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ARTICLE *in* BIOCHEMISTRY · JULY 1998

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Shiga Toxin Attacks Bacterial Ribosomes as Effectively as Eucaryotic Ribosomes†

Jung-Keun Suh,‡ Carolyn J. Hovde,§ and Jon D. Robertus*,‡

Institute of Cellular and Molecular Biology, Department of Chemistry and Biochemistry, University of Texas, Austin,Texas 78712, and Deparment of Microbiology, Molecular Biology, and Biochemistry, University of Idaho, Moscow, Idaho 83844

Received February 23, 1998; Revised Manuscript Received May 1, 1998

ABSTRACT: Several pathogenic bacteria, including *Shigella dysenteriae* and certain strains of *Escherichia coli*, produce potent class 2 ribosome inhibiting proteins (RIPs) termed Shiga toxins (Stx). The toxins are bipartite molecules composed of a single A chain (StxA) noncovalently associated with a pentamer of receptor-binding B subunits (StxB). StxA and Stx1A from *E. coli* are protoxins. Proteolysis generates an A1 enzyme (28 kDa) and an A2 fragment (3 kDa), which remain bound, inactivating the enzyme, until a disulfide bond linking them is reduced. Efforts to express active recombinant Stx1A1 in the cytoplasm of *E. coli* were very difficult and led to the hypothesis that Stx1A1 is toxic to *E. coli*. We created the gene for a His-tagged Stx1A1 (cStx1A1) and expressed it in *E. coli* from a tightly controlled expression vector. About 1−2 mg of protein can be purified in a one-step isolation from 1 L of culture. cStx1A1, RTA, and PAP exhibited similar high toxicity against the *Artemia* ribosomes with IC_{50} values near 1 nM. Surprisingly, Stx1A1 had an IC_{50} of 0.8 nM against *E. coli* ribosomes, about the same as it had for *Artemia* ribosomes. This is about 250 times more active than PAP against bacterial targets, making Stx1A1 the most powerful RIP toxin presently known against *E. coli* ribosomes.

A variety of higher plants and some bacteria contain ribosome inhibiting proteins (RIPs). There are many reviews of this copious literature (*1*−*4*). RIPs have been categorized into two classes based on their ability to bind to target cells. Class 1 RIPs are roughly 30 000 molecular weight N-glycosidases. The RIP enzymes catalytically remove a single adenine from a conserved stem and loop sequence of rRNA, inactivating the ribosome (*5*). Examples of class 1 RIPs are pokeweed antiviral protein (PAP), gelonin, and tritin. Class 2 RIPs contain an enzyme homologous to class 1 RIPs, called A chain, that is associated with one or more B chains. The B chain(s) facilitate toxin binding and uptake into cells. Class 2 RIPs are usually lectins with an affinity for surface glycoproteins or glycolipids. Because class 2 RIPs bind target cells, they are potent cytotoxins, generally 10^3−10^4 times as cytotoxic as class 1 enzymes. Ricin is the best understood of the class 2 plant RIPs and is the archetype for the toxin family. Its X-ray structure has been solved (*6*), numerous site-directed mutations of active site residues have been analyzed (*7*−*9*), and a mechanism of substrate binding and catalysis has been postulated (*10*).

Several bacteria produce potent class 2 RIPs termed Shiga toxins. These include Shiga toxin (Stx), produced by *Shigella dysenteriae* type I (*11*); Shiga toxin type 1 (Stx1), Shiga toxin type 2 (Stx2), and Stx2 variants (Stx2c or Stx2e), produced by certain strains of *Escherichia coli* (*12*, *13*). The most infamous serotype among the Stx-producing *E. coli* is O157:H7. This serotype or other enterohemmorhagic *E. coli* have been implicated in outbreaks of hemorrhagic colitis, neonatal and adult diarrhea as well as two life-threatening sequalae, the hemolytic uremic syndrome, and thrombotic thrombocytopenic purpura (*14*, *15*). Stx1 and Stx are 99% identical and immunologically cross-reactive, while Stx1 and Stx2 are immunologically distinct and are about 56% identical in amino acid sequence (*16*). Toxins in the Stx family have also been called Shiga-like toxins or Vero toxins (*13*).

Members of the Stx family are bipartite molecules composed of a single A chain (StxA) noncovalently associated with a pentamer of receptor-binding B subunits (StxB). The enzymatic StxA is activated by mild proteolysis generating an active A1 enzyme (28 kDa) and an A2 fragment (3 kDa), which remain bound until a disulfide bond linking them is reduced (*17*). StxA1 or the essentially identical Stx1A1 (from *E. coli*) are sequence homologues of ricin A chain (RTA), and a hypothetical model of Stx1A1 based on the RTA structure suggests that the three-dimensional structures are probably very similar (*18*). StxA1 catalyzes the same depurination reaction as ricin (*19*) and the activity is dependent upon the same active site residues (*19*, *20*). StxB chains are more closely related to those of cholera toxin than to ricin. The X-ray structure solution of Stx suggests that it is an inactive proenzyme and confirms that the structure of StxA1 is very similar to RTA. The holotoxin crystal structure shows that the catalytic center in A1 is physically blocked by side chains of A2. Toxin activation involves proteolysis of the chain, forming a loop at the C terminus of StxA. Reduction of the disulfide bond between cysteines

† This work was supported by Grant GM 30048 from the National Institutes of Health, by grants from the Foundation for Research and the Welch Foundation, by Public Health Service Grant AI 33981 from the National Institutes of Health, and by the U.S. Department of Agriculture Grant 92-04350.

* To whom correspondence should be addressed. Telephone: 512-471-3175. Fax: 512-471-8696. E-mail: jrobertus@mail.utexas.edu.

‡ University of Texas.

§ University of Idaho.

S0006-2960(98)00424-3 CCC: $15.00
Published on Web 06/11/1998

242 and 261 forms the A1 and A2 peptides, with A2 blocking the active site of A1. Enzyme action results from the dissociation of the two chains (*21*).

The action of RIPs as specific N-glycosidases was initially determined using ricin against rat liver ribosomes (*22*). These studies revealed that the enzyme removes a single adenine base, A4324 in rat ribosomes, from the 28S rRNA (*5*). The scissile adenine is found as part of a GAGA sequence within the loop of a stem and loop structure of rRNA that has been conserved in most species from *E. coli* to man. RTA can attack RNA containing a GAGA target in naked RNA or in synthetic nucleotides (*5, 23*), but the hydrolysis was at least 10 000 times slower than the attack on intact eucaryotic ribosomes. Other plant RIPs have similar action against eucaryotic ribosomes (*24*) as does Stx (*19*). Ricin does not attack intact bacterial ribosomes, although naked bacterial rRNA can be hydrolyzed at a very slow rate. This is consistent with the ability to express recombinant wild-type RTA and many site-specific RTA mutants in *E. coli*.

Conversely, efforts to express recombinant Mirabilis antiviral protein (MAP), a single-chain RIP, in *E. coli* were very difficult and led to the discovery that MAP has measurable activity against bacterial ribosomes (*25*). It was subsequently discovered that several plant RIPs can attack both bacterial and eucaryotic ribosomes, although the enzymes are generally about 100 times more efficient against eucaryotic targets (*26*). In this paper, we present evidence that Stx1A1 not only attacks bacterial ribosomes but also does so with essentially the same efficiency as it attacks eucaryotic ribosomes.

MATERIALS AND METHODS

Materials. Oligonucleotides for polymerase chain reaction (PCR) were purchased from Integrated DNA Technologies, Inc. (Coralville, IA). All restriction enzymes were obtained from New England Biolabs (Beverly, MA). T4 DNA ligase and *E. coli* S30 extract system were from Promega Corp. (Madison, WI). TA cloning kit was purchased from Invitrogen Corp. (Carlsbad, CA). *Taq* DNA polymerase was from Perkin-Elmer (Norwalk, CT). PAP was kindly provided by Dr. J. Irvin. The Ni−NTA resin was purchased from Qiagen Inc. (Santa Clarita, CA).

Bacterial Strains and Plasmids. E. coli strains INVαF′ (Invitrogen Corp.) [F′ *end*A1 *rec*A1 *hsd*R17(rk⁻, mk⁻) *sup*E44 *thi*-1 *gyr*A96 *rel*A1 *ϕ*80*lac*Z15D(*lac*ZYA-*arg*F)-u169] and BL21(DE3)pLysS (*27*) (Novagen Inc., Madison, WI) [F⁻ *omp*T *had*SB (rb⁻, mb⁻)*gal dcm* (DE3)pLysS] were used for cloning and expression experiments. The expression plasmid, pAII17 (*28*), was obtained from New England Biolabs (Beverly, MA), and pREP4groESL (*29*) was from Dr. M. Stieger (Hoffman-La Roche, Basel, Switzerland).

Construction of an Expression Vector for Cytoplasmic Stx1A1 (pcSTA1). For cytoplasmic expression, PCR was used to amplify the Stx1A1 gene minus the signal sequence and with three additional histidine residues at the C-terminus. pRD500 (20), an expression vector encoding the signal peptide and the first 255 amino acid of Stx1A1, was used as the template for amplification. Briefly, two primers were used: a sense strand primer (5′-GCCATATGGAATTTAC-CTTAGACTTC-3′) containing the N-terminal first amino acid codon after the signal sequence and an antisense primer (5′-CCACTAGTGGTGGTGATGATGATGACAATTCA-GTAT-3′) having three extra histidine codons and the stop codon at the C-terminal end of the protein. Twenty-five cycles of amplification were carried out using *Taq* DNA polymerase (Perkin-Elmer), and the resultant DNA product was cloned into the TA cloning vector pCRII (Invitrogen Corp.). The recombinant plasmid pTASTA1 having the coding sequences for Stx1A1, minus the signal sequence, and three C-terminal His residues was digested with appropriate restriction enzymes. The resulting DNA fragment was ligated into the expression vectors, and the ligation mixtures were transformed. The transformants having the Stx1A1 coding sequences were selected and confirmed by restriction analysis and sequencing. The resultant recombinant plasmid, pcSTA1, was transformed into the expression *E. coli* host BL21(DE3)pLysS. To co-express bacterial chaperones (GroEL and GroES) pREP4groESL was co-transformed with the pcSTA1. This recombinant cytoplasmic form of Stx1A1 is referred to as cStx1A1 throughout.

Expression of Recombinant cStx1A1 and RTA from E. coli. A single colony expressing cStx1A1 or RTA (*8*) was inoculated into 10 mL of 2xYT medium. This culture was grown overnight at 37 °C and added to 1 L of 2xYT supplemented with appropriate antibiotics. The culture was grown at 37 °C for 4−5 h with shaking. IPTG was added to a final concentration of 0.5−1 mM for Stx1A1 and 0.1 mM for RTA to induce expression. To co-express GroEL and GroES with cStx1A1, ATP was added to a final concentration of 1 mM. The culture was grown at 30 °C for 3 more hours, and the cells were harvested. The cell pellet for cStx1A1 was resuspended in 1/20 volume of lysis buffer (20 mM Tris-Cl, pH 8.0, and 0.3 M NaCl), and the cell pellet for RTA was resuspended in 1/20 volume of 5 mM sodium phosphate (pH 6.5). The resuspended cells were broken in a precooled French Press Cell (SLM Aminco, Urbana, IL) at 20 000 psi twice. The lysed cells were centrifuged at 3000*g* for 20 min. The supernatant was saved and subjected to ultracentrifugation in a Ti 60 rotor (Beckman) at 4 °C for 1 h at 100000*g*. The supernatants (S100) were saved for the further purification.

Isolation of the Recombinant cStx1A1 and RTA from E. coli. To purify cStx1A1, the S100 fraction was applied to a 5-mL Ni−NTA column that had been previously equilibrated in lysis buffer with 5 mM imidazole. After application of toxin, the column was washed with the same buffer and with wash buffer (20 mM Tris-Cl, pH 8.0, 0.3 M NaCl, and 60 mM imidazole) until the A280 of the effluent was less than 0.05. The bound proteins were eluted with 0.5 M imidazole in wash buffer and collected in 2-mL fractions. Fractions containing cStx1A1 were pooled and dialyzed into storage buffer (20 mM Tris-Cl, pH 8.0, and 10% glycerol). To purify RTA, the S100 fraction was applied to a carboxymethyl-Sepharose (Pharmacia) column equilibrated in the same buffer. Unbound proteins were washed with the same buffer and the buffer containing 0.1 M NaCl. RTA was eluted with a linear gradient of 0.1−0.3 M NaCl. RTA-containing fractions were pooled and stored at 4 °C.

Growth Inhibition of E. coli. E. coli cells expressing cStx1A1 or control cells containing the pAII17 vector were inoculated into 2xYT and grown overnight. These overnight cultures were used to initiate cultures in 2xYT containing 1

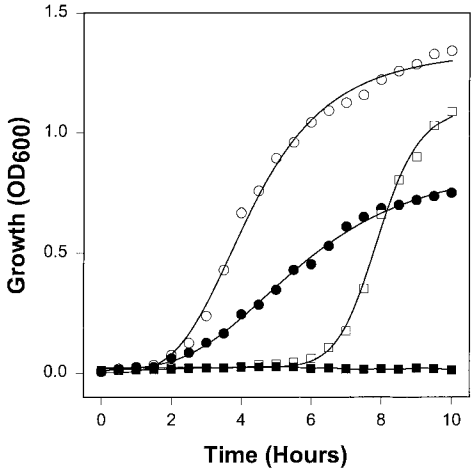

FIGURE 1: Growth curves of *E. coli* transformants with control vector, pAII17 (circles) or cStx1A1 expression vector pcSTAl (squares) in the absence of (open symbols) or in the presence of IPTG (solid symbols).

mM IPTG. The OD_{600} of the cultures was measured every 30 min.

Protein Synthesis Inhibition Assay. The inhibition of in vitro protein synthesis by toxins was assessed using eucaryotic *Artemia salinas* ribosomes. Polyuridylate mRNA was used to direct synthesis of radiolabeled polyphenylalanine (*30*) or prokaryotic *E. coli* ribosomes obtained in the *E. coli* S30 extract system (Promega). Briefly, toxins were incubated for 5 min at 25 °C with ribosomes, the reactions were stopped by the addition of antibodies, and the residual ribosomes activities were measured as previously described (*31*).

RESULTS

Expression and Purification of Soluble cStx1A1 in E. coli. When the coding sequence for cStx1A1 (StxA1 modified with three C-terminal His residues) was inserted into the nonexpressing TA system, transformation was efficient. However, when the construct was moved to leaky high-level expression vectors such as pGEX4T3 (Pharmacia) or pCYB1 (New England Biolabs.), no transformants were obtained.

It had been shown that the T7 promoter of pAII17 gives high and tightly controlled expression after IPTG induction (*28*). Using pAII17 together with *E. coli* strain BL21(DE3)-pLysS gives even more stringent conditions for expression by preventing leaky expression of T7 RNA polymerases (*27*). Moving the cStx1A1 construct into this system allowed efficient transformation. Subsequent induction of the promoter system gave modest, but useful, expression levels of the active enzyme. These results suggest that cStx1A1 may be toxic for *E. coli*.

To test this hypothesis, the influence of protein expression on *E. coli* growth was observed. The 20-mL cultures were initiated from overnight cultures, and the growth was measured (Figure 1). As shown by circles, induction of the parent pAII17 vector system with 1 mM IPTG retarded cell growth slightly. Cells with the inducer showed the same 2 h lag as the uninduced cells, but the subsequent growth rate and total growth density was about 60% of the control. Presumably, this is due to the added expression load of nontoxic proteins from the vector, including the T7 polymerase, GroEL, and GroES. Cells with the vector carrying the toxin increased the lag time from 2 to about 6 h but eventually reached a normal growth density. The reason for the increased lag time is not clear. However, analysis of stationary cells showed that active toxin could be induced by the addition of IPTG, suggesting that no mutations had occurred in the toxin gene or in the induction system during the extended lag period. As shown in Figure 1, cells carrying the gene for cStx1A1 could not grow in the presence of inducer (1 mM IPTG). Since toxin induction prevented any measurable bacterial growth, it seemed reasonable that the enzyme was lethal to the bacterial host cell.

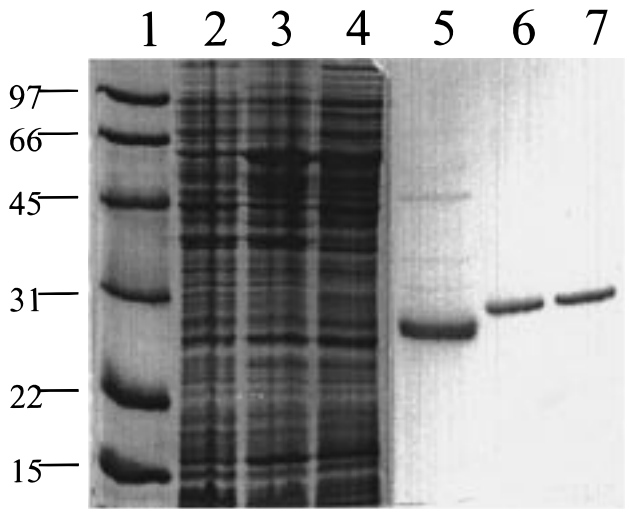

FIGURE 2: Isolation of recombinant cStx1A1 having a C-terminal His-tag. Lane 1, protein molecular weight standards; lane 2, total protein from uninduced *E. coli*; lane 3, total protein after IPTG induction; lane 4, the soluble fraction from induced cells; lane 5, eluent from the nickel column; lane 6, purified PAP; and lane 7, purified RTA.

The engineered cStx1A1 has 248 amino acids, including three extra histidine residues and a stop codon added after histidine 245 of the wild-type Stx1A1 enzyme. The construction therefore has six histidine residues at the C-terminus, forming a polyhistidine tag to facilitate purification.

After disrupting the cells, the extract was applied to a Ni−NTA column and eluted with 0−0.5 M gradient of imidazole; the recombinant cStx1A1 was eluted at 0.12 M imidazole. Figure 2 shows the purity of the isolated cStx1A1 with an estimated molecular weight of 27 400, based on polyacrylamide gels. Applying this one-step purification protocol, 1−2 mg of highly purified cStx1A1 was obtained from a 1-L culture.

Inhibition of in Vitro Protein Synthesis by cStx1A1, Stx1A1, PAP, and RTA. Purified toxins, cStx1A1, PAP, and RTA show >95% purity after SDS−PAGE (Figure 2). Compared with the wild-type, cStx1A1 has three extra histidine residues at the C-terminus that may affect enzyme activity. To test the effect of the His tag, the enzymatic activity of cStx1A1 was compared with Stx1A1 lacking the His tag (*32*). Both Stx1A1 forms showed the same activity against *Artemia* ribosomes, having an IC_{50} of 0.8 nM (Figure 3). This result shows that the three extra histidine residues did not affect the enzyme activity.

The enzymatic activity of cStx1A1 was measured against both *Artemia* and *E. coli* ribosome systems and compared with the activities of two plant RIPs: RTA and PAP (Figure 4). A dose response assay shows that the cStx1A1 had an

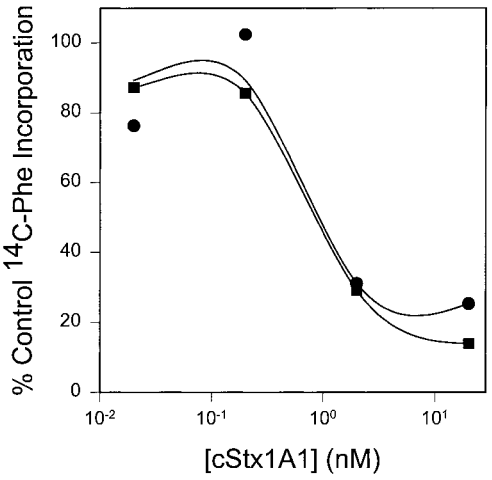

FIGURE 3: Inhibition of in vitro protein synthesis against *Artemia salinas* ribosomes with recombinant His-tagged cStx1A1 (square) and Stx1A1 having 1−255 residues (circles).

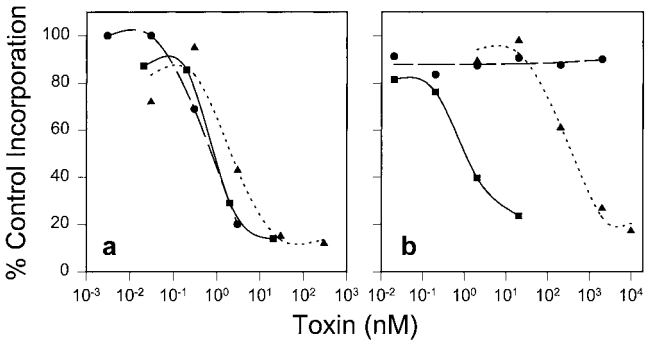

FIGURE 4: Inhibition of in vitro protein synthesis against *Artemia salinas* (a) and *E. coli* (b) ribosomes with recombinant His-tagged cStx1A1 (squares), PAP (triangles), and RTA (circles).

IC_{50} of 0.8 nM against *Artemia* ribosomes, very similar to values for RTA and PAP with values of 0.75 and 1.1 nM, respectively (Figure 4a).

The response against *E. coli* ribosomes was quite different. The cStx1A1 attacked the bacterial substrates with an IC_{50} of 0.8 nM, about the same as for the eucaryotic *Artemia* ribosomes. The IC_{50} of PAP against *E. coli* ribosome was 200 nM. This value is similar to MAP, another type 1 RIP (*25*), and about 250 times higher than the IC_{50} for eucaryotic ribosomes. The class 2 RIP (ricin) shows no detectable activity against *E. coli* ribosomes (Figure 4b).

DISCUSSION

Stx1 is synthesized as an inactive precursor with an A chain of 293 amino acids. The protein is activated by proteolysis, which cleaves the A-chain precursor into a 28-kDa A1 and 3-kDa A2 fragment that can be separated after reduction of the disulfide bond linking the peptides (*1*). The A1 fragment is at least 6 times more active that the intact protein (*33*) and may be as much as 400 times as active (*32*). It has recently been reported that site-directed mutations separating A_2 fragment from the active-site increased N-glycosidase activity by about 50-fold (*34*). The A2 peptide from the C-terminus of the proenzyme was thought to act as an inhibitor of the Stx1A enzyme activity, and the binding of that peptide into the active site has been confirmed by crystallography of Stx, an essentially identical toxin (*21*). As part of an ongoing interest in the structure and action of RIP toxins, we hoped to avoid the ambiguities of proenzyme activation and purification through protein engineering methods.

We engineered the toxin gene to add three His residues after position 245 of the A1 chain, creating a hexaHis tag on the C terminus. The His tag permitted a one-step purification procedure that produced milligram quantities of Stx1A1. Although the toxin has been expressed and secreted to the periplasm of *E. coli* (*32*), this is the first report to our knowledge of expression and cytoplasmic retention of the active toxin.

We tried several different vectors to express and retain Stx1A1 in the cytoplasm of *E. coli*. These included GST-fusion system and intein-fusion system using the *tac* promoter, both known to be leaky. Using these vectors, no transformants were obtained, suggesting that the newly synthesized toxin could attack the host ribosomes and kill the cell. The pAII17 parent of expression vector pcSTA1 has four copies of *rrn* terminator just upstream of the T7 promoter. The system gives high expression and tight regulation for the heterologous protein production in *E. coli*.

Induction of the tightly controlled vector produced a modest level of cStx1A1 expression; about 1−2 mg of recombinant Stx1A1 was obtained from the 1-L culture without the need to activate and repurify the A1 fragment. These expression levels contrast with the expression of RTA, which has been reported to give a good yield of the product in *E. coli*, usually 40−50 mg of RTA/L of culture (*8*, *34*). These results are consistent with the notion that cStx1A1 is toxic to *E. coli* cells while RTA is not. A leaky promoter system releases sufficient toxin to interfere with cell viability and account for the low transformation efficiencies we observed with such vectors. The tightly controlled system allows the transformed cells to attain reasonable growth densities before induction of the toxin begins to kill the host culture and limits overall production as compared with RTA.

To quantify Stx1A1 toxicity to *E. coli*, two different experiments were performed. First, the effect of expression and cytoplasmic retention of Stx1A1 on *E. coli* growth was tested. Cells carrying the uninduced Stx1A1 gene exhibit a longer lag time than control cells with just the pAII17 parent. It is unclear why this is so, but it is reasonable to assume that even this tightly controlled system may express a very low level of toxin that retards the metabolism of the passenger cells but does not kill them. If the IPTG inducer is present, however, no growth is observed, suggesting that the increased toxin production is lethal to each cell. Clearly, these results are consistent with the notion that Stx1A1 is toxic to *E. coli*.

Second, in vitro experiments were done to compare the potency of cStxA1 with other toxins in their ability to inhibit protein translation by eucaryotic (*Artemia*) and prokaryotic (*E. coli*) ribosomes. It was first shown that the genetically engineered toxin cStxA1 was indistinguishable in activity from the wild-type toxin. Next, cStx1A1, RTA, and PAP were shown to exhibit similarly high toxicity against the *Artemia* ribosomes with IC_{50} values near 1 nM.

It has long been known that RTA is unable to attack *E. coli* ribosomes, a fact that has facilitated expression and mutagenesis studies of the enzyme (*7*−*9*). It is also known that some class 1 plant RIPs, like PAP and MAP, can inactivate prokaryotic ribosomes (*25*, *26*). These enzymes

have IC_{50} values around 200 nM against *E. coli* ribosomes, generally around 200 times higher than for their action against eucaryotic ribosomes.

Surprisingly, Stx1A1 had an IC_{50} of 0.8 nM against *E. coli* ribosomes, about the same as it had for *Artemia* ribosomes. Therefore it was about 250 times more active than PAP against bacterial targets. This result makes Stx1A1 the most powerful RIP toxin presently known against *E. coli* ribosomes. This rationalizes the observation that the toxin is synthesized as an inactive precursor and is activated only when it is transferred away from the host ribosomes.

Although the key catalytic residues in the active site of the three RIPs used in this study are conserved and the mechanism of depurination is the same, substrate specificity is clearly different in the sense that the three have very different abilities to attack bacterial ribosomes. It has been proposed that this difference in specificity may result from modest structural differences at noncatalytic positions on the RIP protein (*35*). There is also reason to believe that the differences may result from differing interaction between the RIP and the ribosomal proteins. For example, intact *E. coli* ribosomes are insensitive to ricin, but the deproteinized *E. coli* 23S rRNA is depurinated by RTA. The rate is at least 1000 times slower than for intact ribosomes but is roughly equivalent to the rates that other toxins attack naked RNA (*22*). This suggests that, in the absence of ribosomal proteins, prokaryotic rRNA has a structure suitable for depurination (*36*). Comparison of the crystal structure of RTA and PAP suggested that several regions show enough difference to warrant investigation as the possible cause for the specificity differences (*35*). Peptide swap experiments between RTA and PAP show that some structural differences in the amino-terminal half of the proteins do affect ribosome specificity, but to date no detailed information about the nature of this interaction is available (*36*).

ACKNOWLEDGMENT

We thank Paula R. Austin for technical expertise.

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BI980424U