PROTEINS: Structure, Function, and Genetics 10:270–278 (1991)

Site-Directed Mutagenesis of Ricin A-Chain and Implications for the Mechanism of Action

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ABSTRACT **Ricin A-chain is an N-glycosidase that attacks ribosomal RNA at a highly conserved adenine residue. The enzyme is representative of a large family of medically significant proteins used in the design of anticancer agents and in the treatment of HIV infection. The x-ray structure has been used as a guide to create several active site mutations by directed mutagenesis of the cloned gene. Glu177 is a key catalytic residue, and conversion to Gln reduces activity 180-fold. Asn209 is shown to participate in substrate binding by kinetic analysis. Conversion to Ser increases Km sixfold but has no effect on kcat. Conversion of Tyr80 and Tyr123 to Phe decreases activity by 15- and 7-fold respectively. A mechanism of action is proposed that involves binding of the substrate adenine in a syn configuration that resembles the transition state; the putative oxycarbonium ion is probably stabilized by interaction with Glu177.**

Key words: ricin A, site-directed mutagenesis, mechanism of action

INTRODUCTION

Ricin, the cytotoxin from *Ricinus communis,* is a heterodimeric protein of form AB, in which the B-chain (RTB) is a cell surface, receptor binding protein that mediates entry of the A-chain (RTA) into the cytoplasm.[1] RTA is representative of a major group of plant ribosome inhibiting proteins, or RIPs, which inactivate eukaryotic ribosomes by catalyzing the deadenylation of a specific adenosine on the 28S rRNA.[2,3] Ricin, containing both an A- and B-chain, is a so-called class II RIP; this class includes other toxins such as abrin[4] and modeccin.[5] Class I RIPs resemble the A-chain of ricin but lack a B-chain; they are therefore only slightly cytotoxic. Examples include trichosanthin,[6] pokeweed antiviral protein,[7] and dodecandrin.[8] These proteins, like RTA, are known to be N-glycosidases and attack the same ribosomal target.[9] Finally, the A-chains of several bacterial toxins such as Shiga toxin and the Shiga-like toxins of *Escherichia coli* are homologous to plant RIPs and catalyze the same reaction.[10–12] RIPs are of interest not only because of their enzymology, but also because of their medical significance. Ricin is a common component of immunotoxins targeted against tumor cells[13] or in the treatment of graft versus host disease.[14] Trichosanthin has been used in China since antiquity as an abortifacient; more recently, it has been shown to inhibit replication of HIV-1 in cultured cells.[15] Other RIPs are currently being tested for activity against AIDS-infected cells. RIP genes might also be introduced into agriculturally significant plants to eliminate virus sensitivity.

The x-ray structure of ricin has been solved by conventional methods to 2.8 Å resolution[16] and refined to 2.5 Å.[17] A detailed molecular description of both RTA[18] and RTB[19] has been presented.

As stated above, RTA acts as a site-specific RNA N-glycosidase, catalyzing the cleavage of a conserved adenine (A_{4324} in the rat rRNA) from the 28S rRNA of the eukaryotic ribosome.[2,3] RTA typically shows four orders of magnitude less activity against plant ribosomes than against animal ribosomes and virtually no activity against prokaryotic ribosomes. However, naked rRNA from all sources is attacked with the same efficiency, though at least three orders of magnitude less than for intact ribosomes.[3] The target sequence is among the most rigorously conserved sequences in nature, being almost invariant in all organisms; 35 base synthetic RNA containing this sequence can be attacked, but only at a very slow rate.[20] This suggests that the enzyme does not solely recognize the target RNA sequence; specificity is likely determined by the higher order structure of the ribosome. We might therefore expect a number of different effects from modification of critical residues. Because the native substrate is substantially larger than the enzyme, a number of surface residues distant from the catalytic site might play a role in forming a loose complex and placing the RTA molecule in the correct orientation for binding the target RNA sequence. Once the RTA is properly oriented on the ribosome, very specific interactions might be expected to form a tight complex with

Received July 20, 1990; revision accepted February 4, 1991.
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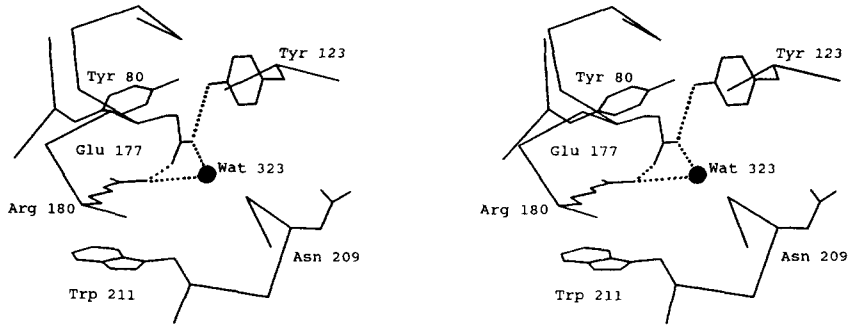


Fig. 1. The active site of RTA. A stereo pair displays the geometric relationship of conserved active site residues, which are suspected to be involved in substrate binding or catalysis.

the target sequence. A few residues are expected to play specific chemical roles in catalyzing the base hydrolysis.

Are there any likely catalytic models for base hydrolysis by ricin A-chain on which experiments might be based? Acid hydrolysis is the typical means for chemical cleavage of N-glycosides.[21] In an acid-catalyzed model, protonation of the adenine base induces electron flow from the ribose ring oxygen into the ring, forming an oxycarbonium intermediate with partial bonding between the ribose and the adenine. Attack by water at C_1 of the sugar completes the reaction. Initial rationalization of targets for site-directed mutagenesis was made on the basis of the above models for binding and catalysis.

Recently Schramm and coworkers have analyzed the mechanism of action of the AMP nucleosidase from *Azotobacter vinlandii*. Their work suggests that the transition state for the N-glycosidase shows oxycarbonium ion character on the ribose ring, a bond order of roughly 0.2 between the sugar and leaving adenine, a likely attack by water on C1 of ribose and likely protonation of N_7 of adenine.[22]

The structure of RTA reveals a mushroom-shape protein with a prominent cleft that we proposed to be the active site.[18] Comparison of known RIP amino acid sequences shows that, although the RIPs are a divergent group of proteins, their sequences contain several invariant and highly conserved residues.[18,23] These residues tend to cluster in the proposed active site cleft. An enlarged drawing of the active site cleft, with the side chains of the conserved residues highlighted, is shown in Figure 1. We have undertaken a program to modify selected residues by site-directed mutagenesis in order to elucidate what roles these residues play in the action of ricin A. The gene coding for RTA has been cloned and expressed in *E. coli*.[24] We have obtained that gene from Cetus Corporation and engineered it to express RTA under the *lac* promoter. Our expression system and the effects on catalysis of modifying several residues in the active site are described in this study. In particular we have converted Tyr80 to Phe (Y80F), Tyr123 to Phe (Y123F), Glu177 to Gln (E177Q), and Asn209 to Ser (N209S).

MATERIALS AND METHODS

Materials

E. coli JM105 (Δ (lac proAB) thi, gyrA96, endA1, hsdR17, relA1, supE44, λ^- / F′ traD36, proAB, lacI^{q}ZΔM15) and JM107 (Δ (lac proAB) thi, rpsL, strA, endA, sbcB15, hspR4 / F′ traD36, proAB, lacI^{q}ZΔM15) were originally obtained from Bethesda Research Laboratories (Bethesda, MD). Strain CJ236 (dut1, ung1, thi1, relA1/pCJ105) was a gift from Gloria Vaaler. M13mp18 and 19 and the pUC series of plasmids were obtained from Bethesda Research Laboratories, and pRAP229[24] was a gift from Cetus Corporation.

Assembly of the pUTA Expression Vector

pRAP229 was digested with Hind3 and BamH1 and electrophoresed on a 0.8% agarose gel using standard methods.[25] The fragment containing the coding sequence for ricin A-chain, including the initiator Met in front of Ile1 and a stop codon after Phe267 as engineered by Cetus Corporation, was excised from the gel and electroeluted in a Schleicher and Schuell Elutrap using Tris-borate buffer. pUC18 was digested with EcoR1 and BamH1, and the linearized vector isolated in similar fashion.

A synthetic linker was constructed to link the Hind3 site of the RTA coding sequence to the EcoR1 site of the vector. The linker was synthesized on an Applied Biosystems 380A DNA synthesizer as two 19mers having a mutual 15 bp overlap. These were annealed by heating equimolar quantities of each oligomer together at 95°C for 5 minutes, then slow cooling to room temperature. Polyacrylamide gel electrophoresis followed by staining with acridine orange showed essentially quantitative conversion to the double-stranded form. A twofold molar excess of the annealed linker was ligated to the purified vector and insert by standard techniques. A Bcl1 site within the peptide facilitated construction of the plasmid. After ligating the vector, linker, and in-

sert, the resulting mixture was digested with Bcl1. Several possible ligation products thus resolve to a linear DNA containing single vector, insert, and linker sequences. This linear fragment was electrophoresed, electroeluted, and religated in dilute solution prior to transformation to maximize the yield of correct transformants. The ligation mix was then transformed into *E. coli* JM105 by the $CaCl_2$ method and transformants plated on YT medium containing 80μg/ml ampicillin.

Screening of Transformants for RTA Expression

Several ampicillin resistant transformant colonies were picked and grown overnight in 10 ml liquid culture of 2 × L broth supplemented with 0.2% glucose and 80 μg/ml ampicillin. Cells were pelleted by centrifugation and resuspended in 0.5 ml 10 mM HEPES-0.2 M NaCl pH 7.6. 20 μl toluene and 20 μl 1% Na deoxycholate were added. The mixtures were then vortexed vigorously and incubated 15 minutes at 37°C. Cells and debris were pelleted and the clarified supernatants tested for the presence of RTA by ELISA,[25] using rabbit anti-RTA and horseradish peroxidase conjugated goat antirabbit (BioRad Laboratories) as the first and second antibodies, respectively. 2, 2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) was used as the colorometric indicator for peroxidase activity. The culture exhibiting maximum RTA expression was propagated and the plasmid vector named pUTA1.

Site-Directed Mutagenesis of RTA

The Hind3 / BamH1 fragment from pRAP229 containing the RTA coding sequence was cloned into M13mp19. THe resulting construct was transformed into JM107 cells; transformants were selected carrying the complete insert, the presence of which was confirmed by partial sequencing of the ssDNA and restriction analysis of the RF form DNA. M13 phage stocks were prepared by standard methods.[25] Uracil containing DNA template was prepared by infecting CJ236 cells with the recombinant phage and growing for 8 hours in the presence of 0.5 μg/ml uridine and 40 μg/ml chloramphenicol. Mutagenesis was performed according to the Kunkel method[26] using synthetic DNA primers containing the appropriate mismatches. Typically, mutagenic primers were 22–30 bases long; they were designed to contain two mismatches to the wild-type sequence near the middle of the primer. Primer extension products were transformed into *E. coli* JM107 by standard methods. Plaques were screened for the presence of the correct mutation by sequencing of the phage ssDNA. Based on the gene sequence for RTA,[27,28] a family of sequencing primers was constructed so that they would generate overlapping sequence information along the entire length of the gene. The mutant gene or appropriate restriction fragment was then excised from the RF form of the bacteriophage DNA and substituted into the pUTA vector for expression.

Isolation of Mutant Proteins

Cells were grown in 6 l of 2 × L Broth supplemented with 0.2% glucose and containing 80 μg/ml ampicillin and 30 μM isopropyl-β-thiogalactopyranoside (IPTG). After overnight growth at 30°C, cells are harvested by centrifugation and resuspended in 100 mM Tris-Cl pH 8.5-500 mM NaCl-25 mM EDTA-0.1% βME. Cells were broken in a Bead Beater (BioSpec Products) using 0.1 mm glass beads for a total of 16 minutes in 2-minute increments; 1/100 volume of 1 mg/ml phenylmethylsulfonyl fluoride in dimethyl sulfoxide was added to the decanted extract. Cellular debris was removed by centrifugation at 14,000 × g for 30 minutes. RTA was precipitated in the 0–50% $(NH_4)_2SO_4$ fraction. After pelleting by centrifugation, the precipitate was resuspended in phosphate-buffered saline + 0.1% βME and applied to the Phenyl Sepharose column.

Phenyl Sepharose Chromatography

The 0–50% ammonium sulfate fraction in PBS was applied to a 2.5 × 30 cm column of phenyl sepharose 4B (Pharmacia). The column was then washed with about 500 ml of PBS-0.1% βME and bound material eluted with a 1,000 ml gradient from the starting buffer to 55% propylene glycol in 5 mM sodium phosphate pH 7.2–10 mM NaCl-0.1% βME. RTA containing fractions were determined by ELISA and their relative purity determined by 10.5% SDS-PAGE. Appropriate fractions were pooled for further purification by one of several means.

AffiGel Blue Chromatography

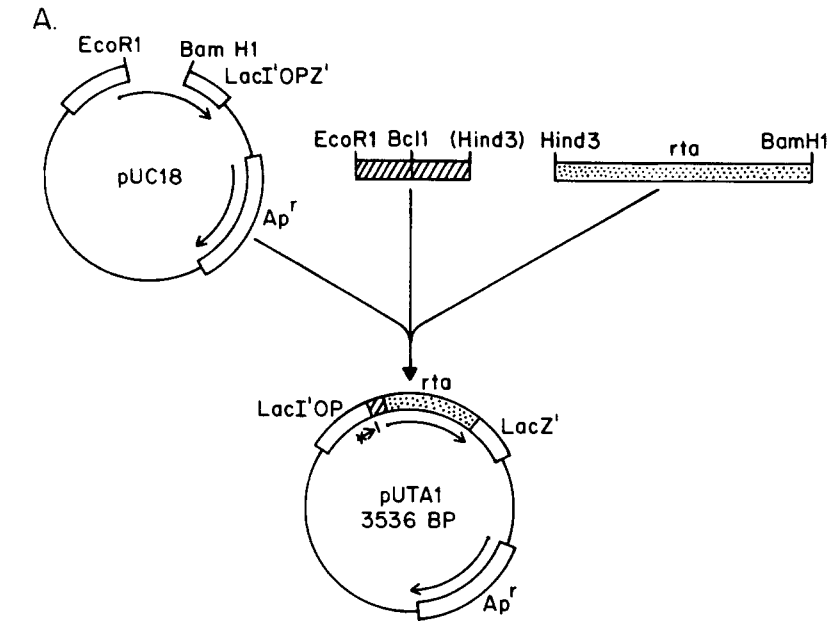
The pooled phenyl sepharose fractions were dialyzed into 20 mM Tris-Cl pH 8.5-200 mM NaCl-0.1% βME and applied to a 30-ml column of AffiGel Blue (Pharmacia) equilibrated in the same buffer. After washing with several column volumes of the starting buffer, RTA was eluted with a 200–1,000 mM NaCl gradient in the same buffer.

Q-Sepharose Chromatography

Alternatively, the pooled phenyl sepharose fractions were dialyzed into 10 mM CHES pH 9.5–1 mM EDTA-0.1% βME and applied to a 2.5 × 30 cm column of Q-sepharose equilibrated in the same buffer. After washing the column, RTA was eluted with a 0–250 mM NaCl gradient in the CHES buffer.

Protein Synthesis Inhibition

The antiribosomal activity of RTA was measured by RTA directed inhibition of poly-U dependant polyphenylalanine synthesis on *Artemia salina* ribosomes. *A. salina* ribosomes were prepared and polyphenylalanine synthesis assays carried out as pre-



B.

M T M I T N S *Opl* **S. D.** M I F
ATGACCATGATTACG|AATTCGTGATC**AGGAGG**CG|AGCTTATGATATTC
TACTGGTACTAATGCTTAA|GCACTAG**TCCTCC**GCTCGA|ATACTATAAG

pUC18 Linker rta

Fig. 2. Cloning strategy for assembling the pUTA expression vector. **(A)** The large EcoR1/BamH1 fragment from pUC18 and the rta-containing Hind3/BamH1 fragment of pRAP229 were ligated with the synthetic linker as described in Materials and Methods. DNA was precipitated, resuspended, restricted with BclI, electrophoresed, and a 3536 bp fragment eluted. This was religated and transformed into *E. coli* JM105. **(B)** The sequence through the linker connecting the *lacZ* coding region to that for RTA. The linker inserts an opal stop codon into the β-galactosidease gene and is followed immediately by a consensus Shine-Dalgarno ribosome binding site. The single letter amino acid code is written over the bases of the linker.

viously described.[29] The RTA reaction was stopped by addition of anti-RTA before adding the ingredients required for protein synthesis; the two reactions were thus decoupled and were not occurring simultaneously over the course of the assay. This is essential to guarantee that kinetic measures reflect true initial rate velocities.

RESULTS AND DISCUSSION

Expression of RTA

The RTA expression vector pRAP229 was obtained from Cetus Corporation. This vector contains the coding sequence for RTA linked to the inducible *phoA* promoter.[24] The coding sequence has been engineered to contain an amber stop codon after the last RTA residue, Phe267, and an initiator Met codon before the N-terminal Ile1. A Hind3 site has also been inserted immediately upstream of the methionine codon.

Although *phoA* is a strong promoter, the nature of its induction by low phosphate concentration made it inconvenient to use. We therefore undertook to place RTA expression under control of the easily regulated and induced *lac* promoter. Since the commonly used, high copy number pUC series of plasmids contain the alpha complementing fragment of β-galactosidase under the *lac* promoter, we decided to link the RTA coding sequence to that of β-galactosidase to produce a hybrid mRNA. The strategy for this manipulation is seen in Figure 2. The linker joins the EcoR1 site at the 5′ side of the pUC 18 multiple cloning region to the Hind3 site at the 5′ end of the RTA coding sequence. As can be seen in Figure 2, the linker introduces an opal stop codon into *lacZ*, causing the nascent galactosidase to be truncated after seven residues. Immediately after the opal codon is a consensus Shine-Dalgarno sequence followed at the correct distance by the initiator Met. A ribosome that halts at the opal codon can therefore immediately reinitiate and begin synthesis of the RTA coding sequence without producing a fusion protein. The linker also removes the Hind3 site at the 5′ end of the RTA coding sequence, leaving a unique Hind3 site in the construct derived from the multiple cloning region of the pUC vector.

Transformants containing the correct plasmid

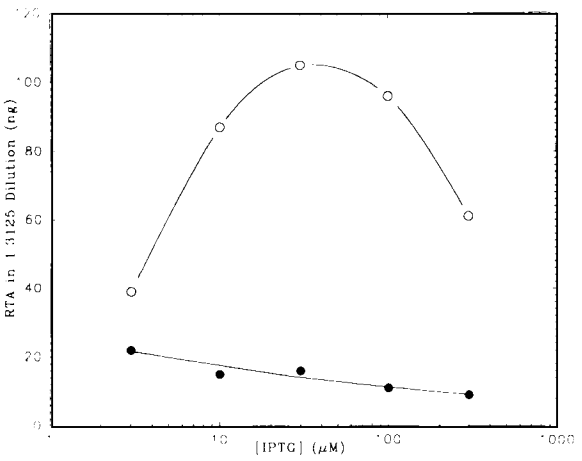


Fig. 3. RTA expression as a function of induction and temperature. Expression is plotted in response to varying level of IPTG, an inducer of the lac promoter, which governs RTA expression in pUTA. Expression is also a function of temperature plotted here at 30°C (open circles) and 37°C (solid circles).

construct were tested for expression of RTA by ELISA of toluene permeabilized cells. The clone giving the greatest level of expression was selected for further use. In order to determine the conditions producing maximum expression of protein, 10 ml cultures were grown overnight with varying concentrations of IPTG at 30° and 37°C. Cell extracts were subjected to ELISA and the results are shown in Figure 3. Maximum expression is obtained at 30°C with 30 μM IPTG. Higher inducer concentrations produce a decrease in both RTA expression and total protein, indicating a probable inhibitory effect on cell growth. Expression at 37° is markedly inhibited, although cells grow quite well at this temperature. Other workers have shown that growth at elevated temperatures causes some heterologous proteins to be rapidly degraded or packaged in insoluble protein bodies;[30] RTA would seem to be a case in point.

Piatak et al. purified recombinant RTA expressed under control of the *phoA* promoter by hydrophobic interaction chromatography on phenyl Sepharose followed by chromatography on CM sepharose.[24] Our initial purifications of wild-type RTA followed this scheme with good result; electrophoretically pure RTA was obtained at a yield of 15–30 mg/l of culture. SDS-PAGE revealed a single band with M_r = 30,000; which reacted strongly with anti-RTA antibody on Western blots. Sequencing of the purified protein showed that 55% possessed the N-terminal sequence IFPKQYP . . . typical of native ricin, whereas 45% had the sequence MIFPKQYP . . . ; RTA isolated from the pUTA vector crystallized in the same space group and under the same conditions as RTA previously crystallized in collaboration with Cetus Corporation.[31]

Mutant RTA tended to be far less well behaved than wild-type in terms of expression level and stability. The E177Q, Y123F, and N209S proteins eluted later than wild-type from the phenyl Sepharose column and were displaced into a large peak of contaminating protein eluting at 50% propylene glycol. In addition, the mutant proteins tended to be far less stable at the low pH required for fractionation on CM Sepharose. Several changes in the basic scheme were therefore developed, as discussed in Materials and Methods. Since CM Sepharose chromatography could not be used, two approaches were employed in the final purification of the mutant proteins. Since the protein is most stable at high pH, Y80F, Y123F, and E177Q were fractionated on Q Sepharose at pH 9.5. This produced protein of high purity for the two tyrosine mutants; E177Q was expressed at such low yield that even low absolute amounts of contaminating protein result in a preparation of rather poor purity. The activity of E177Q was therefore always assayed relative to an equivalent E177Q preparation treated with anti-RTA to control for inhibitory or stimulatory effects by the contaminating proteins.

RTA, like many nucleotide binding proteins, is known to bind the dye Cibacron blue.[32] The phenyl Sepharose fractions of the N209S mutant protein were therefore applied to a column of AffiGel Blue as described in Methods and eluted with a NaCl gradient. This resulted in electrophoretically pure protein.

Activity of Wild-Type and Mutant Proteins

Ricin A has previously been reported to have a K_m for reticulocyte ribosomes of 0.1–0.2 μM and a k_{cat} of 1,500 min^{-1}.[33] We have previously shown for another RIP, pokeweed antiviral protein (PAP), that kinetic parameters depend on the salt concentration of the assay and the presence or absence of postribosomal supernatant factors and hydrolyzable ATP.[29] At pseudophysiological salt concentrations (4 mM Mg^{+2}, 90 mM Na^+) and in the presence of ATP and supernatant, PAP has a K_m of about 0.2 μM; at these salt concentrations without cofactors, PAP is essentially inactive. At low salt concentrations (2 mM Mg^{+2}, 20 mM Na^+) the enzyme is active with a K_m of about 1 μM. In both cases k_{cat} is about 300–400 min 1.

We find a similar cofactor dependency for RTA. In the presence of the complex supernatant fraction and ATP, wild-type RTA has a K_m for *Artemia salina* ribosomes of 0.4 μM and a k_{cat} of 1,000 min 1. In their absence the K_m rises to 1.3 μM while k_{cat} drops to about 300 min^{-1}; a double reciprocal plot reflecting these latter conditions is shown in Figure 4. Our standard assay system employs the low salt conditions in order to minimize the effects of other components on the RIP reaction.

In analyzing the effect of mutations, it is important to recall that enzymes frequently produce rate

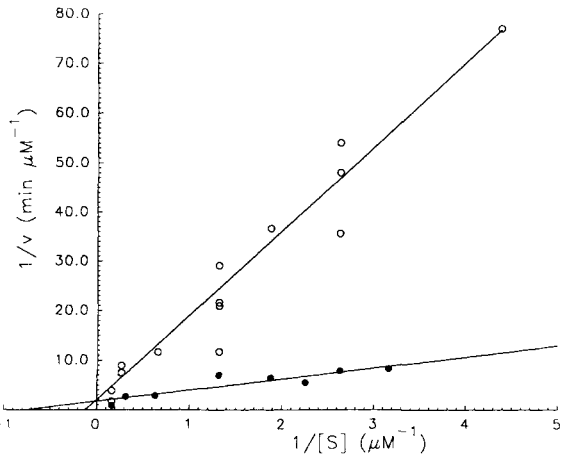


Fig. 4. Kinetic plots for RTA. Toxin was at 1.87 nM; ribosome concentrations were varied from 0.23 to 6.1 μM. Wild-type RTA is shown as solid circles and N209S protein as open circles.

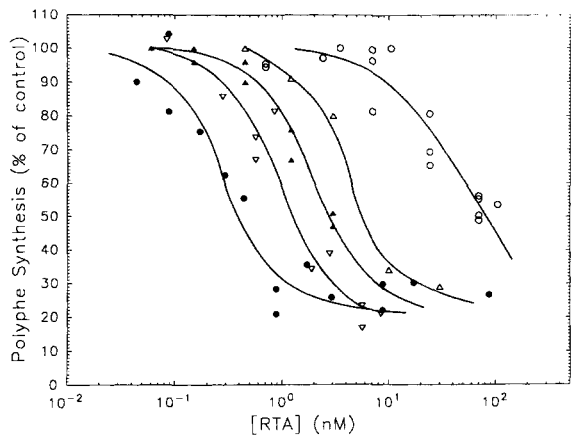


Fig. 5. Dose response curves for wild-type and mutant RTAs. In each case the concentration of *A. salina* ribosomes is 380 nM. The RTA reaction was carried out in the absence of ATP and soluble factors. Curves for the various proteins are: wild type (●), N209S (▽), Y123F (▲), Y80F (△), and E177Q (○).

enhancements of 10^8–10^{12} over uncatalyzed reactions. Several residues may play roles in the process, binding the transition state or facilitating group transfers. Depending on the details of the mechanism, alteration of these residues may have a marked effect on enzyme rates. For example, subtilisin causes a 10^{10} enhancement in the rate of hydrolysis of peptide anilides. Conversion of the active site serine to alanine reduces activity a million fold.[34] This may well represent an extreme for the contribution of a single residue to the overall mechanism.

Egg white lysozyme is an O-glycosidase and might be expected to have some mechanistic similarities to the N-glycosidases. In particular, lysozyme Asp52 is thought to stabilize the oxycarbonium intermediate of the glycosidase reaction; conversion to asparagine reduces enzyme activity against defined substrates by 100 to 1,000 fold.[35] Glu35 acts to protonate the leaving group; conversion to Gln drops the catalytic rate by at least three orders of magnitude.

Activity of RTA mutant proteins was compared to wild-type activity by dose-response plots and, where feasible, by kinetic analysis. Activity assays were typically carried out at low salt in the absence of supernatant factors. Dose response curves are presented graphically in Figure 5; the enzyme concentrations giving 50% ribosomal inhibition (ID_{50}) for the wild-type and mutant proteins are given in Table I. Since RTA shows a maximum inhibition of *A. salina* ribosomes of about 80%, ID_{50}s represent the amount of RTA needed to give 50% of this maximum inhibition under standard conditions.

The N209S mutation increases the ID_{50} by approximately one-half log as shown in Figure 5 and Table I. Initial rate kinetic analysis indicates that this is due primarily to a change in K_m to about 8 μM, with essentially no change in k_{cat}. A normalized Lineweaver-Burke plot comparing wild-type and N209S RTA is shown in Figure 4. The K_m difference between N209S and wild-type is consistent with the notion that the amide group, located on the periphery of the active site cleft shown in Figure 1, is involved in substrate recognition and binding. This difference in K_m corresponds to a free energy increase for the enzyme substrate complex of about 1 kcal/mol. The substitute serine hydroxyl is apparently capable of bonding the substrate though less strongly than the asparagine of wild-type. Amide residues are frequently used by proteins for specific recognition of nucleic acid bases. For example, five amides are used by phage 434 repressor in binding its operator sequence.[36]

Tyrosines 80 and 123 are found at the top of the proposed active site. Both residues are invariant in all known RIPs. In their study of barley protein synthesis inhibitor (BPSI), Asano and Svennson[37] showed that nitration of a single tyrosine residue with tetranitromethane reduced enzyme activity tenfold. The single modified tyrosine was determined to be Tyr86, the homologous residue to Tyr80 in RTA.

Conversion of Tyr80 to Phe results in a 15-fold loss of activity compared to wild-type, an effect reflected in both lower K_m and k_{cat} values. The Km for ribosomes is about 8 μM with a turnover number of about 100 min^{-1}.

Conversion of Tyr123 to Phe (Y123F) results in a sevenfold reduction in enzyme activity. Because Tyr123 is involved in a network of hydrogen bonds at the presumed catalytic center, it was considered more likely than Tyr80 to be involved in catalysis. Removing the hydroxyl group clearly has little effect on the enzyme, however. Tyr123 may interact with substrate and contribute to the apparently

TABLE I. Toxicity of Various RTAs

Protein	ID_{50} (nM)	Wild-type ID_{50} / Mutant ID_{50}	K_m (μM)	k_{cat} (min^{-1})
Wild-type	0.3	1	1.25	300
N209S	1.0	3	8	300
Y123F	2.0	7	N.D.	N.D.
Y80F	4.5	15	8	100
E177Q	55.	180	N.D.	N.D.

tight binding of RTA to the ribosome, but neither it nor Tyr80 are major players in the catalytic mechanism.

The most striking mutation results from changing Glu177 to the corresponding amide (E177Q). This results in a 180-fold decrease in overall activity, consistent with a key role in catalysis. Frankel and coworkers have recently converted Glu177 to an aspartate with an 80-fold loss in activity,[38] whereas Collier's group has shown that changing the corresponding Glu167 of Shiga-like toxin to aspartate decreases specific activity by about three orders of magnitude.[39] Interestingly, conversion of RTA Glu177 to alanine results in only a 20-fold loss in activity.[38] It has been proposed that the small size of the alanine side chain may allow the nearby Glu208 residue to shift into position and fill the same role as Glu177.[40] The E177Q mutation reported here, nearly isosteric with the wild-type, probably gives the best estimate of the contribution of Glu177 to the RTA mechanism.

In addition to Glu177, alteration of Arg180 can produce significant catalytic effects.[40] X-ray analysis shows the two residues are hydrogen bonded to one another and to a water residue that may be the ultimate nucleophile of the N-glycosidase reaction.[18] What, then, is a likely mechanism for RTA catalyzed N-glycosidation? As noted in the introduction, Schramm and coworkers have described the transition state for AMP nucleosidase as having strong oxycarbonium ion character, with the likely attack of an enzyme bound water molecule on C_1 of ribose, and possible adenine protonation.[22] They have also shown that a nonhydrolyzable AMP analog, formycin monophosphate, binds tightly to the enzyme as a transition state analog and acts as an inhibitor.[41] The crystal structure of formycin monophosphate shows the base in the syn conformation, leading to the suggestion that the AMP transition state, like the inhibitor, is bound in a torsionally strained conformation.[42] Hydrogen bonding of the base by one or more active site residues induces sufficient electron flow to initiate reaction.[22]

Results of mutagenesis of RTA by ourselves and others can be interpreted in terms of a similar mechanism, shown schematically in Figure 6. Modification of Tyr80, Tyr123, Asn209, and Trp211[43] have little effect on enzyme activity, decreasing activity

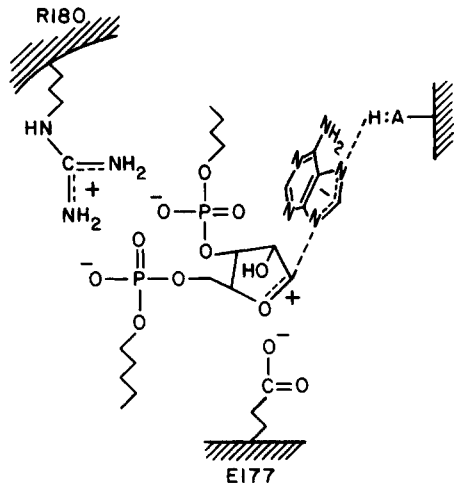


Fig. 6. Schematic diagram of the proposed ricin A-chain mechanism. The substrate adenine is shown in the syn conformation, with partial breakage of the N-glycoside bond and oxocarbonium ion development on the ribose. Glu177 stabilizes the positive charge on the ribose ring while Arg180 is shown binding the phosphate backbone of the RNA. Tha attacking water molecule is positioned close to the scissile glycoside linkage. Other residues, not shown, such as Trp211 and the aromatic rings of Tyr123 and Tyr80 are likely to interact with ribose and purine rings of the substrate. H:A represents an unspecified residue that hydrogen bonds to the leaving adenine. This partial protonation facilitates N-C bond cleavage.

by factors of 3 to 15. These residues likely interact with the substrate and contribute additively, along with other groups, to the tight binding of RTA to the ribosome. The total energy of such interactions permit the substrate adenosine to be bound in a strained syn configuration resembling the transition state, with partial breakage of the N-C bond. The positive charge at Arg180 may act to hold this configuration by ion pairing to the phosphate backbone as indicated in Figure 6. This is consistent with the minor effect seen by conversion of Arg180 to lysine and the major loss of activity by conversion to histidine, a shorter and less highly charged residue.[40] Glu177 probably acts to stabilize the positive charge developing on the ribose ring (oxycarbonium), in a manner similar to the role of Asp52 in egg white lysozyme. The destabilized N-C bond would then be displaced by nucleophilic attack by water at C_1 of the ribose moiety. Quite possibly this water is the bound active site water 323. Unlike lysozyme, N-glycosidases such as RTA and AMP nucleosidase may not require a strong protein acid to protonate the leaving group. Partial protonation by hydrogen bonding at N_1, N_3 or N_7 could all stabilize the leaving group in this conjugated system.[42] Indeed Asn209, Tyr80, and/or Tyr123 may fulfill this role in some additive fashion.

Despite some frustrations, efforts are continuing to bind substrate analogs into the ricin active site for analysis by x-ray diffraction. Such studies should

ultimately allow a clearer description of the roles played by various active site residues.

It is a reasonable question why residues such as Tyr80, Tyr123, and Asn209 are so highly conserved in nature when they play no critical role in catalysis. It must be remembered that a five- or tenfold loss in activity leaves an effective enzyme with a very significant rate enhancement over uncatalyzed reactions. In vivo, however, such an enzyme is only 10–20% active compared to the enzymes of competitors in the struggle for existence; such a difference is easily operated on by natural selection. It is also possible that these residues may play critical roles in the overall stability of the folded protein; indeed, the effects of several mutations on expression level seem to equal or exceed those on catalysis. Ricin is capable of crossing the cell membrane and is thought to undergo at least partial unfolding during this process.[44] It is likely that the protein in the folded state is in a rather delicate balance, which can be disrupted by relatively small changes in the side chains of key residues.

ACKNOWLEDGMENTS

We are grateful to Dr. Vern Schramm for helpful discussions and to Raquelle Smalley for her help in preparing the figures. This work was supported by grants GM 30048 and GM 35989 from the National Institutes of Health and by a grant from the Foundation for Research.

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