Cleavage of nicotinamide adenine dinucleotide by the ribosome-inactivating protein from *Momordica charantia*

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Received 22 March 2015

Accepted 14 July 2015

Edited by W. N. Hunter, University of Dundee, Scotland

Keywords: ribosome-inactivating protein; *N*-glycosidase; nicotinamide; crystal structure; momordin.

PDB references: momordin, co-crystallized with nicotinamide, 4yp2; co-crystallized with NADP⁺, 5cf9

Supporting information: this article has supporting information at journals.iucr.org/f

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The interaction of momordin, a type 1 ribosome-inactivating protein from *Momordica charantia*, with NADP⁺ and NADPH has been investigated by X-ray diffraction analysis of complexes generated by co-crystallization and crystal soaking. It is known that the proteins of this family readily cleave the adenine–ribose bond of adenosine and related nucleotides in the crystal, leaving the product, adenine, bound to the enzyme active site. Surprisingly, the nicotinamide–ribose bond of oxidized NADP⁺ is cleaved, leaving nicotinamide bound in the active site in the same position but in a slightly different orientation to that of the five-membered ring of adenine. No binding or cleavage of NADPH was observed at pH 7.4 in these experiments. These observations are in accord with current views of the enzyme mechanism and may contribute to ongoing searches for effective inhibitors.

1. Introduction

Momordin (MOM), also known as α -momorcharin (EC 3.2.2.22), is a type 1 ribosome-inactivating protein (RIP) from the seeds of the bitter gourd *Momordica charantia*. RIPs are predominantly plant-derived toxins which possess *N*-glycosidase activity. They depurinate a specific conserved adenine residue from a GAGA hairpin loop of the 28S ribosomal RNA of the 60S component of eukaryotic ribosomes and inactivate them. Type 1 RIPs such as MOM consist of a single A chain of about 250 amino acids with *N*-glycosidase activity, while type 2 RIPs such as ricin (RTA) contain an additional B chain with lectin properties, linked to the A chain by a disulfide bridge and enabling penetration of cells (Virgilio *et al.*, 2010; Stirpe, 2013). RIPs probably contribute to the survival of plants by virtue of their antifungal, antiviral and insecticidal properties. There has been considerable interest in harnessing and regulating the highly potent cell-killing properties of these toxins as immunoconjugates capable of targeting tumour cells. There is also concern regarding the accessibility of such potent toxins from commonly available plant products. Thus, the mechanism of action of these *N*-glycosidases remains of great interest as a foundation for the development of effective inhibitors as antitoxins. Recently, progress has been made in the design of tetranucleotide transition-state mimic inhibitors and in fragment-based searches for smaller inhibitors (Ho *et al.*, 2009; Bai *et al.*, 2009, 2010; Pruet *et al.*, 2011). A persistent feature of these investigations is the variability in the properties of these homologous toxins. This led us to investigate the interaction

Table 1

Data and refinement statistics for momordin co-crystallized with nicotinamide and NADP⁺.

Values in parentheses are for the highest resolution shell.

	MOM–nicotinamide	MOM–NADP ⁺
PDB code	4yp2	5cf9
Source	Beamline I04, DLS	Beamline I03, DLS
Wavelength (Å)	0.979	0.917
Space group	<i>H</i> 3	<i>H</i> 3
Unit-cell parameters		
<i>a</i> (Å)	130.10	130.51
<i>b</i> (Å)	130.10	130.51
<i>c</i> (Å)	37.57	37.98
γ (°)	120	120
Resolution (Å)	37.56–1.34 (1.39–1.34)	62.5–1.52 (1.56–1.52)
<i>R</i> _{merge}	0.027 (0.81)	0.058 (0.91)
<i>R</i> _{meas} [†]	0.043 (1.05)	0.066 (1.13)
<i>R</i> _{p.i.m.} [†]	0.022 (0.57)	0.023 (0.47)
Multiplicity	3.3 (3.3)	7.9 (5.6)
Completeness (%)	99.4 (99.6)	99.9 (99.9)
Average <i>I</i> / σ (<i>I</i>)	16.8 (1.4)	15.3 (1.5)
Reflections observed	173299 (12702)	292456 (15408)
Unique reflections	51799 (3869)	37103 (2759)
Wilson <i>B</i> factor (Å ²)	17.90	17.89
Solvent content (%)	45	46
Molecules in asymmetric unit	1	1
Refinement		
<i>R</i> _{work}	14.10	13.83
<i>R</i> _{free} (5%)	16.80	16.80
<i>MolProbity</i> score	1.01	1.27
Ramachandran outliers	1	—
R.m.s.d., bonds	0.004	0.008
R.m.s.d., angles	0.975	1.11
No. of nicotinamides	1	1
No. of residues	246	246
No. of waters	272	202

[†] Diederichs & Karplus (1997).

between MOM and a variety of molecules that include adenine in their composition as surrogates of the ribosomal interaction. Prior work on the ricin A chain, MOM and other RIPS have shown that the adenine–ribose bond of nucleotides and nucleosides is cleaved in the crystalline protein, but NADPH was reported to bind intact to tricosanthin (Xiong *et al.*, 1994) and this led us to investigate the interaction between MOM and NADPH.

It has been well established from earlier crystallographic studies on RTA and MOM (Monzingo & Robertus, 1992; Ren *et al.*, 1994) that the cleaved adenine moiety remains bound to the enzyme in a deep active-site pocket by stacking with Tyr80 in RTA (MOM 70) and forms the following hydrogen bonds: N1–Ile81 N (MOM 71), N3–Arg180 NH1 and NH2 (MOM 163), N6–Ile82 O (MOM 71) and N6–Gly121 O (MOM 109). Tyr80 is a mobile residue in the active site and changes its conformation upon binding substrate, optimizing the stacking interactions with the adenine ring. The mechanism of hydrolysis of the *N*-glycosidic bond was informed by studies of acid hydrolysis, which involves protonation at N3 and N7, stabilizing an adenyl cation leaving group, and attack by water at the weakening C1–N1 bond and the evolving ribocation intermediate (Zoltewicz *et al.*, 1970). The water molecule is bound to the protein rather than deriving from bulk solvent. Conflicts have been presented in the roles of the critical

residues Arg180 and Glu177 (MOM 160) and in the identity of the attacking water molecule (Huang *et al.*, 1995). Nucleoside analogues where the glycosidic bond is replaced by a C–C bond (formycin) are not cleaved and bind intact (Ren *et al.*, 1994). In the most recent description of a ricin-bound tetranucleotide transition-state inhibitor, N7 and N6 of adenine interact with Gly21 O. A water molecule close to the glycosidic bond and hydrogen-bonded to Glu177 implicates them as the significant nucleophile and a general base, respectively. The nucleotide bases interleave with Tyr80 and Tyr123 (MOM 111) to give an aromatic stack flanked by Arg134 (Ho *et al.*, 2009).

In this paper, we describe the surprising finding that momordin cleaves the nicotinamide base from the NADP⁺ molecule (see Fig. 1) and forms a stable complex with the product nicotinamide instead of binding the adenine moiety as in other related enzymatic reactions.

2. Materials and methods

MOM was purified from *M. charantia* seeds as described previously (Husain *et al.*, 1994). It was crystallized from an 8 mg ml^{−1} stock protein solution in 20 mM phosphate buffer pH 7.4 by hanging-drop vapour diffusion with polyethylene glycol 4000 precipitation [10–15% (w/v)] and drops consisting of 2 μ l protein solution and 2 μ l reservoir solution at 293 K. NADPH and NADP⁺ were purchased from Sigma and nicotinamide was purchased from Aldrich, and they were either included in well solutions at 20 mM (a 75-fold excess) for co-crystallization or added to the mother liquor of preformed crystals of the protein and soaked for 6 d at 277 K. Data were collected at 100 K from crystals cryoprotected in 30% (v/v) glycerol on beamlines I03 or I04 at Diamond Light Source (DLS) using Pilatus detectors (Trueb *et al.*, 2012). Images were processed with *XDS* (Kabsch, 2010) within the *xia2* pipeline (Winter, 2010). The momordin molecule (PDB entry 1mom; Husain *et al.*, 1994) was positioned with *Phaser* (McCoy *et al.*,

Figure 1

The structure and numbering of nicotinamide adenine dinucleotide phosphate, which is cleaved by momordin.

2007) and restrained refinement was carried out with *phenix.refine* (Afonine *et al.*, 2012) and modelling with *Coot* (Emsley & Cowtan, 2004). Rounds of model building and refinement to optimize the fit of protein and water atoms preceded ligand fitting. A randomly selected portion of the data (5%) was excluded from the refinement for validation (Brünger, 1993). The structures were validated with *MolProbity* (Chen *et al.*, 2010). Data statistics are presented in Table 1. HPLC employed a Varian 5000 LC using a 300×4.6 mm Vydac 218TP54 reversed-phase column and UV detection at 254 nm. A gradient from 5 to 10% methanol in 10 mM phosphate buffer pH 5.8 was used to resolve peaks for the various nucleotide components to ensure that neither contamination nor chemical instability contributed to the results.

3. Results and discussion

Rhombohedral crystals of MOM grew within 5–10 d at room temperature. X-ray data collected from MOM crystals soaked or co-crystallized with NADP⁺ showed a strong electron-density feature located in the same general position as the five-membered ring of adenosine as described in previous work, but it rather resembled nicotinamide. Co-crystallization with nicotinamide reaffirmed this observation with clear, well defined electron density in a high-resolution map (see Figs. 2*a* and 2*b*). The structures obtained by co-crystallization with NADP⁺ or nicotinamide are reported here as evidence of the cleavage. Data from crystals soaked in NADPH showed a vacant active site. No crystals were obtained by co-crystallization with NADPH under the same conditions.

The structures of MOM determined include electron density for 246 residues of the mature protein and the first

N-acetylglucosamine of an oligosaccharide attached to Asn227. The fold includes an extended region of six β -strands packed against a domain of eight α -helices and a further region of two antiparallel strands as described previously (Husain *et al.*, 1994; see Fig. 2). The two structures reported here are essentially identical, showing an r.m.s.d. over 3276 atoms of 0.1 Å.

The bound nicotinamide ring is positioned between Tyr70 and Tyr111 (see Figs. 2 and 3). The density does not define the nicotinamide orientation, but we have selected the orientation with hydrogen bonds between the ring N atom and Arg163 NH1, the carboxamide O atom and Ile71 NH, and the carboxamide NH2 and Gly109 CO. The glycine carbonyl O atom takes up two positions splayed either side of the nicotinamide. None of the hydrogen bonds can form in the alternate nicotinamide orientation. There is a network of well defined water molecules beyond the nicotinamide, some of

Figure 2
Cartoon of the momordin molecule, showing β -strands in yellow, helices in red and bound nicotinamide in blue, sandwiched between Tyr70 (green) and Tyr111 (pink).

Figure 3
(*a*) Electron-density map ($2mF_o - DF_c$) at 1.35 Å resolution for momordin co-crystallized with nicotinamide, contoured at 1.5σ and carved at 1.5 Å with *PyMOL* (DeLano, 2002), showing a preferred hydrogen-bonded orientation for nicotinamide in the active site of momordin. Nicotinamide is shown with yellow C atoms and protein with green C atoms. Waters are shown as red spheres. (*b*) Electron-density map ($2mF_o - DF_c$) at 1.5 Å resolution contoured as described above but for momordin co-crystallized with NADP⁺. The nicotinamide has not yet been included in the refinement producing this map ($R_{\text{work}} = 18.39\%$, $R_{\text{free}} = 20.23\%$).

which would be displaced on binding of the ribose constituent of the substrate. One of the waters makes a short hydrogen bond (2.66 Å) to Glu160 and is only 3.87 Å from N1 of nicotinamide, and may represent the catalytic water. In contrast to some other structures including bound adenine, Tyr70 is well ordered and in close contact over its entire area with the nicotinamide ring. On the other side, the nicotinamide ring contacts Tyr111 and Ile155 but there is no aromatic stack. There is an extended region of weak unassigned difference electron density within 4 Å of the other side of Tyr70 in a position analogous to the location of guanine in the tetranucleotide complex of Ho *et al.* (2009) (PDB entry 3hio). The validation report from *MolProbity* indicated a single marginal Ramachandran outlier ($\varphi, \psi = 62.1, 20.4^\circ$) at position Val69 in one structure and the same residue as a κ_1 outlier (212.8°) in the other. The electron density strongly supports these similar conformations, perhaps implying some structural strain in this region, which is adjacent to the ligand-binding site and the mobile residue Tyr70.

These observations are in keeping with expectations of the cleavage mechanism involving ribocation and adenine-cation intermediates and the influence of substituents on their stability. The dinucleotide substrate presents two glycosidic bonds: one to adenine and one to nicotinamide. In its oxidized form, NADP⁺, an explicit charge on the nicotinamide ring as well as hydrogen bonding in the active site is sufficiently influential to achieve bond breakage to nicotinamide by MOM. Phosphorylation at the 2' position of the ribose ring appears to limit ribo-cation formation and disable adenine cleavage. This phosphorylation, together with the loss of the charge on the nicotinamide, prevents cleavage of both glycosidic bonds in NADPH, as we see no cleavage product in the active site of MOM. Cleavage was not observed in earlier work with tricosanthin (PDB entry 1tcs), although intact NADPH was bound to the protein and hydrogen-bond interactions attributed to enhancing the leaving-group characteristics of adenine were formed (Xiong *et al.*, 1994). These may be necessary but not sufficient to achieve breakage of the glycosidic bond. The 2' phosphate group is positioned in an accessible polar region in 1tcs but very close to the 5' phosphate. The discrepancy between the results for MOM and tricosanthin may be owing to their individual characteristics or to differences in the crystallization conditions, packing and refinement.

When the 2' substituent is a hydroxyl as in adenosine, the glycosidic bond is readily cleaved and leaves adenine bound to the protein. The same product results from cleavage of ATP and dATP by the RIP from *M. balsamina* (Kushwaha *et al.*, 2012). It is of note that Tyr70 in the enzyme–adenine product complex from dATP cleavage occupies a distinct rotamer from that observed on ATP cleavage. This rotamer has previously been observed in the intact RIP–formycin complex and is interpreted as a trapped intermediate state, indicating slower degradation of DNA substrates. A contrary influence of the 2'

ribose hydroxyl substituent is well known in the sense that hydrolytic depurination of RNA is relatively harder to achieve than it is for DNA. The active sites of various RIPs appear to modulate the chemical susceptibility of substrates in different ways, so that while RTA shows high specificity for a particular base in ribosomal RNA, the homologous saporin from *Saponaria officinalis* also readily degrades DNA substrates (Barbieri *et al.*, 2000).

In conclusion, the cleavage of nicotinamide from NADP⁺ and its retention in the active site of MOM *via* a selection of hydrogen-bond interactions indicates that it may be a useful small fragment for elaboration into the adjacent ordered water space and the discovery of effective new RIP inhibitors.

Acknowledgements

We acknowledge access to the Diamond Light Source.

References

- Afonine, P. V., Grosse-Kunstleve, R. W., Echols, N., Headd, J. J., Moriarty, N. W., Mustyakimov, M., Terwilliger, T. C., Urzhumtsev, A., Zwart, P. H. & Adams, P. D. (2012). *Acta Cryst.* **D68**, 352–367.
- Bai, Y., Monzingo, A. F. & Robertus, J. D. (2009). *Arch. Biochem. Biophys.* **483**, 23–28.
- Bai, Y., Watt, B., Wahome, P. G., Mantis, N. J. & Robertus, J. D. (2010). *Toxicon*, **56**, 526–534.
- Barbieri, L., Valbonesi, P., Govoni, M., Pession, A. & Stirpe, F. (2000). *Biochim. Biophys. Acta*, **1480**, 258–266.
- Brünger, A. T. (1993). *Acta Cryst.* **D49**, 24–36.
- Chen, V. B., Arendall, W. B., Headd, J. J., Keedy, D. A., Immormino, R. M., Kapral, G. J., Murray, L. W., Richardson, J. S. & Richardson, D. C. (2010). *Acta Cryst.* **D66**, 12–21.
- DeLano, W. L. (2002). *PyMOL*. <http://www.pymol.org>.
- Diederichs, K. & Karplus, P. A. (1997). *Nature Struct. Biol.* **4**, 269–275.
- Emsley, P. & Cowtan, K. (2004). *Acta Cryst.* **D60**, 2126–2132.
- Ho, M.-C., Sturm, M. B., Almo, S. C. & Schramm, V. L. (2009). *Proc. Natl Acad. Sci. USA*, **106**, 20276–20281.
- Huang, Q., Liu, S., Tang, Y., Jin, S. & Wang, Y. (1995). *Biochem. J.* **309**, 285–298.
- Husain, J., Tickle, I. J. & Wood, S. P. (1994). *FEBS Lett.* **342**, 154–158.
- Kabsch, W. (2010). *Acta Cryst.* **D66**, 125–132.
- Kushwaha, G. S., Pandey, N., Sinha, M. S., Singh, B., Kaur, P., Sharma, S. & Singh, T. P. (2012). *Biochim. Biophys. Acta*, **1824**, 679–691.
- McCoy, A. J., Grosse-Kunstleve, R. W., Adams, P. D., Winn, M. D., Storoni, L. C. & Read, R. J. (2007). *J. Appl. Cryst.* **40**, 658–674.
- Monzingo, A. F. & Robertus, J. D. (1992). *J. Mol. Biol.* **227**, 1136–1145.
- Pruet, J. M., Jasheway, K. R., Manzano, L. A., Bai, Y., Anslyn, E. V. & Robertus, J. D. (2011). *Eur. J. Med. Chem.* **46**, 3608–3615.
- Ren, J., Wang, Y., Dong, Y. & Stuart, D. I. (1994). *Structure*, **2**, 7–16.
- Stirpe, F. (2013). *Toxicon*, **67**, 12–16.
- Trueb, P., Sobott, B. A., Schnyder, R., Loeliger, T., Schneebeli, M., Kobas, M., Rassool, R. P., Peake, D. J. & Broennimann, C. (2012). *J. Synchrotron Rad.* **19**, 347–351.
- Virgilio, M. de, Lombardi, A., Caliendo, R. & Fabbrini, M. S. (2010). *Toxins*, **2**, 2699–2737.
- Winter, G. (2010). *J. Appl. Cryst.* **43**, 186–190.
- Xiong, J.-P., Xia, Z.-X. & Wang, Y. (1994). *Nature Struct. Mol. Biol.* **1**, 695–700.
- Zoltewicz, J. A., Clark, D. F., Sharpless, T. W. & Grahe, G. (1970). *J. Am. Chem. Soc.* **92**, 1741–1750.