Structure of a Ricin Mutant Showing Rescue of Activity by a Noncatalytic Residue†

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ABSTRACT: Ricin A chain is an *N*-glycosidase which removes a single adenine base from a conservative loop of 28S rRNA, thereby inactivating eukaryotic ribosomes. The mechanism of action has been proposed to include transition-state stabilization of an oxycarbonium ion on the substrate ribose by interaction with Glu 177. Conversion of Glu 177 to Gln reduces activity nearly 200-fold [Ready, M. P., Kim, Y., & Robertus, J. D. (1991) *Proteins: Struct., Funct., Genet. 10*, 270–278] while conversion to Ala (E177A) reduces activity only 20-fold [Schlossman, D., Withers, D., Welsh, P., Alexander, A., Robertus, J., & Frankel, A. (1989) *Mol. Cell. Biol. 9*, 5012–5021]. X-ray analysis of the latter mutant protein shows that a residue at the edge of the active site, Glu 208, rotates into the space left vacant by the mutation. Its rearranged carboxylate partially substitutes for that of Glu 177. This is equivalent to the rescue of enzyme activity by a second-site reversion. Kinetic analysis shows the E177A mutation affects k_{cat} and not K_m, consistent with the notion that the carboxylate serves in transition-state stabilization.

The castor seed toxin ricin is a heterodimeric protein, consisting of a 32 000-M_r A chain glycoprotein (RTA) linked by a disulfide bond to a 32 000-M_r B chain (RTB) which facilitates cell uptake. The biochemistry of the toxin has been reviewed (Olsnes & Pihl, 1982). RTA acts as an *N*-glycosidase which attacks the 60S ribosomal subunit (Endo & Tsurugi, 1988). It hydrolyzes a specific adenine, A_{4324} in rat. This single depurination prevents elongation factors from binding normally and disrupts protein synthesis. Depending on the source of ribosomes and assay conditions, RTA has a k_{cat} = 300–1500 min^{-1} and a K_m = 0.1–1.3 μM (Olsnes et al., 1975; Ready et al., 1991).

We have refined the X-ray model of ricin to 2.5 Å (Rutenber et al., 1991) and described the chain structures (Katzin et al., 1991; Rutenber & Robertus, 1991). In addition, we have made a number of site-directed mutants of RTA (Schlossman et al., 1989; Frankel et al., 1990; Ready et al., 1991). A mechanism of action has been proposed on the basis of the X-ray structure and the results of mutagenic studies (Ready et al., 1991). It is likely that the depurination reaction proceeds through an oxycarbonium intermediate, with positive charge building up on the ribose as the bond to adenine breaks. Such a mechanism has been seen measuring kinetic isotope effects for another *N*-glycosidase, AMP nucleosidase (Mentch et al., 1987). The enzyme provides an anion to stabilize this charge in the transition state; in RTA Glu 177 appears to fill this role. Because the X-ray model had suggested a role for Glu 177, the position was investigated by site-directed mutagenesis in a number of systems. The A chain of Shiga-like toxin-I is a ricin A chain homologue. Conversion of its Glu 167 to Asp caused a nearly 1000-fold decrease in activity (Hovde et al., 1988). Conversion of RTA Glu 177 to neutral Gln reduces enzyme activity nearly 200-fold (Ready et al., 1991), while conversion to Asp decreased activity roughly 80-fold (Schlossman et al., 1989). Surprisingly, conversion to Ala (E177A) reduced enzyme activity only 20-fold in the same type of test (Schlossman et al., 1989).

To explain this seeming anomaly, we suggested that the carboxylate of Glu 208 could rotate into the position normally occupied by Glu 177 and substitute for it (Frankel et al., 1990). This would only be possible in the Ala mutant; the steric bulk of a Gln residue at position 177 would prevent Glu 208 from moving into position. To test this hypothesis, we crystallized the E177A mutant protein and report its structure in this paper.

Materials and Methods

Wild-type RTA was expressed from the pUTA plasmid and purified from a clone in *Escherichia coli* as described earlier (Ready et al., 1991). The mutant gene for E177A was initially created to code for a fusion protein (Schlossman et al., 1989). The 351-bp *NsiI–BglII* restriction fragment from the original mutant plasmid, containing the mutant at residue 177, was isolated and used to replace the corresponding fragment in pUTA. This created a gene coding for the E177A protein, but with a native amino terminus instead of the collagen-sensitive linker.

Dose response enzyme assays and initial rate kinetic experiments were carried out as described previously (Ready et al., 1983, 1991). Ribosomes from *Artemia salina* in a low salt buffer (2 mM Mg^{2+}, 20 mM K^+, 30 mM HEPES, pH 7.6) served as substrate.

Both the wild-type and E177A proteins were crystallized as described previously (Robertus et al., 1987); E177A crystals were isomorphous with wild type. X-ray diffraction data for wild-type RTA were collected to 1.9-Å resolution on an SDMS multiwire area detector, and the data were processed as described previously (Howard et al., 1985). Data for the E177A crystal were collected to 2.8-Å resolution.

The structure of the ricin heterodimer has been refined to 2.5 Å (Rutenber et al., 1991). The A chain from that model was used as a test structure in a molecular replacement scheme. The ricin A chain was oriented in the RTA cell using rotation, translation, and rigid body refinement. A subsequent crystallographic refinement of the model is essentially complete. The crystallographic R factor is <18%, and a manu-

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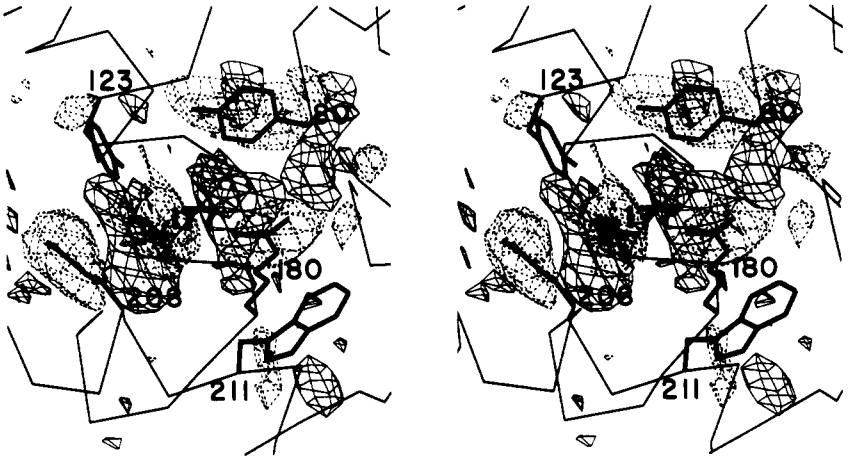

FIGURE 1: Difference electron density map between native and E177A RTA. Positive difference density is shown as solid lines, and negative density is shown as broken lines. The density baskets are contoured at 3σ level. The side chains of key active site residues from native RTA are superimposed as heavy dark lines extending from the thinner lines of the Cα trace.

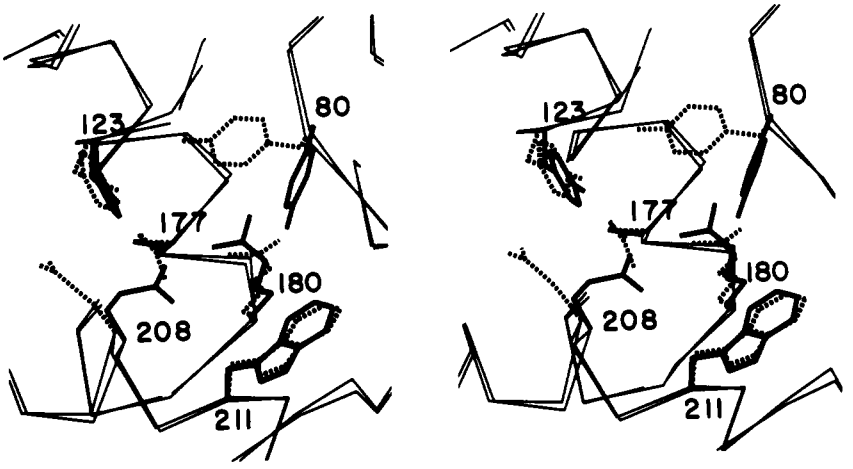

FIGURE 2: Superposition of the active sites of nature and E177A RTA. The Cα traces of the two models are shown as nearly superimposable thin lines. The side chains of the native model are shown as broken heavy lines, while the mutant model side chains are shown as heavy solid lines.

script describing the molecular replacement, crystallographic refinement, and molecular structure is in preparation. This model serves as the parent protein for the present comparison with the E177A protein.

Maps and molecular modeling of E177A were carried out as described previously (Rutenber et al., 1991). The E177A model was refined using the molecular dynamics option of XPLOR (Brunger et al., 1987).

Results

A difference Fourier at 2.8-Å resolution between native and E177A showed essentially all strong peaks to be in the active site area. Figure 1 shows the active site area of the difference map, with the native model superimposed. It is clear that, as expected, the side chain at position 177 has been reduced to the methyl group of Ala. In addition, the side chain of Glu 208 sits in negative density, indicating it has moved. An appropriate basket of positive density clearly shows the new position. Arg 180 also appears to move upward, so that an ion pair can be formed with Glu 208 in its new position. Finally, Tyr 80 has rotated outward toward the solvent by roughly 65°.

The model for E177A was refined using XPLOR (Brunger et al., 1987) to an R factor of 21.8%. Refinement of the occupancy for Glu 208 and Tyr 80 suggests that the movement occurs in roughly 85% of the molecules, while about 15% retain the wild-type configuration. A superposition of the active site residues of the refined native protein and E177A is shown in Figure 2.

As described in Materials and Methods, the E177A mutation was moved into the pUTA plasmid to produce a mutant protein with a native amino terminus. Dose response assays against *A. salina* ribosomes indicated an ID_{50} = 12 nM, roughly 40 times higher than that of wild type (data not shown). To dissect this reduced activity, initial rate experiments were carried out. Data were analyzed using nonlinear regression analysis and double-reciprocal plots (Figure 3). Both methods gave essentially the same results. K_m for E177A is 1.2 μM, essentially the same as the 1.3 μM value for wild type. In marked contrast, k_{cat} for the mutant is 5 min^{-1} versus 300 min^{-1} for wild type.

Discussion

Glu 177 is invariant in the eight plant and bacterial toxins sequenced from this class of enzymes (Robertus, 1991). We have hypothesized that it functions to stabilize the oxycarbonium ion which is likely to develop on the ribose during the depurination reaction (Ready et al., 1991). The importance of the residue is seen by several site-directed mutagenesis experiments. The conservative conversion of Glu 177 to Asp decreases activity nearly 100-fold (Schlossman et al., 1989). When the equivalent Glu in Shiga-like toxin-I is converted to Asp, activity drops nearly 1000-fold (Hovde et al., 1988). Such mutations clearly retain the negative charge thought to be

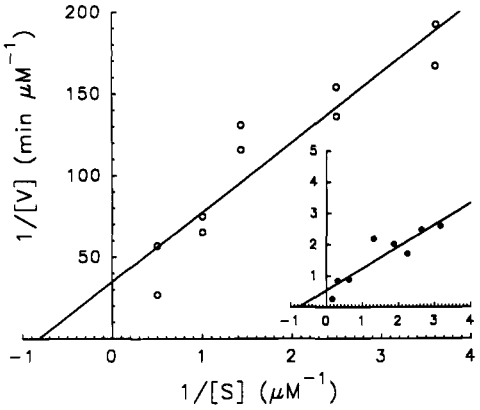

FIGURE 3: Double-reciprocal plots of kinetic data for wild-type and E177A RTA. The large plot indicates the quality of initial rate data for E177A, and the inset shows that for wild-type RTA. Enzyme concentrations were 6 nM. K_m, as measured by the x-axis intercepts, is essentially identical, while V_{max}, and therefore k_{cat}, for wild type exceeds that of the mutant by roughly 60-fold.

important in transition-state stabilization but probably move it from the optimum position. Conversion of Glu 177 to the neutral amide to Gln reduces activity 200-fold (Ready et al., 1991). Gln could occupy the same space as Glu, but the amide would clearly lack the formal charge to counter a cation. Conversion to Ala might be expected to show the full effect of losing this active site residue, but in fact the mutant exhibits only 20-fold (Schlossman et al., 1989) to 60-fold inactivation. The X-ray study presented here clearly confirms our hypothesis (Frankel et al., 1990) that the active site hole left by the E177A mutation can be adventitiously filled by the neighboring residue Glu 208. Although not in the optimum position, the relocated Glu 208 can provide a negative charge to stabilize the transition-state oxycarbonium ion.

In wild-type RTA, Glu 208 forms part of the active site wall (Katzin et al., 1991). It is a fairly conservative residue, but it has been replaced by either Thr or Val in some toxins (Robertus, 1991). As a result, Glu 208 does not appear to be a crucial active site residue. This is also borne out by site-directed mutagenesis which shows Glu 208 can be altered to Asp (E208D) with no measurable effect on activity (Frankel et al., 1990). However, if Glu 208 is converted to the shorter and less flexible Asp, it cannot rescue enzyme activity if Glu 177 is then converted to Ala. The double mutant of E208D and E177A is reduced in activity at least 1000-fold (Frankel et al., 1990).

The kinetic data are completely consistent with our hypothesis that transition-state stabilization is the normal role for the carboxylate of Glu 177. The removal of the Glu 177 side chain, and the adventitious substitution of Glu 208, has little if any effect on K_m, but it causes a 60-fold decrease in k_{cat}. In the simplest interpretation of kinetic parameters, this suggests that Glu 177 is not involved in substrate binding but does help control the rate-limiting step in the reaction. An effect on the catalytic rate is equivalent, in the simplest view, to an effect on transition-state stabilization (Kraut, 1988). The position of the Glu 208 carboxylate in E177A is probably not optimum for the depurination reaction, and it may provide less stabilization to the oxycarbonium ion transition state than does Glu 177.

In principle, the rescue of activity we have seen represents a model for second-site revertants. The usual definition of a second-site revertant is one in which activity is lost due to a mutation at a key residue. Activity is then rescued by a compensating mutation at another site. This is not the case in RTA which possesses an apparent "backup" residue in Glu 208. However, we have seen that an RTA with Asp at position 208 is perfectly functional but it cannot rescue activity if Glu 177 is converted to Ala (Frankel et al., 1990). In such a protein, the second-site conversion of Asp 208 to Glu would constitute a true second-site reversion. We predict that conversion of Glu 167 in the Shiga-like toxin A chain (SLTA) to Ala would result in a large decrease in activity. In SLTA, the residue corresponding to Glu 208 is Thr 200 (Calderwood et al., 1987; Robertus, 1991), and so it could not rescue the enzyme from the loss of the active site carboxylate. In that case, conversion of Thr 200 to Glu should create a true second-site revertant.

As far as we know, this is the first example of an effective second-site revertant for which an X-ray structure exists. The structure provides useful information about these proteins. It shows that steric as well as chemical constraints must be considered in rationalizing mutagenic results. It is interesting that changing the key active site Glu 177 to Ala may allow more options for activity rescue than does the conservative change to Asp or Gln.

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Registry No. Glu, 56-86-0; Gln, 56-85-9.

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