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Biochemical Aspects of Ricin-Ribosome Complex: A Short Review

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Review Article

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Abbreviations:
RNA: Ribonucleic acid;
RIP: Ribosome inactivating proteins;
RTA: Ricin toxin A;
RTB: Ricin toxin B;
SRL: Sarcin ricin loop;
RCA: Ricinus communis agglutinin;
EF-Tu: Elongation factor – temperature unstable;
EF-G: Elongation factor – G;
GTPase: Guanosine triphosphatase;
BFA: Brefeldin A;
ER: Endoplasmic reticulum;
NMR: Nuclear magnetic resonance

ABSTRACT

The large ribosomal subunit contains a highly conserved RNA sequence (GAGA tetra loop) which is crucial for the binding of the elongation factors during translation. Ribosome- inactivating proteins (RIPs) like ricin and alpha sarcin binds at this sequence and discourage the interaction of the elongation factors thus, inhibiting the translation process. Ricin is a heteromer composed of two subunits RTA and RTB linked by a single disulphide bond. The B subunit does not interact with the ribosome but is only a carrier of the catalytically active subunit A. In this paper we have tried to name some of the ricin inhibitors and their mechanism of action. Also, we have suggested strategies to select an appropriate and effective method amongst the various methods.

INTRODUCTION

Ricinus communis or castor bean plant produces a highly noxious protein called ricin. It belongs to a family of proteins called the ribosomal inactivating proteins (RIP)-Type II [1,2]. It is composed of two monomers viz. Ricin Toxin A (RTA) and Ricin Toxin B (RTB), attached to each other by a single disulfide bond between Cys 259 of RTA and Cys 4 of RTB [3,4] **(Figure 1)**. Out of the two moieties, RTB or ricin B chain attaches to the galactose - rich glycoprotein receptors on the cell surface and is responsible for the entry of the toxin into the cell while the other subunit (RTA) ceases the protein synthesis, [2,5,6]. RTA binds and depurinates the α-Sarcin/Ricin Loop (SRL) of 28S rRNA on 60S eukaryotic ribosomal subunit [2,7]. The proteolytic cleavage of RTA and RTB is of great importance for enzymatic activity of RTA [7]. After the attachment of ricin B to the cell surface, the holotoxin is endocytosed and undergoes retrograde transport from Golgi to endoplasmic reticulum [8].

Ricin Synthesis in the Plant

Plants producing ribosome -inactivating proteins (RIPs) develop several schemes to protect themselves against their own toxins. One such scheme is producing the toxin in its inactive form [9]. Ricin is synthesized as preproricin from a single mRNA sequence in its seed stage. The inactive nascent ricin consists of 576 amino acids, the first 35 residues comprise of the signal peptide for the entry of ricin into ER. RTA comprises of 267 residues and RTB 262 residues. The two monomers are joined with a

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12 peptide long linker sequence. Also, a propeptide of unknown length is present which is later removed in the ER for the formation of mature ricin [10]. Five disulphide bonds are created [11], 4 of which are intra-RTB bonds and one that links the carboxyl end of RTA and amino end of RTB. The folded and glycosylated proricin is delivered through the dictyosomes to the storage vacuoles [12] where removal of the linker peptide takes place. This releases the matured disulfide-linked RTA-RTB heterodimer. The enzyme responsible for the conversion of proricin to mature ricin is cysteine proteases [13,14].

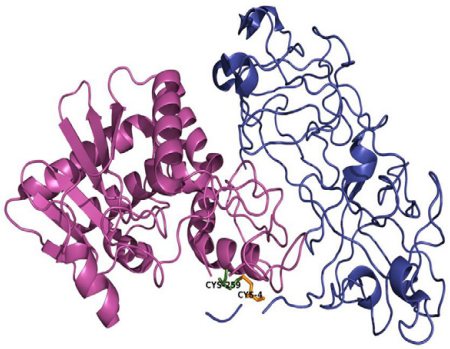

Figure 1. RTA (pink) and RTB (blue) linked by a disulfide bond. PDB Id: 2AAI.

Ricin Toxin Structure

Ricinus communis seeds contain a concoction of varied isoforms of ricin viz. Ricin E, Ricin D and Ricinus communis agglutinin (RCA). RCA, a closely related lectin is a tetramer composed of two ricin-like subunits. RCA is a weak cytotoxin but a resilient haemagglutinin, whereas ricin is a strong cytotoxin. In ricin molecule both RTA and RTB are structurally different and, therefore, exhibit sharp disparity in their biological activity [15].

Ricin Toxin A (RTA)

The A chain or RTA is composed of 8 alpha helices (A-H) and 8 beta sheets (a-h). The 117 residues at the aminoacyl end form an intact folding region. Beta sheet (1-f) and helices (A and B) form the base of the molecule. Most of the RTA is formed of alpha helices; these helices form a packed structure and rest over the beta pleated sheets. The non-polar Helix E (length more than 5 turns) travels through the molecular structure and accommodate two paramount active site residues Glu 177 and Arg 180 **(Figure 2)** towards its carboxyl terminal. About 8 amino acid residues are universally conserved in all RIP's and are mostly present at the active site. These residues include Tyr 80, Tyr 123, Glu 177, Arg 180, and Trp 211 [16].

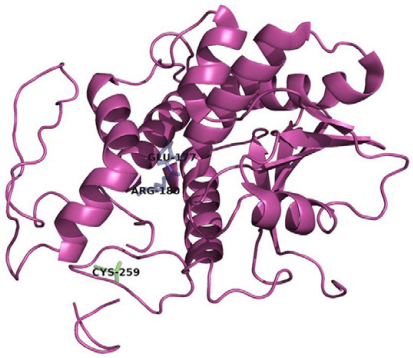

Figure 2. Structure of RTA extracted from whole ricin molecule (PDB Id: 2AAI) showing the active site residues.

Ricin Toxin B (RTB)

RTB has a homologous two domain structure that is a result of gene duplication. Domain one is towards the amino end and domain two towards carboxyl terminal. Each domain is a resultant structure of four sub-domain units; each of these contains a 17 residue long linking peptide (lambda) and 40 residue core of homologous structures (alpha, beta, and gamma) [17]. The galactose binding site on RTB is superficial and gets into contact with less than half of the sugar. The base of the galactose binding site is formed of 3 residues. The apex of the binding site is an aromatic side chain (Trp 37 and Tyr 248) and stays in contact with the hydrophobic region of the sugar. Definite hydrogen bonds are formed between the sugar and RTB, which accounts for the specific binding. The C3–OH group of the bound galactose forms a strong bond with Asn46 and Asn255 [18,19] **(Figure 3)**.

Ribosome as Target of Ricin Toxin A (RTA)

Ribosomes are famous for their performance in the intricate polypeptide synthesis process. This process employs multiple factors at various stages for enhanced protein production, most important of them being the guanosine triphosphatase factors (GTPase) which mechanizes the crucial steps of translation. These factors also networks with the Sarcin/Ricin loop (SRL) [20]. The

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momentous site for the binding of RTA is the SRL on the large subunit of the ribosome, which also facilitates the binding of the elongation factors (EF-Tu and EF-G) [21,22]. EF-Tu helps in the binding of charged tRNA to the ribosomes and EF-G helps in the translocation step of peptidyl-tRNA from A site to P site during the process of translation of mRNA to polypeptides. Binding of EFs is subdued by the ribotoxins like ricin which leads to changes in the SRD domain and consecutively in the inactivation of the protein machinery [23]. Helix 95 in the 23S rRNA, ranging from nucleotides 2653–2667 (in prokaryotes-E.coli) consists of the Sarcin/ricin loop (SRL) **(Figure 4)**.

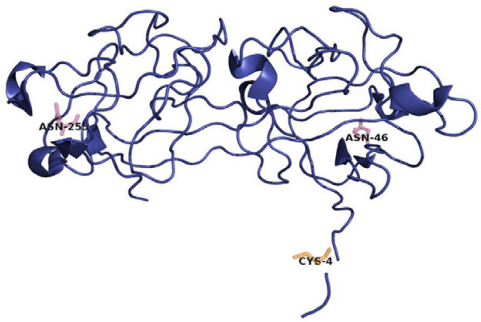

Figure 3. Structure of RTB extracted from ricin molecule (PDB Id: 2AAI) showing the residues involved in bond formation with galactose.

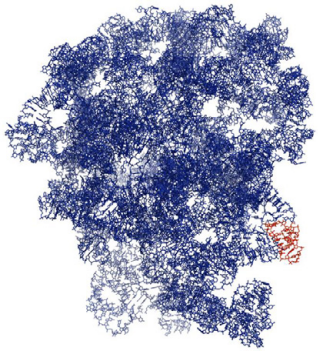

Figure 4. 23S rRNA (PDB Id: 1c2w) having the Sarcin/ricin loop (red).

This region is the highly conserved rRNA sequence [24]. The crystal structure studies reveal that the sarcin/ricin loop networks to the surface of the large ribosomal subunit (prokaryotic) through the carboxyl end terminal of the ribosomal protein leucine6 and the loop to loop tertiary communication with helix91 of the on the 23S rRNA [25]. Two motifs on the SRL that are recognized by both, the ribotoxins and the factors employed in polypeptide elongation, are the GAGA tetra loop and the bulged G portion. The G2659, A2660, G2661, A2662 (in prokaryotes) **(Figure 5)** tetraloop comprises of the cleavage sites for the toxins [26-29]. The G2655, i.e., the bulged G is the crucial site for the elongation factors [28].

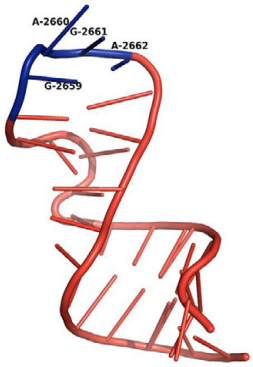

Figure 5. Sarcin/ricin loop (red) showing the GAGA tetraloop (blue). PDB Id: 1Q9A.

It is found that the RTA binds to a constituent of the large subunit of the ribosome known as the stalk [30,31]. The ribosomal stalk of the complex eukaryotes contains two identical dimers of the acidic phosphoproteins, i.e., P proteins (P1/P2). This dimer is bound to P0 in a pentameric form [P0-(P1/P2)2]. The proteins remain the same in both prokaryotes and eukaryotes, but their organization differs. In prokaryotic ribosomes, a pentameric organization of single P0 protein with the heterodimer of Pα/Pβ and Pβ/Pα proteins is observed [32]. The P1 protein binds the P1/P2 dimer to two independent locations on P0 [33,34] and forms a stable complex [35]. The N-terminals of the P1/P2 proteins are accountable for dimer formation and binding of P1 to P0. While the C- ter-

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minals remain free in the cytosol and communicates with GTPases [36,37]. The complexes of these phosphoproteins are not found in the cytoplasm [38]. In mutant ribosomes, with the presence of only P0 and not P1 and P2, the binding of the RTA and ribosomes was found to be low [39,40].

The binding of RTA with ribosomes is a two-step process. The AB1 type of interactions exhibited very quick rates of association and dissociation and it required an intact ribosomal stalk. On the other hand, the AB2 interactions had slow association and dissociation rates and an intact ribosomal stalk was not necessary. Both the interactions are electrostatic in nature, but type AB1 is stronger than type AB2. The AB2 interaction directs the RTA towards the ribosome and aids its transport to the stalk and promotes the more specific and fast AB1 interaction [30], depurinates the ribosome bound 28S rRNA more efficiently than free 28S rRNA [2]. Also, RTA is not efficient in depurination of the prokaryotic ribosome [41]. This clearly indicates that the rRNA conformation and ribosomal proteins play a crucial role in binding of RTA [42]. The ribosomal proteins are different in the case of prokaryotes. The N- terminus of the heterodimer of protein L7/L12 stays in contact with the L10 protein. This L10 in turn, through its N-terminus, communicates to the rRNA [43,44].

The Attack Mechanism of Ricin Toxin A (RTA)

The internalization of the holotoxin occurs due to the attachment of RTB to the galactosyl residues present on the cell surface. This toxin then follows a retrograde route from Golgi to ER. An enzyme which plays the vital role of breaking the bond between RTA and RTB is the protein disulphide isomerase. It is a multifunctional enzyme which binds to RTB by the formation of a disulphide bond to mediate the entry of the Ricin holotoxin in the ER lumen. Once the holotoxin has reached the ER it is reduced and activated. The glycosylated membrane proteins [45] and glycosylated lumen proteins [46] of the ER can be sent to the cytosol for degradation. This finding led to the conclusions that RTA may facade as an infectious protein and employ such a pathway to reach its cytosolic target (ribosomes) [47]. After the binding of RTB to galactosides on the cell surface, the journey of the holotoxin to the cytosol may proceed through either clathrin-coated vesicles or non-clathrin coated vesicles [48]. Both the routes coincide to the formation of endosomes. It is known that for the toxicity of Ricin, the endosome stage should be surpassed and membrane translocation should be reached [49]. Even after translocation, an appreciable amount of ricin is accumulated in the Golgi Network. Treatment of cells with brefeldin A (BFA), a Golgi stack disrupting agent, indicated that ricin was not translocated through the Golgi Network [50]. This gave rise to the hypothesis that transfer of Ricin to endoplasmic reticulum was crucial for its toxicity [51]. RTA is non-glycosylated initially but the RTA found in the cytosol was glycosylated, this gave clear indications that the toxin monomer undergoes retrograde translocation to the ER [52].

To reach the high depurination rate of ribosomes, RTA should have relatively high local concentrations. Also, the reason for the high catalytic activity of the RTA is the electrostatic surface of the ribosome [53]. This is because the ribosome is nearly 120 times larger than the RTA [53,54]. The interactions of RTA to the ribosome are facilitated by the 7 Arginine residues through electrostatic forces [30] Glu177 and Arg180 are crucial for the catalytic activity of RTA [55,56] and Tyr80, Tyr123, Asn209, and Trp211 aids the binding of RTA to the target of depurination (Adenine 4324 in eukaryotes and Adenine 2660 in prokaryotes) [57,58]. Also, Asn209 participates in hydrogen bond formation with the GAGA tetraloop on the sarcin/ricin domain, according to the crystal structure of RTA [16]. Modeling analysis done on the RNA-RTA interactions reveal that Arg48, Arg134, Arg213, and Arg258 are the residues that help in the binding to the phosphate backbone on rRNA [16,59].

Ricin Toxin A (RTA) Inhibition Strategies so far

The depurination mechanism of the adenine residue in the GAGA nucleotide sequence involves an exceptionally dissociative transition state and an oxocarbenium ion intermediate [60,61]. Proteins involved in accelerated formation or cleavage of linkages between nitrogenous bases and sugar group exhibit various prominent pathways [60,62-66], one of the important being stabilization of the oxocarbenium ion intermediates. Experimental and quantum mechanical studies have shown that RTA forms oxocarbenium ion which is finally neutralized by water [65,67,68].

Many RNA-based inhibitors were designed which were able to mimic this state [60,61]. These inhibitors being functionally potent were not considered as useful drugs because they were highly unstable and witnessed difficulties in crossing the cell membrane [68]. Therefore other inhibitors of RTA should be given preference [61,69-72]. The open conformation of RTA has two binding pockets-primary and secondary, partitioned by side chain of a tyrosine 80. The primary pocket has adenine specificity and secondary pocket, which is a little larger; harbors a Guanine of the GAGA tetraloop [16,73]. The space between the two pockets is fulfilled by several positively charge Arginine residues. The phosphate backbone of the ribosomal RNA occupies this space. So, while designing new inhibitors, all these points should be taken under consideration. Till date, the most popular methods for synthesizing many anti-RTA molecules are virtual screening and structure-based design. There have been difficulties in designing a powerful inhibitor that have the capability to accommodate both the primary and secondary pockets, because of the difference in the polarities of the two. Out of the many compounds made, Pterin series were found to be most propitious [72,74,75]. Pteroic acid, a 6-substituted pterin and 7-carboxy pterin (7CP) have reported success as RTA inhibitors. The interactions and effects of 7CP were studied by kinetic and temperature analysis and X-ray crystallography [74].

Structure based designing of drugs is a striking perspective for the characterization of micro-molecular inhibitors for the treatment of inebriation by ricin. The foundation of this approach is studying the interaction between the inhibitor and the target by

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techniques like X-ray crystallography and NMR spectroscopy [17,76,77]. RTB appears to be an easy target. It would be quintessential if small inhibitors would bind to the RTB tightly and debar the cellular uptake. However, it is not a useful scheme [78]. As stated above RTB has a two domain structure and each domain contains 4 sub-domains. Only one of the sub-domains of the major domains interacts with the galactose residues on the cell surface. These binding sites lie on the opposite ends of the B chain about 50 Å away [16,79,80]. This makes it difficult for small molecular inhibitor to bind both the active sites concurrently [68]. The basis of binding of the RTA to the SRL lies in the arrangement of the target Adenine residue into the specificity pocket of RTA [16]. The data revealed by the crystallographic studies shows that RTA exhibits a closed conformation of its specificity pocket in the absence of substrate, i.e., the side chain of the residue Tyr80 is rotated to block the pocket [79]. However, if a substrate is provided the RTA attains an open conformation where, Tyr80 allows the interaction with the substrate by establishing π- stacking interactions with Tyr123 and Adenine of the substrate. Also, the substrate forms 6 additional hydrogen bonds to confer specificity to the Adenine base [68].

Also, several aptamers have been reported that determinedly bind to proteins and discourage their activity [81,82]. Aptamers are a novel class of DNA molecules that bind to targeted sites with dissociation constants in the range of picomolar to nanomolar [83-92]. A high affinity RNA Aptamer (ligand) of 31 nucleotides was synthesized *in vitro* which had the proficiency to battle with SRL to bind to Ricin A chain and decrease the depurination of SRL through the A chain. To study the effectiveness of this experiment, Luciferase assay was performed which is a stable and sensitive method for screening the outputs [93].

Landegren et al. designed a modern technology, i.e., Proximity-dependent surface hybridization [17,76]. This technique employs DNAzymes (DNA aptamers having catalytic properties) [94,95] which can be used as biosensors due to their property of stability, easy labeling and flexibility in designing [91,96].

Two molecules of aptamers are used to bind to a single protein molecule forming a closed loop structure. Intensified inhibition has been observed in the case of such protein binding. The binding is reversible and the protein molecule can be restored using near Infrared light. These 2 aptamer based inhibitors are more effective than the single affinity ligand inhibitors [97].

Circular DNA-RNA Hybrids or DNA can also be used as substrates/inhibitors for RTA. They have low molecular weight and thereby avoid the degradation activity by exonucleases. In this way they serve as a better alternative for linear inhibitors with free 5' and 3' ends which are prone to degradation and have high molecular weights [98]. Sturm et al. synthesized closed GAGA tetraloops devoid of stem in which 5' and 3' ends were linked to each other via a phosphorothioate bond or a covalent oxime linker.

Later RTA Inhibition assays were carried out with these circular molecules and finally it was concluded that small circular DNA and DNA/RNA oligonucleotides are promising inhibitors of RTA and also phosphorothioate closed DNA tetraloop served the purpose better than oxime linked circular and linear GAGA tetraloop [99].

These techniques are used to analyze protein activity and being a protein, RTA activity can be determined using these techniques and an effective inhibitor can be chosen.

CONCLUSION

The review covers crucial details regarding a ribosome inactivating protein, Ricin. The ricin holotoxin is a dimer consisting of RTA and RTB linked by a disulfide bond which is cleaved by the surface of the cell and only RTA enters the cell to interact with a highly conserved region on the large ribosomal subunit and inactivate the protein machinery. RTA follows a retrograde transport pathway, i.e., Golgi to ER, to reach its cytosolic target. The conformation of the stalk P proteins is very critical for the binding of the RTA to the ribosome. Disruption of the Golgi stack with chemical agents like Brefeldin A, reported the inhibition of translocation of the A chain. RTB appears to be an easy target for combating the intoxication of ricin, but it is difficult to achieve a molecule to harbors the two distinct binding sites on the surface of RTB. The inhibitors used for RTA should possess a stable conformation and be able to cross the cell membrane easily. The anti-RTA molecules are widely studied using techniques like virtual screening and structure based designing, the latter being very useful for micro molecular inhibitors. Also, novel classes of proteins, aptamers and DNA-RNA hybrids have been used as inhibitors for RTA.

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DECLARATION OF INTEREST

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AUTHORS CONTRIBUTION

All the authors have equally contributed for the completion of this paper.

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