



Crystal structures of a type-1 ribosome inactivating protein from *Momordica balsamina* in the bound and unbound states

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ABSTRACT

The ribosome inactivating proteins (RIPs) of type 1 are plant toxins that eliminate adenine base selectively from the single stranded loop of rRNA. We report six crystal structures, type 1 RIP from *Momordica balsamina* (A), three in complexed states with ribose (B), guanine (C) and adenine (D) and two structures of MbRIP-1 when crystallized with adenosine triphosphate (ATP) (E) and 2'-deoxyadenosine triphosphate (2'-dATP) (F). These were determined at 1.67 Å, 1.60 Å, 2.20 Å, 1.70 Å, 2.07 Å and 1.90 Å resolutions respectively. The structures contained, (A) unbound protein molecule, (B) one protein molecule and one ribose sugar, (C) one protein molecule and one guanine base, (D) one protein molecule and one adenine base, (E) one protein molecule and one ATP-product adenine molecule and (F) one protein molecule and one 2'-dATP-product adenine molecule. Three distinct conformations of the side chain of Tyr70 were observed with (i) $\chi^1 = -66^\circ$ and $\chi^2 = 165^\circ$ in structures (A) and (B); (ii) $\chi^1 = -95^\circ$ and $\chi^2 = 70^\circ$ in structures (C), (D) and (E); and (iii) $\chi^1 = -163^\circ$ and $\chi^2 = 87^\circ$ in structure (F). The conformation of Tyr70 in (F) corresponds to the structure of a conformational intermediate. This is the first structure which demonstrates that the slow conversion of DNA substrates by RIPs can be trapped during crystallization.

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1. Introduction

Ribosome inactivating proteins (RIPs) are *N*-glycosidases (EC 3.2.2.22) which catalytically hydrolyze *N*-glycosidic bond between adenine and ribose in highly conserved sarcin/ricin loop of the large subunits of ribosomes [1,2]. The depurination of this specific adenine results in the prevention of the elongation factor (EF-2) from binding to the large subunits of ribosomes and thus arrests the protein biosynthesis [3]. RIPs are a group of plant toxins and have been classified into three groups according to their structural organizations [4]. Type 1 RIPs, are catalytically active single chain polypeptides with molecular mass ranging from 26 to 31 kDa. Type 2 RIPs with molecular mass ranging from 60 to 65 kDa, consist of two chains, chain A is functionally similar to type 1 RIPs, and is linked through a disulfide bond to lectin-like B chain. Type 3 RIPs are single chain polypeptides containing an extended C-terminal region with an unknown function [5]. The RIPs are known to remove an adenine residue at the 4324th position (according to rat sequence) from the sarcin/ricin loop (SRL) of 28S rRNA of eukaryotic ribosomes. Type 1 RIPs are capable of depurinating both prokaryotic as well as eukaryotic ribosomes [6]. In a contrast, type 2 RIPs have a preference for animal ribosomes [7]. In case of type 3 RIPs, the amino terminal domain resembles with type

1 RIPs. It is linked to an unrelated carboxyl-terminal domain whose function is still unknown [5]. The target site in prokaryotic ribosomes is 2660th adenine residue (according to *E. coli* sequence) of SRL of 23S rRNA. In addition to SRL, these RIPs also have *N*-glycosidase activity on other substrates such as, genomic DNA, poly(A)RNA, viral RNA and mRNA [8,9]. It has been suggested that the RIPs have a considerable potential for medicinal applications [10,11]. They inhibit HIV replication and integration [12,13] and show specific *in vitro* and *in vivo* antitumor activity against breast cancer and melanoma cells [14]. It has been shown to be useful in various clinical conditions [15,16] while further investigations related to its efficacy against different diseases are required to be pursued. In this regard, it is imperative to examine structures and functions of RIPs from different sources as well as its complexes with various compounds including substrate analogs, substrate products and also structural intermediates. Although structures of type 1 RIPs from several species were known [17–25], the precise mode of substrate binding and catalytic mechanism was not yet fully understood. Similarly, structures of several type 2 RIPs, whose A chain is structurally and functionally similar to type 1 RIPs, were also known [26–29] with which a detailed comparison is necessary. We report here crystal structure of a type 1 RIP from *Momordica balsamina* (MbRIP-1) together with structures of its three complexes, one with ribose sugar, two with free bases guanine and adenine as well as two structures of its complexes with product adenine which was obtained from the crystallization conditions using an RNA substrate adenosine triphosphate (ATP) and a DNA substrate, 2'-deoxyadenosine triphosphate (2'-dATP). These structures

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have revealed three distinct conformational states of protein representing its unbound state, when bound to free adenine or product adenine from ATP and when bound to product adenine from substrate 2'-dATP. The last structure represents the first RIP structure of a conformational intermediate.

2. Materials and methods

2.1. Isolation and purification of a type 1 RIP

The dried seeds of *M. balsamina* were obtained from local market. The 50 g of seeds was decorticated and pulverized in the presence of liquid nitrogen in a ventilated hood. The 300 ml extraction solution containing 10 mM sodium phosphate buffer (pH 7.5), was added to the ground tissue. The sample was homogenized by Polytron Homogenizer (Cole Parmer Homogenizer, Vernon Hills, USA) for 15 min. The homogenate was stirred for 12 h at 4 °C. In order to remove insoluble particles the homogenate was filtered through four-layered muslin cloth and then centrifuged at 14,000 g for 15 min at 4 °C. The supernatant was collected.

Further purification of RIP-1 was carried out using three steps. The sample was loaded on Blue Sepharose 6 Fast Flow (GE Healthcare, Uppsala, Sweden) column (10 cm × 2.5 cm), which was pre-equilibrated with 20 mM sodium phosphate buffer pH 7.5. After extensive washing of column using the same buffer, the gradient elution was performed using 0.5 M NaCl on AKTA Prime Plus (GE Healthcare, Uppsala, Sweden). The eluted fractions were analyzed at 280 nm using Varian Cary-100 Bio UV-Visible Spectrophotometer (Varian, Palo Alto, USA) and the elution profile was recorded. The peak containing RIP-1 as indicated by an approximate molecular mass of 30 kDa on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was pooled and concentrated by Amicon ultrafiltration (Millipore, Billerica, USA). The identity of RIP-1 was also confirmed by determining the sequence of the first 20 amino acid residues from N-terminus with an automated protein sequencer PPSQ-21A (Shimadzu, Kyoto, Japan). This type 1 RIP from *M. balsamina* will be called hereafter as MbRIP-1. The concentrated protein sample was further processed through CMC-Sephadex C-50 column (10 cm × 2.5 cm) which was pre-equilibrated with 20 mM sodium phosphate buffer (pH 7.5). In order to remove the unbound proteins, the column was extensively washed with the same buffer until absorbance of the washing solution reduced to <0.05 at 280 nm. The bound protein fractions were eluted with a linear gradient of 0.0–0.2 M NaCl (in 20 mM phosphate buffer, pH 7.5) in a fine gradient maker AKTA Prime Plus (GE Healthcare, Uppsala, Sweden). The peak corresponding to molecular weight of MbRIP-1 was pooled and concentrated using Amicon ultrafiltration device with a membrane of 3 kDa cut off. The concentrated samples of purified protein were lyophilized. In order to remove salt, plant pigments and other alkaloid impurities from the CMC-Sephadex eluted samples, a gel filtration chromatographic step was carried out with Sephadex G-50 column (150 cm × 1 cm) which was washed and pre-equilibrated with 20 mM sodium phosphate buffer (pH 7.5) and the protein was eluted with the same buffer at a flow rate of 6 ml/h. The fractions containing MbRIP-1 as identified using SDS-PAGE were pooled, concentrated and lyophilized. In order to confirm the identification of final sample of protein, it was subjected to N-terminal amino acid sequence determination using an automated protein sequencer PPSQ-21A (Shimadzu, Kyoto, Japan) whereby the sequence of the first 20 amino acid residues showed more than 50% sequence identities with type 1 RIPs [17–25] and about 30% sequence identities with type 2 RIPs [26–29].

2.2. Complete amino acid sequence determination

In order to obtain the complete amino acid sequence of MbRIP-1, the fresh seeds of *M. balsamina* were homogenized in 4 M guanidine

isothiocyanate (GITC) buffer (pH 5.0) in ice cooled conditions and stored at –70 °C. The total RNA was extracted using the phenol/chloroform extraction method [30]. The poly (A) RNA was isolated from the total RNA using Oligo(dT)-Cellulose Type 7 (GE Healthcare, Uppsala, Sweden) as per manufacturer's instructions. The small syringe column packed with oligo (dT) was washed with 0.1 M NaOH. The TE buffer (10 mM Tris-HCl, pH 7.5 containing 1 mM EDTA) was used for equilibrating the column. The total RNA was redissolved in 0.5 ml of TE buffer that contained 1 M NaCl, heated at 65 °C for 5 min, cooled immediately by placing it in ice. The chilled RNA was loaded on the above column. The column was washed with 3 ml of washing buffer (0.2 M NaCl, 10 mM Tris-HCl, pH 7.5 and 1 mM EDTA). Then RNA was eluted using TE buffer. The denatured poly (A) RNA was used as a template for cDNA synthesis. The cDNA was prepared using RevertAid™ First Strand cDNA Synthesis Kit (Fermentas, Glen Burnie, USA) as per manufacturer's instructions. The 12 µl of reagents consisting of 1 µg poly (A) RNA, 1 µl oligo dT primer and diethyl pyrocarbonate (DEPC) treated sterile water were added into a sterile, nuclease-free tube on ice to which 4 µl 5 × reaction buffer (250 mM Tris-HCl (pH 8.3), 250 mM KCl, 20 mM MgCl₂, 50 mM DTT), 1 µl RiboLock™ RNase inhibitor (20 U/µl), 2 µl of 10 mM dNTP (dATP, dCTP, dGTP and dTTP) Mix, 1 µl RevertAid™ M-MuLV (Moloney murine leukemia virus) reverse transcriptase (200 U/µl) were added. The reaction was set in a total of 20 µl volume, mixed gently and centrifuged. The reaction mixture was incubated in thermal cycler (Applied Biosystem, Foster City, USA) at 42 °C for 1 h. PCR reactions were performed with cDNA as the template from the RT-PCR reactions. For amplifying the mature polypeptide gene, the primers were designed using conserved sequences from type 1 RIPs [17–24]. The primers used were 5'-GATGTTAGCTTTCGTTTGTCG-3' (forward primer) and 5'-AATATTTTTTGTGTTTAAACAATAACTGTAT-3' (reverse primer). These corresponded to amino acid sequences of Asp¹-Val-Ser-Phe-Arg-Leu-Ser⁷ and Ile²³⁷-Gln-Leu-Leu-Leu-Asn-Thr-Lys-Asn-Ile²⁴⁶ in type 1 RIPs [31]. The PCRs were carried out with Taq polymerase (Promega, Madison, USA) using thermal cycler (Applied Biosystem, Foster City, USA). The nucleotide sequence of MbRIP-1 was determined using cloned double-strand DNA (pGEM-T) with automatic sequencer model ABI-377 which has been submitted to GenBank with accession code of HM367595.

2.3. N-glycosidase activity assay

The N-glycosidase depurination assay was carried out on both prokaryotic and eukaryotic ribosomes. All reagents and buffers were made using DEPC treated water. The ribosomes were isolated from two organisms, *E. coli* DH5α and rat liver, (i) *E. coli* cells were grown in Luria-Bertani broth (Hi-Media, Mumbai, India) for overnight at 37 °C. The culture was harvested by centrifugation at 4000 g for 20 min. The pellet was resuspended in extraction buffer and subjected to lysis by ultrasonication for 3 min with 1 min for each cycle. In order to remove the undissolved debris, it was centrifuged at 12,000 g at 4 °C for 20 min. (ii) A similar procedure was used with fresh rat liver tissue for extraction of ribosomes. The tissue was sliced and minced in extraction buffer. The sample was homogenized in Polytron homogenizer (Cole Parmer Homogenizer, Vernon Hills, USA) for 10 min in ice. The cell lysate was centrifuged at 12,000 g at 4 °C for 20 min for removing the undissolved parts. The supernatants obtained from above two procedures were laid separately over a cushion of 10 ml sucrose (1 M sucrose, 20 mM KCl and 5 mM MgCl₂ in 25 mM Tris-HCl, pH 7.5) in 70-Ti tubes (Beckman Coulter, Brea, USA) and centrifuged at 200,000 g for 4 h at 4 °C. The resulting pellets were suspended in RIP buffer (25 mM Tris-HCl, pH 7.5 containing 20 mM KCl and 5 mM MgCl₂).

The depurination reactions were prepared for a final volume of 100 µl. A total of 6 sets of reactions were set up, each containing 10 µl of ribosome, 10 µl of 10 × RIP buffer (250 mM Tris-HCl, pH 7.5

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