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Determination by systematic deletion of the amino acids essential for catalysis by ricin A chain

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ABSTRACT **The A chain of ricin is a RNA N-glycosidase that inactivates ribosomes by depurination of a single adenosine in 28S rRNA. Of the 267 amino acids in the protein, 222 (83%) could be deleted from one or another of 74 mutants without loss of the capacity of the protein to recognize a single nucleotide from among the 7000 in ribosomes or to catalyze hydrolysis.**

Ricin, a heterodimeric glycoprotein with an A chain of 267 amino acids linked by a disulfide bond to a B chain of 262 residues, is a cytotoxin that inactivates mammalian ribosomes (1). The ricin B chain recognizes galactose-containing receptors on sensitive cells, whereas the separate ricin A chain (RA) catalyzes the cleavage of the N-glycosidic bond of the adenosine at position 4324 in 28S rRNA (2, 3). Depurination of this single nucleotide from among the 7000 in the organelle accounts for the irreversible inactivation of ribosomes apparently by disrupting the binding site for the elongation factors (4, 5).

An understanding of the action of the toxin requires identification of the nucleotides in 28S rRNA and of the amino acid side chains in RA that contribute to recognition and to catalysis. The identity elements in the ricin domain stem and loop in 28S rRNA are being determined by analyzing the effect of the toxin on mutants of a synthetic RNA that reproduces this structure (6, 7). Complementary information for the protein is needed. Examination of the crystal structure of the ricin heterodimer (8) refined to 2.5 Å (9, 10) (the three-dimensional structure of the A chain alone has not yet been solved) has led to the proposal that the active site lies in a prominent cleft that contains residues that are invariant in seven plant and bacterial toxins that share a common mechanism of action with RA (Fig. 1). At the bottom of the cleft there is an arginine and a glutamic acid that with a water molecule are thought to catalyze base excision (14). However, analysis of site-specific mutations has not revealed the chemistry of the recognition of, or the binding to, the substrate or of the catalytic mechanism (14–19).

A series of consecutive deletions in RA was constructed using a cDNA clone (13); the purpose was to identify the amino acids essential for function. The unexpected result is that a large number of the deletion mutants retain enzymatic activity.

METHODS

A *Bam*HI–*Hin*dIII restriction fragment of 860 base pairs (bp) containing the RA cDNA was excised from the plasmid pRAP229 (13) and subcloned into pIBI30 to form pRAIBI30. Deletions were made in the RA cDNA using synthetic oligodeoxynucleotides with 12 or 15 bases complementary to the 5′ and 3′ flanking regions (20). Plasmids were purified from single colonies of *Escherichia coli* strain MV1190 in cesium chloride gradients and the deletions were verified from the sequence of nucleotides in the DNA. The assay of the activity of RA mutants was modified from that described by May *et al.* (15). Linearized DNA from pRAIBI30 (250 ng) was transcribed in 20 μl of 40 mM Tris·HCl, pH 7.5/6 mM $MgCl_2$/2 mM spermidine/10 mM NaCl/10 mM dithiothreitol/1 mM (each) ATP, GTP, CTP, and UTP/1000 units of placental ribonuclease inhibitor per ml/1000 units of T3 RNA polymerase per ml. Transcripts (0.25 of the total) were translated in 45 μl of micrococcal nuclease-treated rabbit reticulocyte lysate supplemented with 20 μM each of 19 amino acids and 0.8 mCi of [^{35}S]methionine (1 Ci = 37 GBq). After incubation for 1 hr at 30°C, 5 μl was taken for SDS/PAGE and autoradiography. The reaction was stopped by addition of 160 μl of 0.5% SDS. The RNA was isolated by extraction with phenol and chloroform, precipitated with ethanol, and suspended in 20 μl of water. Sufficient aniline solution (1 M aniline in 2.8 M acetic acid) was added to half of the RNA (10 μl) to give a final concentration of 0.83 mM aniline and a pH of 4.5, and the sample was incubated at 40°C for 10 min and reprecipitated with ethanol. The aniline-treated and untreated RNAs (2.5 μg) were analyzed by electrophoresis in 3.5% polyacrylamide gels and stained with ethidium bromide (2, 3).

RESULTS AND DISCUSSION

Twenty-Amino Acid Deletions. A deletion map was constructed by removing 60 or 63 bp from the cDNA encoding RA; each deletion was adjacent to the preceding one. This systematic approach in which the deletions encompass the entire molecule avoids preconceptions or bias about essential structural elements or of the identity of the active site residues. Wild-type RA cDNA was cloned in pIBI30 downstream from a T3 promoter and the single-stranded form of this plasmid (pRAIBI30) was used as a template for oligonucleotide-directed mutagenesis. Wild-type and mutant cDNAs were transcribed *in vitro* with viral T3 RNA polymerase. Transcripts were used to program a rabbit reticulocyte lysate that had been depleted of endogenous mRNA (15). If a mutant is active, translation of the mRNA yields a protein that inactivates reticulocyte ribosomes; this is a suicide assay. The demise of the ribosomes was assessed by formation of a fragment of 460 nucleotides after treatment of extracted RNA with aniline, which causes scission of the phosphoribose backbone at the site of depurination—i.e., 3′ to A4324 in 28S rRNA (see Fig. 2, lane 3). This fragment was produced when RA (Fig. 2, lane 3) or wild-type cDNA transcript (Fig. 2, lane 4) was added to the lysate. However, depurination did not occur if no transcript (Fig. 2, lane 1) or if globin mRNA (Fig. 2, lane 2) was added. The mRNAs for each of these 13, and for all of the subsequent, deletion mutants were translated; the mutant polypeptides were identified by SDS/PAGE (data not shown). There was in general an inverse correlation between the amount of the mutant protein synthesized (judged by the intensity of the band on the SDS/PAGE radioautograph) and the activity; however,

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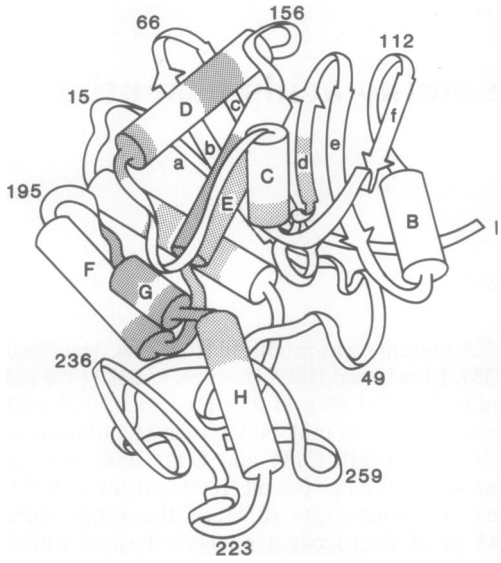
Abbreviation: RA, ricin A chain toxin.



a A
IFPKQYPIIN FTTAGATVQS YTNFIRAVRG RLTTGADVRH
20 40
b c
EIPVLPNRVG LPINQRFILV ELSNHAELSV TLALDVTNAY
60 80
d e B f
VVGYRAGNSA YFFHPDNQED AEAITHLFTD VQNRYTFAFG
100 120
C D
GNYDRLEQLA GNLRENIELG NGPLEEAISA LYYYSTGGTQ
140 160
E F
LPTLARSFII CIQMISEAAR FQYIEGEMRT RIRYNRRSAP
180 200
G H
DPSVITLENS WGRLSTAIQE SNQGAFASPI QLQRRNGSKF
220 240
SVYDVSILIP IIALMVYRCA PPPSSQF
260

FIG. 1. (*Left*) Ribbon representation of the α-carbon backbone of RA viewed approximately down the crystallographic *a* axis. The proposed active site cleft is located at the lower right on the front side. α-Helices are represented by barrels and identified by uppercase letters (A–H); β-strands are represented by arrows and identified by lowercase letters (a–f). The locations of the amino acids that cannot be deleted without loss of the ability to inactivate reticulocyte ribosomes are stippled. (*Right*) Primary sequence of RA (11–13). Amino acids that cannot be deleted without loss of the ability to inactivate reticulocyte ribosomes are underlined and the length of the line is proportional to the number of residues in the deletion. Amino acids in α-helices (A–H) and β-strands (a–f) are designated by coiled and jagged lines and the nine invariant residues in plant and bacterial toxins that have the same mechanism of action as RA are in boldface type (10).

there were too many exceptions for this to be a reliable means for quantifying the activity of the mutants. The 13 mutants lacked either 20 or 21 amino acids and every residue was absent from one, but no more than one, mutant (Table 1). Only the mutant lacking the NH_2-terminal 20 residues (Δ1–20) retained activity (Fig. 2, lane 6; lane 5 has the inactive mutant Δ21–40). These 20 residues span the back of the molecule (Figs. 1 and 3*A*) and include the first 3 amino acids of helix A. There are no surprises here: the 20 residues do not make an important contribution to the structure and do not impinge on the putative active site cleft (10).

Five-Amino Acid Deletions. The resolution of the deletion map was increased by constructing a set of 15- (or, in two instances, 9 and in one 18) bp deletions in the RA cDNA. These 50 deletions of 5 (in two mutants, 3, and in one, 6) amino acids encompassed the 247 residues whose omission had produced inactive mutants in the initial set (Table 1). Twenty-one of the 50 mutants retained enzymatic activity (Fig. 2, lanes 8–12 have several active mutants and lane 7 has an inactive mutant, Δ27–31). A total of 104 residues was deleted in the 21 active mutants. It is noteworthy that the 20 amino acids at the COOH terminus of the protein (residues 248–267) could not be removed in a single mutant (Δ248–267) without loss of activity but their omission was tolerated when they were deleted 5 residues at a time (Δ248–252, Δ253–257, Δ258–262, and Δ263–267). This is one of several examples of the ability of RA to compensate for small perturbations in the structure of a region but not for large ones.

Most of the 5-amino acid deletions that are permitted are on the exterior of the protein (Fig. 3*B*); however, several are in helices (A, B, and F) and some are in β-strands (b and e) (Fig. 1); none of the deletions impinges on the cleft.

Two-Amino Acid Deletions. The resolution of the deletion map was increased again by constructing 6-bp deletions in the RA cDNA. These 75 deletions of 2 amino acids encompassed the 143 residues whose omission had produced inactive mutants in the two previous series (Fig. 1 *Right* and Table 1). Fifty-two of the 75 mutants, having 98 different amino acids, retained enzymatic activity (Fig. 2, lanes 14–18 have several active mutants and an inactive mutant, Δ24–25, is in lane 13).

Many of the 2-amino acid deletions that are permitted are in the central core of the protein (Fig. 3*C*) and, more significantly, several (Tyr-80, Glu-208, and Asn-209) are at the bottom of the cleft where the presumptive catalytic site is located.

Control Experiments. Because of the extraordinary activity of RA (1) the experiments require scrupulous care with the controls. It is especially important to ensure that the mutant transcripts are not contaminated with wild-type mRNA. The smallest amount of contaminating wild-type DNA that would yield enough transcript to result in detectable depurination of 28S rRNA upon translation in the reticulocyte lysate was determined. Transcripts were prepared from mixtures having various ratios of the DNAs (a total of 250 ng) encoding an inactive mutant (Δ166–186) and the wild type; one-fourth of the total was translated in the lysate. Depurination was observed when the amount of wild-type DNA was 25 pg (only 0.04% of the total in the mixture) but not when it was 10 pg. Thus translation of transcripts from extremely small amounts of cDNA can catalyze sufficient depurination to be detected and this amount of cDNA would not be discerned on sequencing gels. Therefore, the quality of the results is conditioned by the purity of the mutant clones.

Single colonies were selected from among transformants and grown overnight; the DNA was isolated and the sequence of nucleotides was determined. Colonies with mutant DNA were streaked on agar plates so that single colonies could be selected easily. This is the colony purification procedure. These cells were grown, the DNA was purified, and the sequence of nucleotides was determined again. As a further precaution, the DNA that had been isolated after colony purification from a set of critical mutants (e.g., Δ1–20, Δ21–23, Δ62–66, Δ79–80, and Δ208–209) was used to transform cells and an additional cycle of selection and colony purification was carried out. None of these mutants lost their activity in the process.

The polymerase chain reaction was used to detect possible contamination with wild-type DNA in a representative set of mutants (Δ1–20, Δ42–46, Δ47–51, Δ62–66, Δ157–161, Δ187–191, Δ218–222). Pairs of oligodeoxynucleotides were synthesized; one hybridized to a sequence in mutant and wild-type

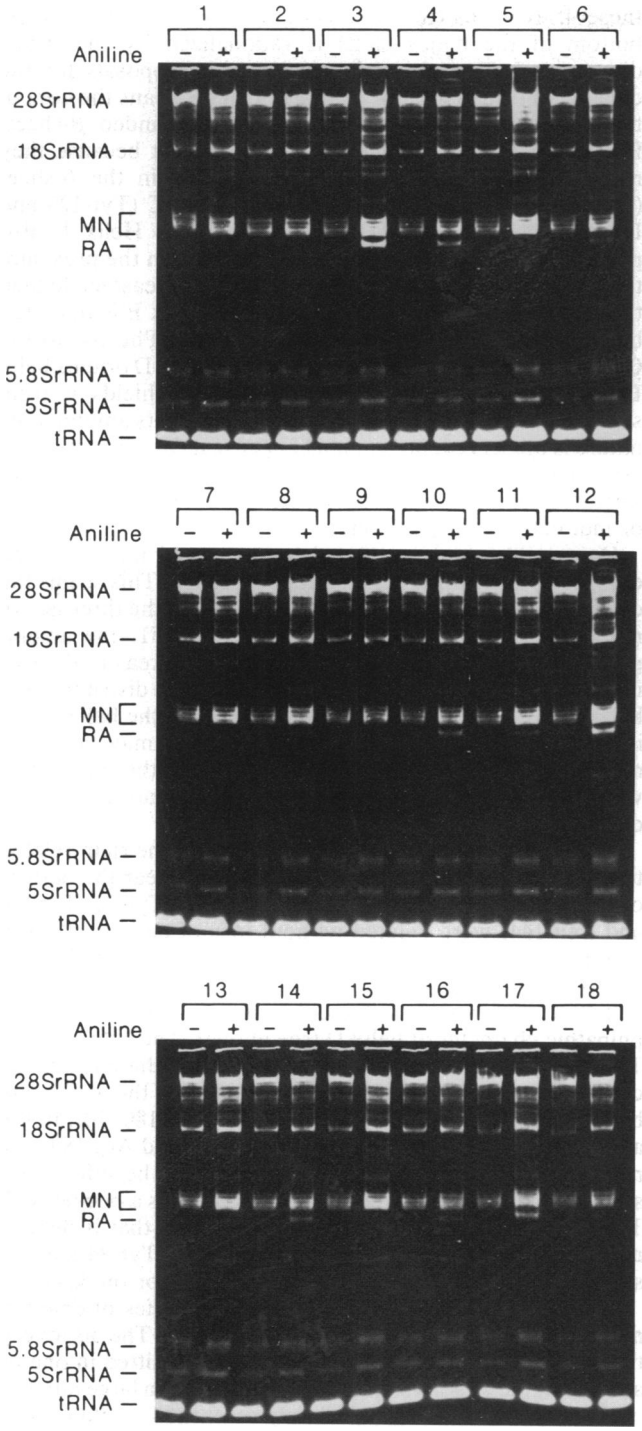


FIG. 2. Activity of RA mutants. The analysis was by electrophoresis in polyacrylamide gels of total RNA extracted from reticulocyte lysate translation reactions and treated with aniline (+) or left untreated (−). The lysate was programed (except in selected controls) with wild-type or mutant RA cDNA transcripts. Lane 1, no addition; lane 2, globin mRNA; lane 3, RA protein (7.4×10^{-5} M); lane 4, wild-type transcript; lane 5, Δ21–40; lane 6, Δ1–20; lane 7, Δ27–31; lane 8, Δ21–23; lane 9, Δ42–46; lane 10, Δ62–66; lane 11, Δ92–96; lane 12, Δ218–222; lane 13, Δ24–25; lane 14, Δ59–60; lane 15, Δ79–80; lane 16, Δ144–145; lane 17, Δ166–167; lane 18, Δ208–209. MN, oligoribonucleotide fragments that result from micrococcal nuclease treatment of the reticulocyte lysate; RA, fragment from the 3′ end of 28S rRNA formed by aniline treatment after depurination by RA.

DNA, the second only to the wild type. In control experiments with mixtures of wild-type and mutant DNA as little as 1 pg of the former could be detected after amplification. No wild-type DNA was detected in the reaction with the mutants specified above. Therefore, contamination is with <1 pg of wild-type DNA, much less than the amount (i.e., between 10 and 25 pg) required after transcription and translation to give detectable depurination. We conclude that the activity of the mutants cannot be accounted for by contamination with wild-type DNA.

Table 1. Activity of deletion mutants of RA

Mutant	ACT	Mutant	ACT	Mutant	ACT	Mutant	ACT
Δ1–20	A	Δ122–126	I	Δ39–40	A	Δ144–145	A
Δ21–40	I	Δ127–131	A	Δ40–41	A	Δ146–147	I
Δ41–60	I	Δ132–136	I	Δ57–58	A	Δ148–149	I
Δ61–81	I	Δ137–141	I	Δ59–60	A	Δ150–151	A
Δ82–102	I	Δ142–146	I	Δ60–61	A	Δ162–163	A
Δ103–123	I	Δ147–151	I	Δ67–68	A	Δ164–165	A
Δ124–144	I	Δ152–156	A	Δ69–70	A	Δ166–167	A
Δ145–165	I	Δ157–161	A	Δ71–72	A	Δ168–169	I
Δ166–186	I	Δ162–166	I	Δ73–74	A	Δ170–171	I
Δ187–207	I	Δ167–171	I	Δ75–76	A	Δ172–173	I
Δ208–227	I	Δ172–176	I	Δ77–78	A	Δ174–175	I
Δ228–247	I	Δ177–181	I	Δ79–80	A	Δ176–177	I
Δ248–267	I	Δ182–186	I	Δ81–82	I	Δ178–179	I
		Δ187–191	A	Δ83–84	I	Δ180–181	I
Δ21–23	A	Δ192–197	A	Δ85–86	A	Δ182–183	I
Δ24–26	I	Δ198–202	I	Δ87–88	A	Δ184–185	I
Δ27–31	I	Δ203–207	I	Δ89–90	A	Δ185–186	A
Δ32–36	A	Δ208–212	I	Δ90–91	A	Δ198–199	A
Δ37–41	I	Δ213–217	I	Δ97–98	A	Δ200–201	I
Δ42–46	A	Δ218–222	A	Δ99–100	A	Δ202–203	I
Δ47–51	A	Δ223–227	A	Δ100–101	A	Δ204–205	I
Δ52–56	A	Δ228–232	A	Δ112–113	A	Δ206–207	I
Δ57–61	I	Δ233–237	I	Δ114–115	A	Δ208–209	A
Δ62–66	A	Δ238–242	I	Δ116–117	A	Δ210–211	I
Δ67–71	I	Δ243–247	I	Δ118–119	A	Δ212–213	I
Δ72–76	I	Δ248–252	A	Δ120–121	A	Δ214–215	A
Δ77–81	I	Δ253–257	A	Δ122–123	I	Δ216–217	A
Δ82–86	I	Δ258–262	A	Δ124–125	A	Δ233–234	A
Δ87–91	I	Δ263–267	A	Δ125–126	A	Δ235–236	A
Δ92–96	A			Δ132–133	A	Δ237–238	A
Δ97–101	I	Δ24–25	I	Δ134–135	A	Δ239–240	A
Δ102–106	A	Δ26–27	A	Δ136–137	A	Δ241–242	A
Δ107–111	A	Δ28–29	I	Δ138–139	A	Δ243–244	A
Δ112–116	I	Δ30–31	A	Δ140–141	I	Δ245–246	A
Δ117–121	I	Δ37–38	A	Δ142–143	A	Δ246–247	A

The mutants are designated by the amino acids that were deleted; the mutants are separated (by a space) into groups of 20-, 5-, or 2-amino acid deletions (see text). Activity (ACT) is the ability to depurinate A4324 in 28S rRNA in reticulocyte ribosomes (compare Fig. 2). A, active; I, inactive.

The Assay. In the assay only the retention of residual detectable activity of the mutants was scored; the amount of residual activity has not been quantitated. A decrease of 10^{-4} but not of 10^{-5} would be scored active. This was determined by serial dilution of wild-type transcripts with transcripts encoding an inactive mutant. Nonetheless, what is remarkable is that so many of these mutants still catalyze a reaction that requires the recognition of a single nucleotide from among 7000 in a very large substrate; this is remarkable even if the activity of some of the mutants is reduced by as much as four orders of magnitude. Indeed, the principal advantage of the assay is its sensitivity, which makes it possible to identify the amino acids essential for the structure and for the function of the enzyme.

Deletions That Are Tolerated. Seventy-four of 138 mutants in RA retained activity; in these, 222 of the 267 amino acids were deleted (Table 1 and Fig. 1). As expected, most of the deletions that were permitted were of amino acids on the

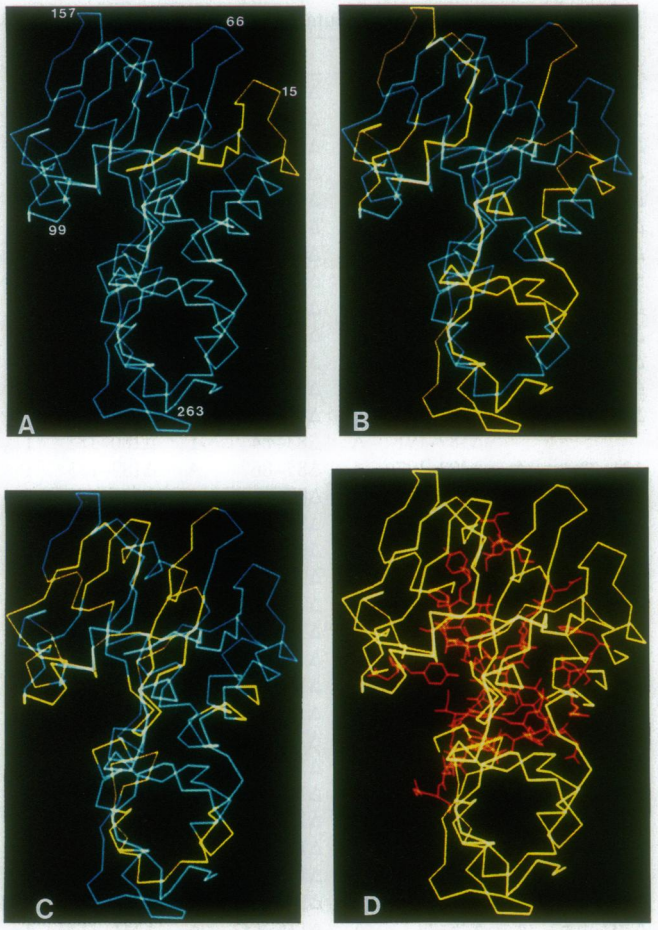


FIG. 3. Three-dimensional structure of RA obtained with the program INSIGHT (21) on an Evans and Sutherland PS300 graphics system using the coordinates (8) from x-ray defraction of the heterodimer. The view of the α-carbon backbones is with the cleft on the left side. The 20-amino acid deletions (*A*), the 5-amino acid deletions (*B*), and the 2-amino acid deletions (*C*) that are tolerated are in yellow. In *D*, the amino acids whose deletion was not tolerated are in red.

exterior of the protein (22–25); however, many deletions had hydrophobic residues that are at least partially inaccessible to solvent (26). Moreover, 16 of the 36 residues that have no access to solvent could be removed. These 16 are in regions of the core further removed from the bottom of the cleft than the 20 others. The average distance from the Cα of Glu-177 (which is at the putative active site) to the 16 residues whose omission is tolerated is 15.1 Å, whereas that of the 20 that cannot be removed is 9.1 Å. No doubt, omission of any one of these 36 residues perturbs the structure; but omission of those more distant may permit, albeit perhaps transiently, a productive structure.

Alkaline phosphatase retained enzymatic activity when short amino acid sequences were inserted into several of its exterior loops; however, no insertions were tolerated within α-helices or β-strands (27). On the other hand, insertion of glycine or alanine into regions of secondary structure in staphylococcal nuclease usually did not inactivate the enzyme, although it had more effect on stability than similar insertions in unstructured regions (28). The results with RA are distinctly different. Deletions, of at least a portion of all eight of the α-helices and of all six of the β-strands, were permitted in one or another of the 74 active mutants (Fig. 1).

Deletions That Are Not Tolerated. The 45 amino acids that cannot be deleted without loss of activity (Fig. 3*D*) form a protein within a protein; the appearance is of the globular RA pregnant with the globular catalytic module. The location of these 45 residues (they are congregated at and behind the bottom of the major cleft) is rationalized by the three-dimensional structure and accords with proposals for the catalytic mechanism. For example, helix E spans the core of the protein and is enfolded by the six-stranded β-sheet. Phe-168 and Ile-172 in helix E are important because they make hydrophobic contacts with residues in the β-sheet (Tyr-84, Phe-93, and Phe-117) and in helix C (Tyr-123 and Leu-126), thereby stabilizing the core (10). Helix E also positions the invariant Glu-177 and Arg-180 in the presumptive catalytic site at the base of the cleft. It is reasonable then that the deletion of the NH_2 terminus of helix E is tolerated but that the omission of the residues from Phe-168 to the COOH terminus is not. The amphipathic helix D orients helix E through local hydrophobic forces and shields it from solvent. Helix D can be shortened, as long as its amphipathic nature is preserved, presumably because it can still serve this essential structural function. The deletions that were not tolerated contain the active site residues or influence, directly or indirectly, their position.

One might anticipate that deletions could inactivate the enzyme by disrupting amphipathic α-helices. This seems the case with deletions in helix D (Fig. 1 *Left*). In the three active mutants, Δ142–143, Δ144–145, and Δ150–151, the amphipathic nature of the helix is maintained, whereas in the two inactive mutants, Δ146–147 and Δ148–149, the distribution of hydrophobic and hydrophilic residues about the helical axis is altered. Thus, for some mutants activity may be determined by the framing of the deletion—i.e., the position at which the deletion starts and ends—rather than the identity of the deleted amino acids *per se*.

The nine amino acids that are invariant in the ricin-related toxins (compare Fig. 1 *Right*) are clustered near the bottom of the major cleft. Three invariant residues (Tyr-21, Tyr-80, and Leu-144) were deleted without loss of activity. This is unexpected and not easy to rationalize. Leu-144 is in helix D relatively far from the cleft (its Cα is 13.8 Å from that of Glu-177); it may ordinarily serve only to preserve the amphipathic character of helix D (see above). The side chain of Tyr-21 is directed into the core and toward the base of the cleft and appears to maintain the structure of the active site by securing the positions of Glu-177 and Arg-180 (10). In the absence of Tyr-21, the positions of Glu-177 and Arg-180 may not be optimal but their polar side chains may be sufficiently solvated to give residual activity. Tyr-80 forms a pocket with Tyr-123 above the cleft where the adenosine that is depurinated is thought to bind; on Tyr-80 removal Tyr-84 may be sufficiently close to Tyr-123 to compensate for the loss.

Conclusions. This is the most extensive series of deletion mutants of an enzyme to have been evaluated. The discovery that 83% of the amino acids in RA can be omitted in one or another of the 74 active mutants indicates that a large number of the residues is neither absolutely essential for folding into an effective conformation nor for catalysis. We recognize that in any single mutant the deletion that was tolerated was short—only in one instance as long as 20 residues and in the others either 5 or 2 amino acids. Nonetheless, it is surprising that residues that are hydrophobic and buried in the interior of the protein, or that are part of α-helices or of β-strands, can be deleted without loss of activity. This unexpected outcome reinforces the conviction that deletion analysis is a valuable procedure for defining the minimal cohort of amino acids necessary for substrate recognition and for catalysis. In addition, the results have bearing on the evolution of proteins, and the mutants provide a data set that may allow formulation or evaluation of theories of the mechanism by which polypeptides fold into their three-dimensional conformation.

The findings indicate that RA has a great capacity for compensatory change in structure and, hence, a great ability

to preserve function; in short, the protein displays considerable plasticity. Can these observations be generalized? There is a large body of experiments (27–40) that indicate that other proteins are plastic. No single experimental example is so dramatic as that provided by nature. The structures of 226 different globins are known; the number of residues varies from 132 to 157 with only 102 sites common to all; there are only two invariant residues and the identities of some pairs of globins are no more than 16% (29, 32). Yet they have similar three-dimensional structures and obviously they all preserve function. If we consider the 226 globins to comprise a single genetic set then the protein has tolerated an enormous number of deletions, insertions, and substitutions. There is additional evidence that alternate amino acid sequences can fold into similar or the same three-dimensional structure (37, 38). Random combinatorial mutagenesis of residues in the hydrophobic core of λ repressor indicates that hydrophobicity is a crucial characteristic of the core but many different hydrophobic or apolar sequences will adopt an acceptable structure (39). The effects of mutations on the three-dimensional structure and on the stability and function of T4 lysozyme (34, 35) have revealed this enzyme to be surprisingly tolerant of amino acid substitutions. Insertions of additional amino acids (usually one or two residues) can be accommodated at many sites in proteins without intolerable perturbations in structure and without great loss of activity (27–31, 34, 35). The common theme is that there are inviolable features of protein structure but within limits there is a good deal of latitude in what is permitted. What distinguishes the set of RA mutants is that it employs deletions rather than substitutions or insertions and, more importantly, that it is systematic and extensive.

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