Radiation inactivation of ricin occurs with transfer of destructive energy across a disulfide bridge

(target analysis)

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ABSTRACT   The ionizing radiation sensitivity of ricin, a disulfide-linked heterodimeric protein, was studied as a model to determine the ability of disulfide bonds to transmit destructive energy. The radiation-dependent loss of A chain enzymatic activity after irradiation of either intact ricin or ricin in which the interchain disulfide bond was disrupted gave target sizes corresponding to the molecular size of dimeric ricin or monomeric A chain, respectively. These results clearly show that a disulfide bond can transmit destructive energy between protein subunits.

Ionizing radiation damages molecular structure. Chemical bonds in macromolecules are broken and new ones form, leading to changes in physical, chemical, and biological properties. In a solid, changes in irradiated matter are due to the direct action of radiation on the molecules in question. The initial interaction is a primary ionization of an orbital electron in which large amounts of energy [about 1500 kcal/mol (1 kcal = 4.18 kJ)] are transferred to macromolecules. The subsequent events are only partially understood, but they clearly involve the transfer of energy to other regions of the molecule. In addition to the theoretical interest in these processes, this subject has attracted considerable research because high-energy radiation inactivation can be used to measure the functional size of biologically active macromolecules (see refs. 1 and 2 for reviews). The method is conceptually simple: if a polypeptide chain is "hit" it is completely inactivated; if a "hit" does not occur on a polypeptide it is not affected. By exposing biologically active molecules to increasing doses of radiation and measuring the surviving activity, the functional size of the structure(s) responsible for the activity can be determined by target analysis.

The effects of ionizing radiation depend on the structural requirements for transfer of the energy deposited by a primary ionization. Evidence has been presented that radiation damage in synthetic polymers occurs at considerable distance from the primary ionization (3, 4) and throughout a polypeptide chain no matter where the original hit occurs (5). These data provide no insight into the radiation sensitivity of disulfide bonds, which often play a critical role in the structure and function of proteins. In this report the disulfide-linked heterodimeric protein ricin was studied as a model to determine the ability of disulfide bonds to transmit destructive energy.

Ricin is a toxic plant lectin that consists of two polypeptide chains, A and B, of known amino acid sequence (6, 7) that are joined by a single disulfide bond (Fig. 1). The B chain is a lectin that binds the toxin to cells and facilitates the entry of the A chain into the cytosol. The A chain is an enzyme capable of inactivating the protein-synthesizing capacity of
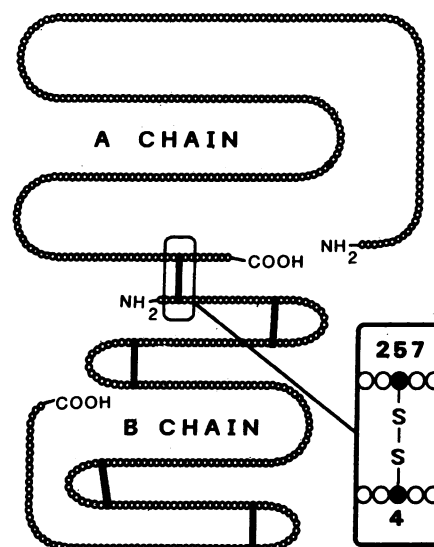
FIG. 1.   Structure of ricin from primary sequence analysis (6, 7).

eukaryotic ribosomes. The enzymatic assay for A chain activity was used to determine if destructive energy could be transferred to the A chain after a primary ionization on the B chain. If transfer occurs, irradiation of intact ricin should give a target size corresponding to the molecular size of dimeric ricin, but if the A and B chains are energetically isolated with regard to ionizing radiation, the expected target size should correspond to the size of the monomeric A chain. In control experiments the radiation sensitivity of A chain enzymatic activity was measured after irradiation of either isolated A chain or ricin that was reduced with mercaptoethanol to form A and B chains that were not covalently associated.

MATERIALS AND METHODS

Purified ricin was a generous gift from Daniel Cawley (Department Biological Chemistry, University of California, Los Angeles), and isolated ricin A chain was obtained from Worthington. Reduced ricin was prepared by incubating ricin (0.1 mg/ml) in Tris·HCl (20 mM, pH 8.0) containing 2-mercaptoethanol (120 mM) for 45 min at room temperature. Irradiations were carried out at −135°C with a 13-MeV electron beam produced by a linear accelerator as described elsewhere (8) on the following preparations sealed in 2-ml glass ampules: 1.0 μg of intact ricin in 0.3 ml of Tris·HCl (20 mM, pH 7.4) containing transferrin at 1.0 mg/ml as a carrier protein; 1.0 μg of reduced ricin in 0.3 ml of Tris·HCl (20 mM, pH 7.4) containing transferrin at 1.0 mg/ml and 120 mM mercaptoethanol; and 0.1 ml of isolated ricin A chain (0.15 mg/ml) prepared by mixing three parts of the commercial

preparation with seven parts of 0.1 M NaCl containing 20% (vol/vol) glycerol as a cryoprotectant.

The enzymatic activity associated with ricin was determined by measuring its ability to inhibit a rabbit reticulocyte *in vitro* translation system that was a modification of the method of Pelham and Jackson (9). Ricin solutions were diluted to the desired concentration in Tris·HCl (30 mM, pH 7.4) containing transferrin (1.0 mg/ml) and mercaptoethanol (100 mM) and preincubated for 30 min at 30°C. The solution containing ricin (10 μl) was added to a reaction mixture (30 μl) containing 40% reticulocyte lysate, amino acids (0.2 mM each of 19 amino acids but no phenylalanine), phosphocreatine (20 mM), creatine kinase (38 μg/ml), hemin (10 μM), potassium acetate (240 mM), Tris·HCl (4 mM, pH 8.0), and dithiothreitol (10.6 mM). The ricin/lysate mixture was incubated for 20 min at 30°C, then translation was started by adding a solution (20 μl) containing L-[*ring*-2,6-³H]phenylalanine (20 μM, 1.6 μCi, New England Nuclear; (1 Ci = 37 GBq) and of magnesium acetate (7.2 mM). After a 35-min incubation at 30°C, aliquots (15 μl) of the reaction mixture were spotted onto filter paper and placed in 10% trichloroacetic acid. The filter papers were boiled for 10 min, rinsed with 10% trichloroacetic acid, ethanol, and diethyl ether, then digested with 90% Protosol (New England Nuclear) before liquid scintillation counting. Approximately 10,000 cpm was incorporated in the absence of ricin and approximately 1500 cpm was incorporated when translation was maximally inhibited with ricin (1.5 ng per reaction).

Radiation dose-dependent inactivation of ricin activity was measured by comparing the translation-inhibiting activity in the irradiated ricin samples with a standard curve constructed by plotting translation activity versus concentration of unirradiated reduced ricin or ricin A chain. In a typical assay, translation was reduced to 37% of the control by approximately 50 pg of reduced ricin per reaction. The logarithm of the fractional surviving activity was a linear function of radiation dose [in megarads (1 rad = 0.01 gray)] and the slope of this function, K, was determined by least-squares regression analysis constrained to the normalized control value of 1.0 at zero radiation dose. The molecular weight of the functional unit was calculated from the relationship $M_r = 6.4 \times 10^5\, S_t K$, in which S_t is a temperature factor with a value of 2.8 for −135°C (10).

RESULTS

Frozen solutions containing intact ricin, or ricin in which the disulfide bond between the A and B chains had been reduced, or isolated ricin A chain were exposed to increasing doses of high-energy electrons (8). The radiation-dependent inactivation of enzymatic activity in each preparation then was assayed by determining the ability of the irradiated sample to inhibit protein synthesis in a rabbit reticulocyte *in vitro* translation system. Since A chain activity is maximal after reduction of the interchain disulfide bond (11), samples containing intact ricin were reduced with mercaptoethanol after irradiation but prior to the assay.

Irradiation of both reduced ricin and isolated ricin A chain resulted in monoexponential inactivation of enzymatic activity; typical experiments are shown in Fig. 2. Target analysis revealed average target sizes of 28 ± 3 kDa and 31 ± 1 kDa, respectively (Table 1); both of these are in good agreement with the known molecular size of the A chain (Table 1). The experiment using isolated A chain can be considered an internal control, and irradition of the reduced ricin dimer provides insights into the role of noncovalent bonds in energy transfer. Reduction of intact ricin under these conditions did not result in dissociation of the two subunits as assayed by chromatography on a Bio-Rad Bio-Gel P-100 column (12) even though the interchain disulfide bond was reduced;
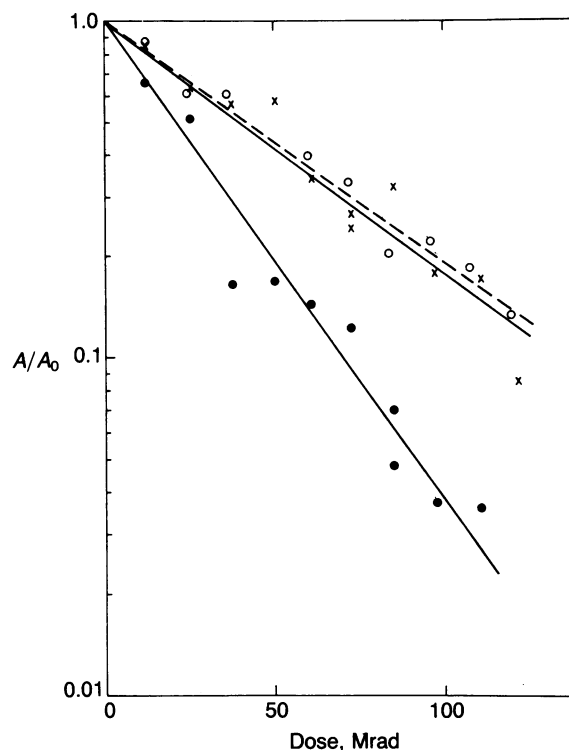

FIG. 2. Radiation-induced loss of enzymatic activity of ricin. Frozen samples of intact ricin (●), mercaptoethanol-reduced ricin (○), and isolated ricin A chain (×) were irradiated and then assayed for the ability to inhibit *in vitro* protein synthesis. Each measurement of activity (A) at a given radiation dose was normalized to the activity (A_0) in the unirradiated control sample. Each data point is the average of duplicate determinations. In these representative experiments the inactivation curves for intact ricin, reduced ricin, and ricin A chain correspond to target sizes of 58, 29, and 31 kDa, respectively.

complete reduction of the bond was confirmed by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (12). Thus, some of the noncovalent interactions between the two chains remain under the conditions in which reduced ricin was irradiated. Since the target size of reduced ricin matches the size of the monomeric A chain, these noncovalent interactions between A and B chains of ricin were not adequate to transfer destructive energy from the B to the A chain.

Irradiation of intact ricin caused a monoexponential inactivation of protein synthesis inhibitory activity (Fig. 2). The inactivation curves from several experiments corresponded to an average target size of 63 ± 4 kDa, in good agreement with the molecular size of dimeric ricin (Table 1). This is so even though the measured biochemical activity resides only in the A chain and the postirradiation assay was performed in

Table 1. Molecular size of ricin

	Molecular size, Da	
Ricin	From sequence analysis	From target size analysis
Intact	57,921	63,000 ± 4000 (3)
2-Mercaptoethanol-treated	57,923	28,000 ± 3000 (2)
A chain	28,376	31,000 ± 1000 (2)

The molecular size of the protein portion of the glycoproteins ricin and ricin A chain is from reported primary sequence data (6, 7). The target sizes were determined by the methods described in the text. The number of separate irradiation experiments is shown in parentheses and the values are the mean ± SD.

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the presence of mercaptoethanol, which disrupts the disulfide bond connecting the two chains.

DISCUSSION

The results indicate that a primary ionization on either the A or the B chain of intact ricin was sufficient to inactivate the A chain. Possible conduits for the transfer of destructive energy from the B chain to the A chain are either the connecting disulfide bond or the noncovalent bonds that join the two chains. The former seems more probable because studies with other enzymes such as glutamate dehydrogenase (12) and other enzymes (2) clearly have shown that destructive energy does not pass between the subunits even if they are closely associated by noncovalent bonds. However, the most convincing argument that the disulfide bond was the conduit is the fact that destructive energy did not pass from the B chain to the A chain when the interchain disulfide bond of ricin was reduced prior to irradiation. The reduction of the interchain disulfide bond in ricin probably disrupts many of the intrachain disulfide bonds of the B chain and thus causes conformational changes. Nonetheless, under these reducing conditions the A and B chains do not dissociate, thus indicating that the noncovalent interactions between the two chains are at least partially intact.

The clear conclusion from these studies is that the interchain disulfide bond between the two subunits of ricin can transmit a significant amount of energy deposited by ionizing radiation. These results are not unexpected because the S—S bond is chemically similar to C—C and C—N bonds in proteins, which have previously been shown to conduct destructive energy (2–4, 13). The radiation sensitivity of disulfide bonds should be a chemical property of the bond rather than a property of the secondary or tertiary structure of the polymer. It is likely therefore that the energy-transferring properties of the interchain disulfide bond of ricin shown here is representative of disulfide bonds in other proteins and nonbiological polymers.

1. Pollard, E. C., Guild, W. R., Hutchinson, F. & Setlow, R. B. (1955) *Prog. Biophys.* **5,** 72–108.
2. Kempner, E. S. & Schlegel, W. (1979) *Anal. Biochem.* **92,** 2–10.
3. Dole, M. & Keeling, C. D. (1953) *J. Am. Chem. Soc.* **75,** 6082–6083.
4. Alexander, P. & Charlesby, A. (1954) *Nature (London)* **173,** 578–579.
5. Aronson, D. L. & Preiss, J. W. (1962) *Rad. Res.* **16,** 138–143.
6. Yoshitake, S., Funatsu, G. & Funatsu, M. (1978) *Agric. Biol. Chem.* **42,** 1267–1274.
7. Funatsu, G., Kimura, M. & Funatsu, M. (1979) *Agric. Biol. Chem.* **42,** 2221–2224.
8. Harmon, J. T., Nielsen, T. B. & Kempner, E. S. (1985) *Methods Enzymol.* **117,** in press.
9. Pelham, H. R. B. & Jackson, R. J. (1976) *Eur. J. Biochem.* **67,** 247–256.
10. Kempner, E. S. & Haigler, H. T. (1982) *J. Biol. Chem.* **257,** 13297–13299.
11. Olsnes, S. & Pihl, A. (1978) *Trends Biochem. Sci.* **3,** 7–10.
12. Lappi, D. A., Kapmeyer, W., Beglau, J. M. & Kaplan, N. O. (1978) *Proc. Natl. Acad. Sci. USA* **75,** 1096–1100.
13. Kempner, E. S. & Miller, J. H. (1983) *Science* **222,** 586–589.