This is in agreement with the fact that the two crystal structures of eEF2 containing sordarin are highly isomorphous. However, the isomorphism could also be explained by sordarin interacting with the three C-terminal domains of eEF2, thereby blocking further rearrangements of the protein. As previously described, the high affinity binding of sordarin to eEF2 results in major conformational changes pulling the three C-terminal domains away from the three N-terminal domains. Additional high-resolution structures and kinetic investigations are required to fully understand and establish the role of the diphthamide and the inhibitory role of the ADP-ribosylation at a molecular level.

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