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Role of Arginine 180 and Glutamic Acid 177 of Ricin Toxin A Chain in Enzymatic Inactivation of Ribosomes

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The gene for ricin toxin A chain was modified by site-specific mutagenesis to change arginine 180 to alanine, glutamine, methionine, lysine, or histidine. Separately, glutamic acid 177 was changed to alanine and glutamic acid 208 was changed to aspartic acid. Both the wild-type and mutant proteins were expressed in *Escherichia coli* and, when soluble, purified and tested quantitatively for enzyme activity. A positive charge at position 180 was found necessary for solubility of the protein and for enzyme activity. Similarly, a negative charge with a proper geometry in the vicinity of position 177 was critical for ricin toxin A chain catalysis. When glutamic acid 177 was converted to alanine, nearby glutamic acid 208 could largely substitute for it. This observation provided valuable structural information concerning the nature of second-site mutations.

Ricin is the peptide toxin found in the seeds of *Ricinus communis*, the castor bean plant. It consists of a 32-kDa A chain linked by a disulfide bond to a 33-kDa B chain (21). The B chain binds to beta-galactoside-terminated oligosaccharides on the surfaces of mammalian cells (1). After binding, the toxin is internalized to an intracellular compartment where the disulfide bond is reduced (18) and the ricin toxin A chain (RTA) enters the cytosol. RTA enzymatically inactivates ribosomes by hydrolysis of the N-glycosidic bond of a conserved adenosine in the 28S RNA of eukaryotic 60S ribosomal subunits (A4324 in rats) (6). RTA has a K_{cat} of 1,500 ribosomes per min and a K_m of 0.1 μmol/liter (5).

The three-dimensional structure of ricin determined by X-ray crystallography contains a putative active-site cleft (19). Comparison of amino acid sequences of ribosome-inactivating proteins has revealed a number of conserved residues (22). Interestingly, some of these residues, including RTA Glu-177, Trp-211, Arg-180, Asn-209, Asn-78, and Tyr-80, have side chains extending into the cleft, and these may play a role in enzyme catalysis. Because of the conservative nature of protein folding, it is likely that corresponding residues in homologous proteins will also be implicated in enzyme activity (22).

In our initial study, RTA was expressed in *Saccharomyces cerevisiae* (8). Yeast strains which survived the induction of RTA and which continued to produce RTA-cross-reactive material were subjected to further study. RTA plasmid DNA was sequenced, and single base mutations were found in each case. All of the mutations analyzed produced changes in amino acid residues with side chains in or near the active-site cleft (Glu-177, Trp-211, Gly-212, Ser-215, and Ile-252).

Mutational analysis has been used to further define the role of cleft amino acid residues in catalysis. Hovde and colleagues changed glutamic acid at position 167 of Shiga-like toxin I (equivalent to RTA Glu-177) to aspartic acid, and the enzyme activity was 0.001 times that of control (11). We expressed RTA in *Escherichia coli* as part of a tripartite fusion protein with beta-galactosidase and a collagen linker (24). RTA was purified and shown to inhibit protein synthesis in a rabbit reticulocyte lysate assay. Glu-177 in RTA was changed by site-specific mutagenesis to aspartic acid or to alanine, and the mutant RTAs had 0.0125 or 0.05 times the activity of the control, respectively. This result led to a reassessment of the importance in catalysis of the carboxylate function at position 177 and to a search for other critical residues in the active site. Several research groups have made changes in amino acid residues remote from the cleft and have not observed major alterations in enzyme activity (3, 16, 25); however, no modifications of RTA cleft amino acid residues have been described.

Another conserved amino acid residue with a side chain extending into the active-site cleft is the arginine at position 180. Chemical modification of RTA arginines suggests that a limited class of arginine residues is critical for RTA inhibition of protein synthesis (26). In this paper, we report site-specific mutagenesis experiments which explore the role of Arg-180 and the role of Glu-177.

MATERIALS AND METHODS

General methods. *E. coli* TG1 [K-12 Δ(*lac-pro*) *supE thi hsd* Δ5/F′ *traD36 proA*$^+$*B*$^+$ *lacI*^{q} *lacZ* ΔM15] was used for plasmid propagation and for single-stranded DNA production. Plasmid DNAs were prepared by the boiling method (10). Restriction endonucleases and other enzymes for DNA manipulation were from Promega Biotec (Madison, Wis.). Site-specific mutagenesis reagents were obtained from Amersham, Inc. (Arlington Heights, Ill.). All enzyme reactions were carried out as specified by the suppliers. Oligonucleotides were purchased from Research Genetics, Inc. (Huntsville, Ala.). Bacteria were grown in L broth or YT broth, and plasmid selection was maintained with ampicillin (100 μg/ml).

Construction of bacterial expression plasmids encoding mutant RTA. A plasmid (pUC119-RTA) containing an RTA *Bam*HI fragment cloned into pUC119 was described previously (24). This plasmid was further modified by site-directed mutagenesis to change the AGA codon of Arg-180 to GCA (alanine), CAA (glutamine), ATG (methionine), AAA (lysine), or CAC (histidine). The modified plasmid containing GCT (alanine) at codon 177 of RTA was previously

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described (24). The GAG codon of Glu-208 was changed to GAC (aspartic acid) by site-directed mutagenesis. The modified plasmids were digested with *Bam*HI, and the 810-bp RTA fragments were isolated and subcloned into pJG200. For each mutant, single-stranded DNA was sequenced by the dideoxy chain termination method (23) with a Sequenase kit (U.S. Biochemical Corp., Cleveland, Ohio) to confirm the desired mutation.

Construction of yeast expression plasmids encoding mutant RTA. pUC119-RTA was previously modified by oligonucleotide-directed mutagenesis to introduce an ATG codon immediately 5′ to the RTA coding sequence and a stop codon (TAA) at the 3′ end of the RTA gene (8). This plasmid was further modified by site-specific mutagenesis to change Arg-180 to lysine (R180K) or histidine (R180H), Glu-177 to alanine (E177A), and Glu-208 to aspartic acid (E208D). The appropriately modified plasmids were digested with *Bam*HI, and the 812-bp RTA fragments were isolated and cloned into the yeast expression vector pBM150 (14). pBM150 is a low-copy-number CEN plasmid containing the *URA3* gene and a *GAL1-10* promoter. *S. cerevisiae* SJ21R (**a** *ade1 leu2-3,112 ura3-52*) (10) was transformed with the RTA-containing plasmids by the lithium chloride method (13), and transformants were selected on uracil-deficient medium.

Growth properties of transformed yeast cells. Yeast cells were grown on YEPD medium or on synthetic complete medium lacking uracil and supplemented with either 2% glucose or 2% galactose (8). Single colonies of yeast strains harboring expression plasmids containing wild-type or mutated RTA genes were transferred as 1-cm^2 patches onto uracil-deficient, glucose-supplemented plates and replica plated onto plates containing either glucose or galactose. Growth was monitored for 48 h and scored as growth or no growth. In addition, single colonies of yeast strains were grown in uracil-deficient liquid broth with either 2% glucose or 2% galactose, and cell density was monitored over time by determination of direct counts with a hemacytometer.

Protein preparation and purification of rRTA. The procedure for bacterial expression and purification of recombinant RTA (rRTA) was previously described (24). The fusion protein was purified on a Pharmacia fast protein liquid chromatography HR5/5 Mono Q anion-exchange column and treated with collagenase as described by Moskaluk and Bastia (20). The collagenase digestion mixture was applied to a Pharmacia fast protein liquid chromatography HR16/50 Superose 6 gel filtration column equilibrated with PBS (150 mM NaCl, 8.5 mM Na_2HPO_4–1.5 mM KH_2PO_4 [pH 7.4], 0.5 mM $MgCl_2$) and was eluted with the same buffer. The protein peak at an elution volume of 75 to 82.5 ml (the volume expected for a globular protein with a molecular mass of 30 kDa) was pooled, concentrated by Centricon 10 ultrafiltration to approximately 1 ml, and stored as aliquots at both 4 and −80°C. The protein concentration was determined by the method of Bradford (2), with bovine serum albumin as the standard. Precautions were taken to maintain the final protein concentration at less than 1 mg/ml.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western immunoblotting. Ten micrograms of rRTA protein and 10-μg samples from the purification procedure were separated on reducing sodium dodecyl sulfate-polyacrylamide gels (15). Either the gels were stained with Coomassie blue R-250 or the proteins were electrophoretically transferred to nitrocellulose with an American Bionetics Polyblot apparatus as described by the manufacturer. Following transfer, the nitrocellulose membranes were blocked with 10% nonfat dry milk, incubated with 10 μg of affinity-purified rabbit anti-RTA antibody per ml (8), probed with a 1:250 dilution of affinity-purified goat anti-rabbit immunoglobulin G conjugated to alkaline phosphatase (Sigma Chemical Co., St. Louis, Mo.), and developed with alkaline phosphatase substrate kit II (Vector Laboratories, Burlingame, Calif.).

Amino acid compositions of RTA mutant proteins. Wild-type, R180K, R180H, E177A, E208D, and E177A-E208D RTA proteins were further purified on a Brownless Labs Aquapore reverse-phase 300 column (7 μm; 0.26 by 22 cm) with two LKB 2150 high-pressure liquid chromatography pumps and an LKB 2152 controller to form a gradient in 0.1% trifluoroacetic acid and in trifluoroacetic acid-water-acetonitrile (0.1:9.9:90 [vol/vol/vol]). The proteins were detected at 214 nm with an LKB 2140 diode array detector, collected manually in polypropylene tubes, and stored at −20°C until assayed. The proteins (1 to 2 nmol) were hydrolyzed in the gas phase for 24 h at 110°C as previously described (7). The hydrolyzed samples were dried in a Speed Vac concentrator (Savant), and the amino acid compositions of the hydrolysates were determined in a Beckman 6300 high-performance amino acid analyzer with sodium citrate buffers provided by the manufacturer.

Molecular graphics of ricin toxin. The molecular model was displayed on an Evans & Sutherland PS390 computer with the program FRODO at the University of Texas Center for Structural Biology and on an Evans & Sutherland PS390 computer with Tripos Sybyl software at the Duke Comprehensive Cancer Center Molecular Graphics Shared Resource Facility.

Enzyme activity of rRTA. The enzyme activity of rRTA was quantitated by a protein synthesis inhibition assay. Increasing amounts of purified rRTA were added to a rabbit reticulocyte lysate protein translation mixture (Promega Biotec). The translation assay was performed with [^{3}H]leucine (140 Ci/mmol; New England Nuclear, Boston, Mass.) and a brome mosaic virus mRNA model substrate in accordance with the manufacturer's instructions. The concentration of rRTA necessary to inhibit by 50% the incorporation of [^{3}H]leucine into protein (ID_{50}) was determined by comparison with the value for the control, purified native plant RTA (Inland Laboratories, Austin, Tex.).

RESULTS

Site-specific mutagenesis to alter RTA arginine residue 180 to alanine, glutamine, or methionine resulted in the production of fusion proteins with a much greater tendency to aggregate and precipitate than the wild-type protein. The majority of the fusion proteins were pelleted by the initial 100,000 × *g* ultracentrifugation of the cell homogenate (Fig. 1). In contrast, mutant RTA with lysine or histidine at position 180 yielded a fusion protein with properties indistinguishable from those of the nonmutated protein (Fig. 1 and 2). We were able to reproducibly prepare high-purity, immunoreactive RTA from the R180K and R180H mutant fusion proteins (Fig. 2). The final yield of Lys-180 mutant RTA was 0.8 mg/liter of induced *E. coli* culture, and that of His-180 mutant RTA was 1.3 mg/liter of induced *E. coli* culture.

Expression of the E177A and E177A-E208D genes yielded fusion proteins with intermediate solubility. The final yield of E177A-E208D RTA was 0.2 mg/liter, and the final yield of E177A RTA was 0.3 mg/liter. The E208D fusion protein was very poorly soluble, but extra efforts were made to purify this control, with final yields of 1 to 10 μg/liter. As with the

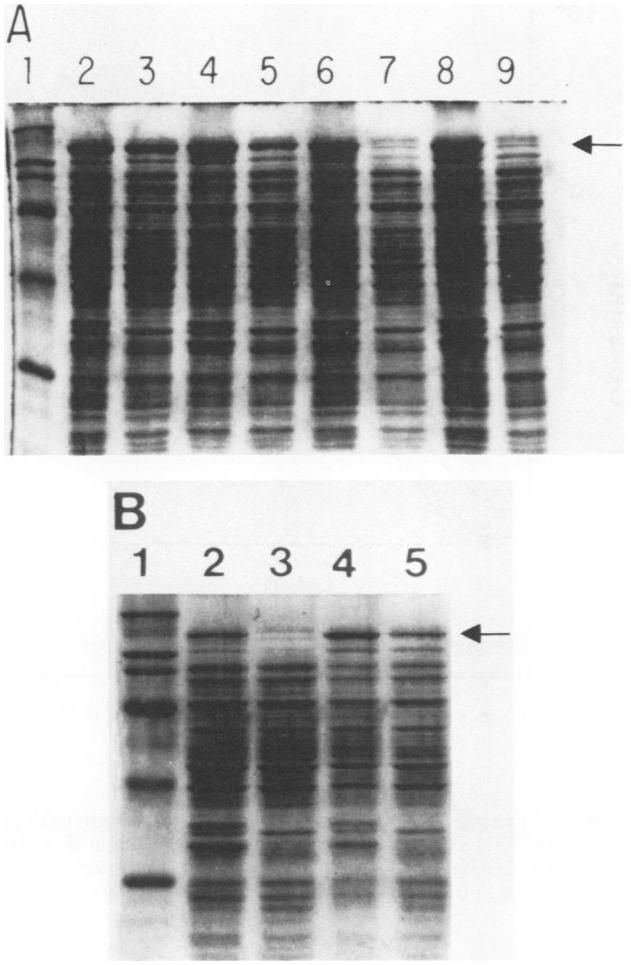


FIG. 1. Coomassie blue R-250-stained reducing sodium dodecyl sulfate-polyacrylamide gels. Electrophoresis (performed as described in Materials and Methods) was done with 10-μg samples of total cell lysate and supernatant after 100,000 × *g* centrifugation. (A) Lanes: 1, molecular mass markers (Sigma) (myosin, 205 kDa; beta-galactosidase, 116 kDa; phosphorylase *b*, 92.5 kDa; bovine serum albumin, 67 kDa; ovalbumin, 45 kDa; and carbonic anhydrase, 30 kDa); 2, total *E. coli* protein after thermal induction of wild-type fusion protein; 3, 100,000 × *g* supernatant of wild-type fusion protein; 4, total *E. coli* protein after thermal induction of Lys-180 mutant fusion protein; 5, 100,000 × *g* supernatant of Lys-180 mutant fusion protein; 6, total *E. coli* protein after thermal induction of Ala-180 mutant fusion protein; 7, 100,000 × *g* supernatant of Ala-180 mutant fusion protein; 8, total *E. coli* protein after thermal induction of Glu-180 mutant fusion protein; 9, 100,000 × *g* supernatant of Glu-180 mutant fusion protein. (B) Lanes: 1, molecular mass markers (as in panel A); 2, total *E. coli* protein after thermal induction of Met-180 mutant fusion protein; 3, 100,000 × *g* supernatant of Met-180 mutant fusion protein; 4, total *E. coli* protein after thermal induction of His-180 mutant fusion protein; 5, 100,000 × *g* supernatant of His-180 mutant fusion protein. The fusion protein bands are indicated with arrows.

residue 180 mutants, the major loss of protein occurred during the initial 100,000 × *g* ultracentrifugation of the cell homogenate for all three mutants.

Amino acid compositions of RTA mutant proteins. Reverse-phase chromatography yielded two protein peaks with 40 and 60% of the 214-nm-absorbing material. The larger peak, which eluted later, had an amino acid composition very close to that predicted for rRTA on the basis of the DNA se-

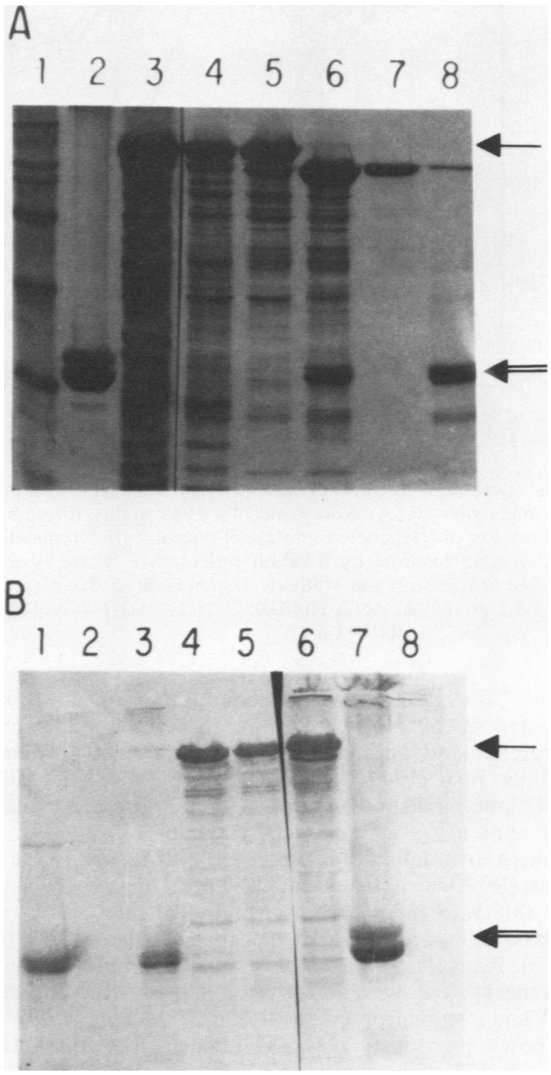


FIG. 2. Analysis of fractions showing Lys-180 rRTA purification. (A) Coomassie blue R-250-stained reducing sodium dodecyl sulfate-polyacrylamide gel. Electrophoresis (performed as described in Materials and Methods) was done with 10-μg samples from each purification step. Lanes: 1, molecular mass markers (as in Fig. 1A); 2, plant RTA; 3, total *E. coli* protein after thermal induction; 4, 40% $(NH_4)_2SO_4$ precipitate of 100,000 × *g* supernatant; 5, fractions containing beta-galactosidase activity after Mono Q chromatography (elution volume, 24 to 25 ml); 6, protein fractions from lane 5 treated with collagenase (note that the fusion protein band disappeared and that a new, lower-molecular-mass band appeared); 7, fractions containing beta-galactosidase activity after Superose 6 chromatography (elution volume, 57 to 60 ml); 8, final purified Lys-180 mutant rRTA. (B) Immunoblot probed with affinity-purified anti-RTA antibody as described in Materials and Methods. Lanes: 1, final purified Lys-180 mutant rRTA; 2, fractions containing beta-galactosidase activity after Superose 6 chromatography (elution volume, 57 to 60 ml); 3, protein fractions from lane 4 treated with collagenase (note that the fusion protein band disappeared and that a new, lower-molecular-mass band appeared); 4, fractions containing beta-galactosidase activity after Mono Q chromatography (elution volume, 24 to 25 ml); 5, 40% $(NH_4)_2SO_4$ precipitate of 100,000 × *g* supernatant; 6, total *E. coli* protein after thermal induction; 7, plant RTA; 8, molecular mass markers (as in Fig. 1A). Fusion protein bands are indicated by single-stemmed arrows, and RTA bands are indicated by double-stemmed arrows.

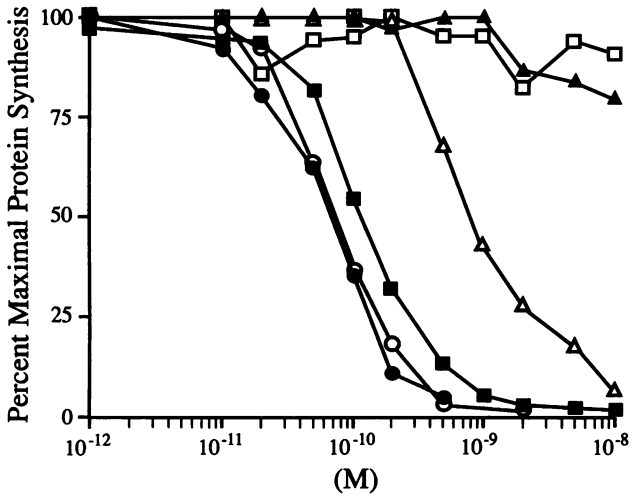


FIG. 3. Protein translation inhibition by wild-type and mutant rRTA molecules. RTAs were tested for their ability to inhibit the incorporation of [^{3}H]leucine into alkali-resistant trichloroacetic acid-precipitable material by a rabbit reticulocyte lysate system as described in Materials and Methods. Symbols: ●, native plant RTA; ■, rRTA Lys-180; ▲, rRTA His-180; □, rRTA Ala-177–Asp-208; ○, rRTA Asp-208; △, rRTA Ala-177.

quence. The R180K and R180H mutant fusion proteins yielded identical results, except for a decrease in the content of arginine and an increase in the content of lysine or histidine, respectively. The E177A, E208D, and E177A-E208D mutant fusion proteins yielded appropriate changes in the contents of glutamic acid and aspartic acid.

Protein translation inhibition by rRTA. Wild-type and mutant rRTAs were tested for enzyme activity by measuring their ability to inhibit the translation of mRNA by rabbit reticulocyte lysates as described in Materials and Methods (Fig. 3). Plant RTA was a potent inhibitor of protein synthesis in this system, with an ID_{50} of 7×10^{-11} M. Nonmutated rRTA had a similar potency, with an ID_{50} of 4.5×10^{-11} M. As shown previously (24) and confirmed here, replacing RTA Glu-177 with alanine (E177A) generated a protein of lower potency, with an ID_{50} of 7.9×10^{-10} M (24). Replacing RTA Arg-180 with lysine (R180K) resulted in a slightly less potent protein, with an ID_{50} of 12×10^{-11} M. Replacing RTA Glu-208 with aspartic acid (E208D) yielded a protein with an ID_{50} of 6.4×10^{-11} M. Conversion of Arg-180 to histidine (R180H) yielded a protein which would not produce 50% inhibition even at 10^{-8} M. The shape of the curve in Fig. 3 suggests that the enzyme activity of R180H was reduced to at least 0.001 times that of the control.

Expression of mutant RTA genes in yeast cells. To confirm the above-described results, we cloned the mutant RTA genes into the yeast expression vector pBM150 and expressed them under the control of the *Gal1-10* promoter. Since yeast ribosomes are sensitive to RTA, these constructs allowed us to determine the relative potency of expressed RTAs by monitoring their effects on yeast growth (Fig. 4). The growth of yeast cells transformed with a plasmid bearing the nonmutated RTA gene, the E208D mutation, or the R180K mutation was totally arrested upon the addition of the inducer galactose to the medium. Transformants with the E177A mutation showed intermediate growth, while the growth of those containing the R180H or the E177A-E208D double mutation was not affected under similar conditions.

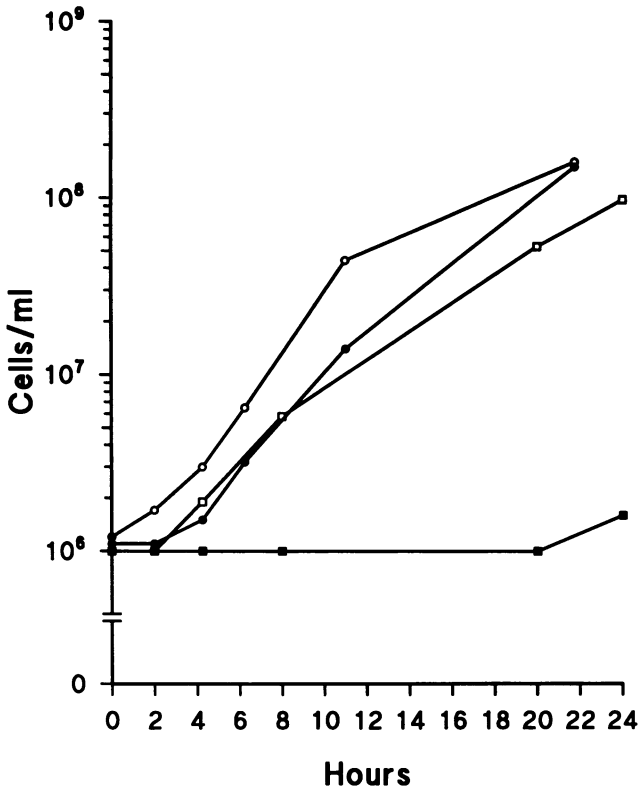
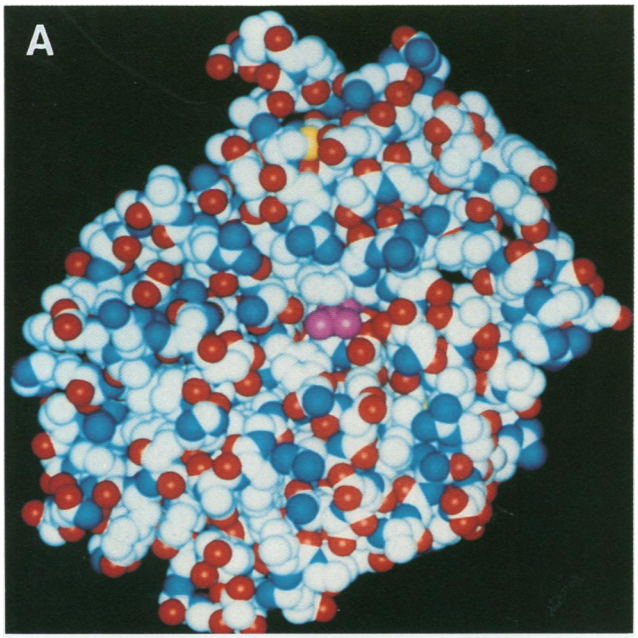


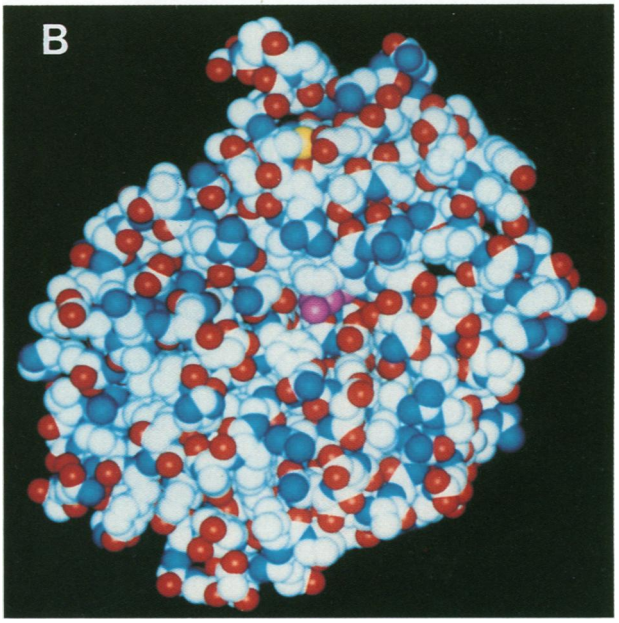
FIG. 4. Yeast growth curves. Cells were inoculated into medium with either 2% glucose (□ and ○) or 2% galactose (■ and ●) at 10^6 cells per ml. Symbols: □ and ■, pBM150-Lys180-transformed yeast cells; ○ and ●, pBM150-His180-transformed yeast cells.

DISCUSSION

There are a number of amino acid residues with polar side chains in the RTA cleft which may participate in N-glycosidic bond hydrolysis (19). Some of these residues, including Asn-78, Tyr-80, Tyr-123, Glu-177, Arg-180, and Asn-209, are conserved among a series of ribosome-inactivating protein toxins (trichosanthin, barley protein synthesis inhibitor, RTA, and abrin A chain) (22). Initial mutagenesis studies focused on one of these residues, Glu-177 (11, 24). Modification of this glutamic acid reduced activity in each case, but significant enzyme activity remained even when the carboxylate function was absent (24). We examined the role of Arg-180 both because it is a conserved amino acid residue, with a side chain protruding into the putative active-site cleft, and because chemical modification studies with phenylglyoxal have suggested an important role for one arginine or a few arginines in the enzyme reaction (26). Furthermore, Arg-180 and Glu-177 coordinate an active-site water which may be the ultimate nucleophile in the N glycosidation reaction (B. Katzin, A. Collins, and J. D. Robertus, unpublished data). Alternatively, Arg-180 may function as a nucleophile in the enzyme reaction. For example, arginine is the acceptor in the ADP-ribosylation of the alpha subunit of RNA polymerase during T4 phage infection of *E. coli* (9).

In this study, we examined a number of substitutions at position 180 of RTA. All three mutants with substitutions of neutral amino acids at position 180 were insoluble, while mutants with positively charged side chains were fully soluble. There are several reasons why the loss of a charge on the side chain of residue 180 could lead to protein





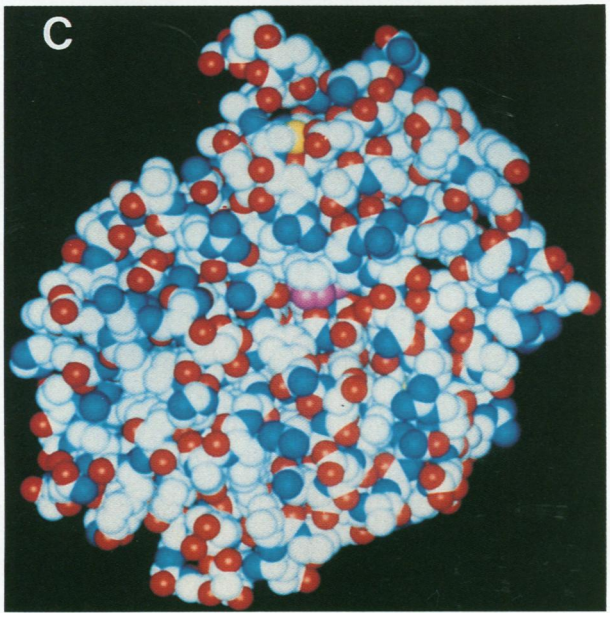


FIG. 5. CPK model of RTA and RTA mutants. Residue 180 is highlighted in magenta. White represents carbon atoms; red represents oxygen atoms; blue represents nitrogen atoms; yellow represents sulfur atoms. Hydrogen atoms are not shown. (A) Wild-type RTA. (B) R180K RTA. (C) R180H RTA. The secondary structure of RTA and the tortional angles for residue 180 are assumed not to change significantly.

precipitation, but the most likely by far is that the positively charged side chain is required for proper folding or stability of the molecule. It has been shown that both Glu-177 and Arg-180 lie on a section of alpha helix which must be bent for the charged residues to reach the solvent of the active-site cleft. A neutral residue may lack the solvation attraction to pull the chain in the proper manner (Katzin et al., unpublished data).

Enzyme activity was assessed for the two soluble mutants. The R180K protein showed, at most, a fourfold decrease in activity. This result rules out a role for the guanidinium moiety as a necessary nucleophile in catalysis. However, the Lys mutation may be able to coordinate the active-site water or perhaps form an ion pair with Glu-177 in a satisfactory way.

A His side chain is substantially shorter than that of either Arg or Lys. A His residue substituted for Arg-180 would have difficulty clearing the imidazole ring of Trp-211, upon which it is stacked, to reach the solvent of the cleft. This structure is shown in the space-filling model in Fig. 5. As a result of its size, the His side chain would probably have difficulty coordinating the active-site water or in forming an ion pair with Glu-177 if that is a role at some step in the catalytic mechanism. It should also be noted that the normal pK_a of the His imidazole is around 6.4. The microenvironment of a folded protein can cause this pK_a to be seriously perturbed, but it is likely that this His is generally nonprotonated at a physiological pH.

The strict requirements for the location of the positive charge in the cleft for catalysis led us to reexamine the results of the mutations at position 177. The E177A mutation appears to remove a key negatively charged residue from the cleft, but the E177A mutant has significant enzyme activity. We evaluated the potential of Glu-208 to replace Glu-177 in position and function. Glu-208 forms an ion pair with Arg-134 and appears to form one wall of the active-site cleft. Modification of Glu-177 to alanine leaves an empty space in the cleft which could be filled via rotation at the alpha carbon with the carboxylate group of Glu-208. Apparently, such a conformation has 0.05 times the activity of the wild-type conformation for ribosome inhibition. Changing Glu-177 to aspartic acid displaces the carboxylate from its optimum position but sterically and electrostatically prevents the Glu-208 carboxylate from moving into the cleft. This conformation has 0.0125 times the activity of the wild-type conformation.

Evidence supporting this hypothesis was obtained by further mutagenesis. When Glu-208 is converted to Asp (E208D), 100% of the wild-type enzyme activity is retained. Presumably, the active site is perturbed only slightly and Asp-208 forms part of the cleft wall structure. When Glu-177

is changed to Ala (E177A-E208D), virtually no activity remains. Unlike the case for the single E177A mutant, the shorter and more stereochemically restrictive Asp residue at position 208 cannot properly fill the catalytic carboxylate site. Efforts are under way to crystallize a number of these mutants to help confirm these notions.

The positive and negative charges at fixed locations in the center of the RTA cleft appear to play a role in folding and also in catalysis. Noncovalent electrostatic catalysis based on charge stabilization of the transition state would explain the behavior of the mutants with mutations at positions 177 and 180. Such a mechanism is used by lysozyme in the hydrolysis of an O-glycosidic bond (4) and by AMP nucleosidase in the hydrolysis of an N-glycosidic bond (17). Lysozyme catalyzes the transfer of C-1 of hexose residue 4 from the C-4 hydroxyl group of residue 5 to water. Glu-35 functions as a general acid catalyst to protonate the departing alcohol. Asp-52 stabilizes the developing positively charged carboxonium ion. Further interactions between the enzyme and substrate stabilize the half-chair form of the sugar. Water then attacks the C-1 atom of the carbonium ion to add a hydroxyl group, and a proton from water is added back to Glu-35 to complete the reaction. AMP nucleosidase has been proposed to require several charged groups which aid in hydrolysis, on the basis of kinetic isotope effects. These include an acidic side chain which protonates the adenine ring at N-7 and a negatively charged side chain which stabilizes the partial positive charge that develops in the ribose (oxycarbonium). It is also possible that a specific base, such as a carboxylate, is needed to ionize an enzyme-bound water molecule for attack on C-1′. In both enzymes, the critical nucleophile is water and the transition state is stabilized by positively and negatively charged side chains in the active site. In RTA, Glu-177 may stabilize the rRNA carboxonium ion or activate water 323. Arg-180 may function to electronically stabilize a catalytic intermediate, to activate water, or to bond to the phosphate backbone of RNA. Further understanding of the mechanism of enzymatic hydrolysis by RTA and other ribosome-inactivating toxins may be obtained by modifying other active-site polar amino acid residues or from X-ray crystallographic analysis of RTA crystals containing bound substrates.

The analysis presented here concerning the ability of Glu-208 to partially fulfill the role of wild-type Glu-177 has interesting implications for our general understanding of second-site mutations. Glu-208 is a conservative but not invariant residue in toxins; it is Gln in barley protein synthesis inhibitor, for example. Glu-208 seems to have a structural role, but its position in space is such that under the correct circumstances it can move into the active site and play a role in maintaining enzyme activity. In barley protein synthesis inhibitor, conversion of Glu-174, the active-site analog of Glu-177, to Ala would presumably reduce activity by several orders of magnitude and would effectively inactivate the enzyme. A second-site mutation converting Gln-208 to Glu would likely restore most of the activity.

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