Crystal Structure of Dinitrogenase Reductase-activating Glycohydrolase (DRAG) Reveals Conservation in the ADP-Ribosylhydrolase Fold and Specific Features in the ADP-Ribose-binding Pocket

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Introduction

Covalent post-translational modification of proteins has a key role in the regulation of the metabolism of virtually all organisms. The chemical nature and the biological functions of these modifications are very diverse. The commonest and the best described modification is the phosphorylation of amino acids such as threonine, serine and tyrosine. Nevertheless, several other modifications, such as glycosylation, acylation and lipoylation, have important functions in several biological systems.¹ Recently, ADP-ribosylation of proteins and other molecules, such as antibiotics and nucleic acids, has started to emerge as an important covalent modification with a plethora of functions.²-⁴

Protein ADP-ribosylation is catalyzed by mono- and poly(ADP)-ribosyl-transferases. These enzymes use β-NAD⁺ as substrate, and covalently link the ADP-ribose group of NAD⁺ to an amino acid side chain of the target protein, releasing nicotinamide as a product of the reaction. These post-translational modifications can be reversed by the action of ADP-ribosyl-hydrolases (ARHs) and poly(ADP)-ribosyl-hydrolases (PARGs) as reviewed by Lin.⁴

Modification of proteins by ADP-ribosylation can result in a change of protein function. This mechanism is used by several bacterial toxins, such as diphtheria and cholera, to alter the metabolism of their hosts.⁵ However, one of the best characterized systems of ADP-ribosylation is the regulation of the NifH component of nitrogenase, which occurs in
Proteobacteria such as *Rhodospirillum rubrum* and *Azospirillum brasilense*. Nitrogenase activity in these bacteria is reversibly down-regulated in the presence of ammonium or upon energy depletion. Under these conditions, an ADP-ribosyl-transferase (designated dinitrogenase reductase ADP-ribosyltransferase, DraT) catalyzes the transfer of an ADP-ribose group from NAD⁺ to the Arg101 residue of one subunit of the dimeric NifH component of nitrogenase. This modification of NifH Arg101 presumably disrupts the docking site between the NifH dimer and the other component of the nitrogenase complex, the NifDK heterotetramer, resulting in nitrogenase inactivation. When the cells return to conditions favorable for nitrogenase activity, an ARH (designated dinitrogenase reductase-activating glycohydrolase, DraG) catalyzes the removal of the ADP-ribose group from NifH, thus reactivating nitrogenase (Fig. 1).

The mechanism underlying the regulation of DraT and DraG activity in response to environmental signals is not completely understood. However, the ammonium response in *A. brasilense* has been shown to involve DraT and DraG forming complexes with GlnB and GlnZ, respectively, which are members of the PII family of signal transduction proteins. In response to elevated levels of ammonium in the extracellular medium, DraT is apparently activated by complex formation with GlnB, and the integral membrane ammonium transporter AmtB. This mechanism is reversible, thereby allowing reactivation of DraG and inactivation of DraT when the extracellular ammonium levels drop.

**Results and Discussion**

**Overall structure**

DraG occurs as a monomer in solution, as indicated by size-exclusion chromatography, and this is most probably the biologically active unit. Full-length *A. brasilense* DraG contains 297 amino acid residues, most of which are well defined in the electron density of both crystallographically independent molecules. Three residues appear disordered at both C-termini and four and three residues at the N-terminus of molecules A and B, respectively. In addition, residues 119 and 120 of molecule B are poorly defined, and the structural perturbation in this chain segment appears caused by nearby crystal contacts. The corresponding chain segment in molecule A shows a different, well-defined structure. Otherwise, the two DraG molecules of the crystallographic asymmetric unit have nearly identical structures and they are not related by a non-crystallographic symmetry axis. The all-α-helix monomer structure comprises 15 α-helices.
(H1–H15) and has molecular dimensions of approximately 45 Å × 38 Å × 38 Å (Fig. 2a). The final structural model contains two magnesium ions bound in the active site of each DraG molecule and a total of 71 water molecules in the asymmetric unit.

**Structural and sequence similarity between A. brasilense DraG and human ADP-ribosylhydrolase 3**

There are four structural homologues of DraG in the Protein Data Bank; namely, human (2FOZ) and mouse (2QTY) ADP-ribosylhydrolase 3, a predicted ribosylhydrolase MJ1187 from *Methanococcus jannaschii* (1T5J) and an ADP-ribosylhydrolase-related protein from *Thermus thermophilus* HB8 (2CWC and 2YZV). These four proteins share levels of sequence identity with DraG of 22.2%, 22.5%, 26.6% and 30.4%, respectively. The human and mouse ARH3 enzymes share 90% sequence identity and have nearly identical structures, and we will not consider the mouse ARH3 separately in further comparisons. Whilst all of the structures are all-α-helix in nature, they are comprised of different numbers of α-helices: DraG, 2FOZ, 1T5J and 2CWC have 15, 19, 16 and 14 α-helices, respectively. A total of 13 of these appear structurally conserved in all homologues and the RMSD values based on superposition with DraG are 1.4 Å (2FOZ), 1.3 Å (1T5J) and 1.2 Å (2CWC) for the corresponding 151 Cα positions. These 13 helices define a common α-helical core structure for the DraG family (Fig. 2). The centrally located helices H3 and H13 carry at their N-termini the highly conserved Asp and Thr residues that are critical for Mg2+ binding. Including the structure and the length of surface loops in the comparison, the prokaryotic homologues are much more similar to each other than they are to the human ARH3 (hARH3) structure. The two proteins from *M. jannaschii* and *T. thermophilus* are, however, unlikely to be true functional DraG homologues, as these two species are not diazotrophs and do not encode a complementary DraT protein. Hence, they are likely to have substrate specificity different from that of DraG.

A more detailed analysis of the structural differences between *A. brasilense* DraG and hARH3 shows that some major differences occur in some of the connections between the 13 conserved core helices (Figs. 2 and 3). Most prominently, the chain connecting H9 with H11 in DraG, including H10, has a very different length and fold compared to the corresponding connection in ARH3 (H10 – H15) which comprises four α-helices and is 37 residues longer. This so-called quasidomain D of hARH3 (H11 – H15) is structurally very different in all three prokaryotic homologues. Other, larger differences between the DraG and hARH3 chain traces are observed in the connections between helices H1/H3, H5/H6 and H11/H12 (DraG numbering). Both H1/H3 connections contain a short but structurally non-equivalent α-helix (H2). DraG residues 46–54 located between H2 and H3 form a hairpin-like loop that reaches with its tip into the active site region. In both mammalian ARH3s there is no equivalent structural element but eight or nine residues between H2 and H3 that potentially could reach into this space are disordered. Connection H5/H6 has an eight residue insertion in the human enzyme and comprises a short, additional α-helix. Finally, the short H11/H12 connection in DraG has an 11 residue insert in hARH3 forming a surface loop.

**The active site**

The active site of *A. brasilense* DraG was expected to be similar to that observed in its mammalian homologues, human and mouse ARH3, and to...
be occupied by two magnesium ions in adjacent sites. Residues Glu28, Asp60, Asp61, Asp243, Asp245, and Thr246 in *A. brasilense* DraG are structurally equivalent to Glu25, Asp61, Asp62, Asp298, Asp301, and Thr301 in hARH3 (Fig. 3, residues marked by an asterisk (*)). Note that amino acid numbering for hARH3 is based on a version of hARH3 that was truncated at the N-terminus.14) These residues are completely conserved in the ARH family and are involved in the coordination of the two magnesium ions in all known ARH structures.

Although the expected bimetal site with its oxygen ligands was not atomically resolved at 2.5 Å resolution in initial difference electron density maps, the arrangement observed in the mammalian homologues provided a very good fit to this density and the refinement results proved very consistent with this interpretation. The presence of the two magnesium ions is also indicated strongly by two density maxima of about 2.0 \( \sigma \) in a simulated annealing omit map (Supplementary Data Fig. S1). The two magnesium ions in the active sites of molecule A and B are 3.54 Å and 3.41 Å apart, respectively. These distances are shorter than that observed in hARH3 (3.8 Å) but very similar to that observed in the structure of a structural homologue from *T. thermophilus* (2YZV). The coordination observed in the bimetal site with its oxygen ligands and by four side chain oxygen atoms of the highly conserved residues: Mg1, Thr59, Asp60, Asp61, and Asp245; Mg2, Glu28, Asp243, Asp245, and Thr246 (see Fig. 5). The Asp245 carboxylate ligates in a bidentate fashion to both ions and one water molecule is in a bridging position. In molecule B, the side chain configuration is essentially the same but the magnesium ions, and particularly the coordinating water molecules, are less well defined as is also apparent from their higher atomic displacement parameters (isotropic B-factors). Although the overall sequence identity among family members is not very high, all residues involved in coordination of the two magnesium ions and their architecture are highly conserved, indicating the precise stereochemistry of the magnesium sites is critical for ARH function (see discussion of mutagenesis data below).

The purified DraG enzyme from both *A. brasilense* and *R. rubrum* can be activated in vitro by Mg\(^{2+}\), Fe\(^{2+}\), and Mn\(^{2+}\).17,18 Mn\(^{2+}\) has been reported to be the most effective ion in vitro at low millimolar concentrations but systematic studies are lacking and there is no experimental evidence for a physiological role of this metal. *A. brasilense* DraG could be crystallized in the presence of Mg\(^{2+}\) but not in the presence of low millimolar concentrations of Mn\(^{2+}\) (data not shown).

### Table 1. Coordination of the magnesium ions

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<th>Distance (Å)</th>
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<tr>
<td></td>
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<td>Asp61-OD1</td>
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<tr>
<td></td>
<td>1.89 (2.07)</td>
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</tr>
<tr>
<td></td>
<td>1.96</td>
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<td>1.98</td>
<td>Wat61-O</td>
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<tr>
<td></td>
<td>1.96 (2.44)</td>
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</tr>
<tr>
<td></td>
<td>2.31 (2.21)</td>
<td>Wat64-O (Wat61-O)</td>
</tr>
</tbody>
</table>

Values are given for molecule A with values for molecule B in parentheses.
Substrate binding

ADP-ribose (ADPR), the product of the reaction catalyzed by DraG, binds to the A. brasilense enzyme with a $K_D$ of 9.7 μM as determined by isothermal titration calorimetry (Fig. 4). For hARH3, a sixfold higher affinity was determined, and in both cases the stoichiometry of binding is 1:1 and the binding appears to be enthalpically driven. We have not been able to experimentally determine the ADPR binding mode to DraG either by crystal soaking or by co-crystallization experiments. Instead, we have modeled ADPR, with an $\alpha$-anomer configuration of the terminal ribose to represent the stereochemistry of the substrate, into the active site of DraG (Fig. 5).

Overall, the modeled interaction of ADPR with DraG is similar to that proposed with hARH3 based on in silico docking. The similarity of the two protein structures is very high at the bimetal-binding site where the terminal ribose is assumed to bind but is much less pronounced in the putative pyrophosphate-binding crevice. In both cases, interactions with the adenosine moiety appear possible but the absence of a binding pocket renders any respective modeling rather uncertain.

The 3'-hydroxyl of the terminal ribose unit is within hydrogen bonding distance of the conserved Asn126 (hARH3-Asn135) in both models but there are differences in sugar pucker and in how its hydroxyl groups substitute for water ligands of the two magnesium ions. In our case, the $\alpha$-glycosidic bond is in an axial position which, unlike an equatorial position, permits unhindered access of an attacking water molecule possibly activated by the conserved Glu28. The glycosidic hydroxyl group is surface-exposed, which is necessary as in the true substrate it is substituted by the arginine-linked dinitrogenase reductase (see below). It has to be noted that we have not considered possible rearrangements in the bimetal site upon substrate binding.

The pyrophosphate moiety makes several very plausible hydrogen bonding interactions with the protein. Its location at the N-termini of $\alpha$-helices, in our case H5 and H6, is typical for anion-binding sites in general. One phosphodiester group makes two hydrogen bonds with main chain N-H groups at the N-terminus of H5 and possibly one with the side chain of Thr102, which is not conserved in hARH3. The other phosphodiester group interacts with the His158 side chain, which is likely to be protonated. Interestingly, His158 (His166 in hARH3) is fully conserved among the family suggesting that this interaction is particularly critical for binding the ADP-ribosyl unit. The other phosphodiester oxygen is within hydrogen bond distance of $O^\gamma$ of Thr101.

Except for the conserved histidine, A. brasilense DraG and hARH3 share no other conserved side chain involved in interaction with substrate in this region. Residue hARH3-Ser132 (equivalent to DraG His123, Fig. 5) has been proposed to hydrogen bond to the second phosphodiester group and has been shown to lack activity when mutated to alanine. In DraG, the local structure at His123 is different and Thr101, located on the opposite side of this phosphodiester group, appears to substitute for the Ser132 interaction.

The proposed ADP-ribose binding mode is in very good agreement with biochemical studies of R. rubrum DraG with substrates of low molecular mass. Synthetic substrate analogues of DraG containing substitution of guanine for the adenine base or modified adenine bases with blocked hydrogen bonding sites were still able to act as substrate for R. rubrum DraG with $K_m$ values similar to those observed with the natural ADP-ribosyl hexapeptide as substrate. Furthermore, substrate analogues lacking the pyrophosphate moiety were not hydrolyzed and addition of free pyrophosphate inhibited DraG activity. The same authors showed also that compounds with an NAD substituent at the glycosidic bond were not cleaved in either the $\alpha$- or $\beta$-anomer configuration. Modeling NAD in place of arginine shows that it is difficult to accommodate this rigid substituent without strongly perturbing the proposed productive binding mode of the terminal ribosyl moiety (data not shown).

The biological substrate of DraG is dinitrogenase reductase (NifH) inactivated by ADP-ribosylation of...
Arg101 in one subunit of the dimeric iron protein.\textsuperscript{20} The structurally best characterized nitrogenase enzyme system is that from \textit{Azotobacter vinelandii}.\textsuperscript{21} Its dinitrogenase reductase shares 74\% identity with \textit{A. brasilense} NifH and can be inferred to represent a close structural model. Arg101 (Arg100 in the \textit{A. vinelandii} homologue) is situated near the center of an extended shallow surface adjacent to the [4Fe:4S] metalloccluster that sits on the twofold axis of the dimer. The spatial closeness of the two equivalent arginines may explain why only one can become ADP-ribosylated by DraT. This shallow surface with two protrusions at its periphery interacts with the molybdenum iron protein in the active nitrogenase complex,\textsuperscript{9} and must face DraG to become de-ADP-ribosylated. While it is tempting to try to dock an ADP-ribosylated homology model of \textit{A. brasilense} NifH onto DraG, there are no data that would define or permit verification of the detailed interactions between the two proteins. Manual docking attempts, positioning one ADP-ribosylated arginine of the dimer into the active site groove of DraG (selected residues in green). The glycosidic substituent R of the terminal ribose is colored blue to indicate the N-glycosidic linkage to an arginine residue of NifH. Putative hydrogen bonds of the pyrophosphate moiety are represented as broken lines in black, interactions of ribose oxygens with the magnesium ions are represented as broken lines in orange. Selected residues of superposed hARH3 (PDB code 2FOZ) are shown in gray. Residue names are put close to the $\alpha$ carbons where possible.

Fig. 5. Model of ADP-ribose binding to \textit{A. brasilense} DraG; a stereo view illustrating the stereochemistry of the docked ADP-ribosyl moiety (magenta) in the active site groove of DraG (selected residues in green). The glycosidic substituent R of the terminal ribose is colored blue to indicate the N-glycosidic linkage to an arginine residue of NifH. Putative hydrogen bonds of the pyrophosphate moiety are represented as broken lines in black, interactions of ribose oxygens with the magnesium ions are represented as broken lines in orange. Selected residues of superposed hARH3 (PDB code 2FOZ) are shown in gray. Residue names are put close to the $\alpha$ carbons where possible.

Structure–function relationships in DraG

Site-directed mutagenesis data that offer insights into structure–function relationships are available for some members of the ARH family, including \textit{R. rubrum} DraG, hARH3 and rat brain ARH.\textsuperscript{14,16,22-24} In the case of the human and rat ARH proteins, the precise biological substrates are unknown and consequently activities were assessed by de-ADP-ribosylation of a generic poly(ADP)-ribosylated substrate. By comparison \textit{R. rubrum} DraG can be assayed with its native substrate, mono-ADP-ribosylated NifH. Mutants of the highly conserved active site residues involved in Mg\textsuperscript{2+} coordination have been studied for hARH3\textsuperscript{14,16} and for rat brain ARH.\textsuperscript{24} They were all found to be inactive or significantly impaired in their activity with the exception of some conservative replacements like Asp to Glu.\textsuperscript{24} Likewise, a D243G variant of \textit{R. rubrum} DraG resulted both in loss of activity and of ability to bind Mn\textsuperscript{2+}.\textsuperscript{22} Hence, as all these mutations are predicted to disrupt coordination of Mg\textsuperscript{2+}, these data support the concept that the magnesium ions are critical for activity.
The docking experiments of hARH3 with ADP-ribosyl-ADPR identified potential residues involved in ADPR binding as Ser132, Tyr133, Asn135 and His166 (Fig. 3, residues marked by +). Changes by mutation of these residues affected the activity in all cases.14 Interestingly, only His166 is fully conserved among the family, suggesting that this residue might be particularly critical for ADPR binding. Mutation of this histidine in R. rubrum DraG (H158N) resulted in both loss of activity and incapacity to bind Mn2+ in vitro.22 hARH3 Ser132, proposed to interact with the pyrophosphate moiety and lacking detectable activity when mutated to Ala, has Asp123 and His123 as its structural equivalents in R. rubrum and A. brasilense DraG, respectively. The structural difference observed between hARH3 and A. brasilense DraG on the N-terminal side of A. brasilense His123 results in a quite different positioning of the respective two side chains with His123 pointing away from the pyrophosphate-binding groove. In R. rubrum DraG, closely related to A. brasilense DraG, the corresponding D123A mutant was affected only partially in enzyme activity and in vitro Mn2+ binding.22

A group of R. rubrum DraG variants that have lost their regulatory properties in response to energy depletion were selected by both random and localized PCR mutagenesis, followed by an in vivo screen for mutants that retained a high nitrogenase activity 60 min after a shift to darkness.23 The variants V98L, N100K and C102S were identified (equivalent residues marked by # in Fig. 3) and these are all located at the N-terminus of H5, which is proposed to be involved in binding the pyrophosphate moiety of ADPR. DraG-mediated regulation of nitrogenase activity occurs in response to either energy depletion or excess extracellular ammonium. Whilst the mechanism of signal transduction for energy depletion is not understood, the response to ammonium is known to be mediated by interaction between DraG and the P1a signal transduction protein GlnZ, which is in turn sequestered to the cell membrane by complex formation with the ammonia channel protein AmtB.10,11,25 Biochemical studies of the three R. rubrum DraG regulatory variants indicated that two of them, V98L and N100K, were impaired in the membrane association typical of wild-type DraG.23 Both these residues are surface-exposed in A. brasilense DraG. Furthermore, one of the variants, N100K, was tested for its response to extracellular ammonium and that was also shown to be significantly impaired. These data could implicate H5, or at least its N-terminal region, in interaction with a regulatory protein, such as GlnZ, whose inhibitory action would then arise from sterically hindering or blocking access to the active site upon complex formation.

Conclusions

A. brasilense DraG, a bacterial type of ADP-ribosylhydrolase that acts specifically towards a mono-ADP-ribosylated substrate, shows an all-α-helix structure and the presence of two magnesium ions in the active site. Comparison of its structure with archael and human orthologues indicates that the ADP-ribosylhydrolase fold is conserved in all domains of life. A. brasilense DraG is the first of these structurally characterized ADP-ribosylhydrolases for which the cognate target protein is known. Modeling of the binding of an ADP-ribosyl moiety suggests that the magnesium ions have a crucial role in positioning and activating the terminal ribose unit for nucleophilic attack by a water molecule. They indicate also the importance of the interactions with the pyrophosphate moiety for substrate binding, in agreement with biochemical data. Knowledge of the structure of DraG will considerably facilitate an understanding of how DraG activity is regulated in response to environmental signals and how it interacts with its target, the nitrogenase NifH protein.

Materials and Methods

Purification and crystallization

The expression plasmid pLHPETDraGwt,11 which encodes wild type A. brasilense DraG and encodes kanamycin resistance, was freshly transformed into Escherichia coli BL21(DE3) cells by heat shock. A single colony was inoculated into 2 ml of LB medium and grown with shaking at 200 rpm at 37 °C for 6–7 h and then inoculated into 100 ml of LB medium which was kept shaking at 200 rpm at 37 °C overnight. Fifteen milliliters of overnight culture was used to inoculate 1 l autoinduction medium.26 After 24 h, cells were harvested and kept frozen at −20 °C. Kanamycin was added to all media at 50 μg/ml.

Two grams cell paste were suspended in 20 ml of lysis buffer (50 mM Tris–HCl pH 8; 0.1 M NaCl; 10% (v/v) glycerol). After cell disruption using a French press (EmulsiFlex C3), the lysate was centrifuged for 30 min at 30,000 g at 4 °C. The clear supernatant was loaded onto a 5 ml Hi-Trap Heparin column (Amersham Bioscience) that was equilibrated with buffer A (50 mM Tris–HCl pH 8, 0.1 M NaCl). After washing with 50 ml of buffer A, a 100 linear gradient with buffer B (50 mM Tris–HCl pH 8, 1 M NaCl) was applied to the column. DraG eluted at 0.5 M NaCl and the eluted fractions were pooled and concentrated to 1 ml using a Vivaspin 10K concentrator (Sartorious Biolab Products). The protein was desalted on a NAP-10 column (Amersham Bioscience) using buffer A, MgCl2 was added to the protein solution to 10 mM, which was then concentrated to 35 mg ml−1 as determined using a nanodrop spectrophotometer.

Before crystallization, the protein sample was centrifuged at 20,000 g at 4 °C for 10 min. Initial screening for crystals was performed with a Phoenix crystallization robot using a drop size of 200 nl. The sample was tested in three commercial screens from Nextal (the pH clear suite, the PACT suite and the PEGs suite), and 10 of 288 tested conditions yielded crystals. The best condition was mixing the concentrated protein solution with reservoir solution (0.1 M Hepes pH 7.5, 15% (v/v) PEG 20000) in a 1:1 ratio. Crystals appeared within two–three days as rods with a size of around 50 μm × 200–300 μm.
Structure determination and refinement

For data collection, crystals were mounted on cryoloops directly from crystallization drops and frozen in a cold nitrogen gas stream at 100 K. Diffraction data were collected on the Pilatus detector at beamline X06SA, Swiss Light Source (SLS), Paul Scherrer Institut, Villigen. A total of 720 images using a rotation angle of 0.25° and an exposure time of 0.5 s per image were recorded. The data were processed using the XDS program.27 Analysis of the data using phenix.xtriage as implemented in PHENIX28 gave no indication of twinning. The structure of DraG was solved by molecular replacement (MR) using as a search model the HB8 protein from T. thermophilus (PDB code 2CWC), predicted to be ADP-ribosylglycohydrolase related, after truncation of some non-homologous parts. Using program Phaser,29 a convincing solution was obtained in space group P65 with two molecules (A and B) in the asymmetric unit. An initial 3.0 Å resolution electron density map based on the MR model phases indicated numerous side chain substitutions corresponding to the A. brasilense DraG sequence and an initial DraG model was fit to this density using the computer graphics program Coot.30 This model was improved and completed through several cycles of restrained refinement using program REFMAC.31 and manual fitting together with real space refinement as implemented in Coot. The density in the putative active site region of DraG, inferred from its homology to hARH3,3,14 indicated the presence of a bimetal cluster as observed in the human enzyme and two magnesium ions together with ligating water molecules were added to each protein molecule and refined. Final refinement statistics are summarized in Table 2. All figures are based on molecule A where the active site is better defined and generally has somewhat lower isotropic Debye-Waller factors than molecule B (average values are 38.1 Å2 and 40.9 Å2 for A and B, respectively).

Determination of ADP-ribose binding constant by ITC

Binding of ADPR to DraG was determined by ITC using an iTC200 Microcalorimeter (Microcal, Northampton, MA, USA) at 16°C. DraG protein was eluted as a monodisperse peak from a Superdex 200 column in buffer A (50 mM Hepes pH 7.5, 0.1 M NaCl, 1 mM MgCl2, 10% glycerol). The sample cell (volume 0.25 ml) was filled with a 50 μM solution of DraG (κ20° = 4.12 × 104 M−1 cm−1) and the reference cell with buffer A. Samples of 1 μl of 1 mM ADPR (Fluka) (buffer A) from a stirred syringe were injected 20 times into the sample and the reference cell leading to a characteristic heat signal with 100 s delay between injections. Integration of the individual calorimeter traces yielded the heat of binding of each injection step. The binding isotherms were fitted via a nonlinear summation over the working set and the test set reflections, respectively.

Table 2. Data collection and refinement statistics

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</tbody>
</table>

We have modeled the binding of ADPR to DraG using the interactive modeling program Moloc35 and its force field for local energy minimization. In our initial placement of ADPR we were guided by the in silico docking experiments of ADP-ribosyl-ADPR to hARH3. From ADPR molecules observed in different complexes deposited in the Protein Data Bank (PDB) we selected one with a conformation fitting the overall shape of the active site cavity (2FAV). To mimic the stereochemistry of an ADP-ribosylated substrate we chose the α anomer form for the terminal ribose. In the initial manual docking, the ribose and pyrophosphate moieties of ADPR could be placed in a deeper groove at the active site while the adenosine moiety had to remain at the surface. All water molecules in the active site groove of the refined structure were removed but their positions were used as guides in the initial placement of the ribose and pyrophosphate moieties of ADPR. Positioning the latter in the narrow cleft extending from the Mg-binding site towards the N-terminus of H6 enabled the formation of several hydrogen bonds of the phosphate oxygens with donors of the protein. The attached terminal ribose with the scissile α-glycosidic bond was then oriented in the putative active site defined by the two bound magnesium ions and their highly conserved ligating side chains. For this, we were guided by (i) trying to make the ribose hydroxyl groups substitute for metal-bound water molecules, (ii) avoiding steric problems when substituting the glycosidic hydroxyl with an Arg-linked protein, and (iii) permitting the attack of a water molecule at the anomeric carbon from opposite the scissile bond. The adenosine moiety at the opposite end of the pyrophosphate was simply modeled in a conformation avoiding steric clashes with the protein. Energy minimization of several such initial ADPR poses, keeping the protein fixed, always pushed the diphosphate moiety out of the groove, as some close contacts with the protein could not be relaxed by conformational adjustments. We therefore permitted two tripeptide segments on opposite sides of the pyrophosphate cavity (124–126 and 99–101) to relax in addition to ADPR during energy minimization. Modest structural adjustments in these segments widened the cavity by about 1 Å and led to a

† http://xds.mpimp-heidelberg.mpg.de/html_doc/XDS.html
stable energy-minimized docking of ADPR, and maintaining most of the initial putative hydrogen bonding and metal ligation interactions.

**Protein Data Bank accession number**

The coordinates and structure factors of *Azospirillum brasilense* dinitrogenase reductase activating glycohydrolase (DraG) have been deposited in the PDB under ID code 3G9D.

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**Supplementary Data**

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jmb.2009.05.031

**References**


