Cross-linking of the Two Constituent Polypeptide Chains of Ricin D with N,N’-o-Phenylenedimaleimide

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Reaction of the reduced ricin D with N,N’-o-phenylenedimaleimide produced a cross-linked ricin D which could not be split into its constituent polypeptide chains with reducing reagents. This cross-linked ricin D was as stable as native ricin D and retained the full cytoagglutinating activity, while the toxicity to mice and cultured cells was remarkably decreased.

The cross-linked ricin D was incorporated into cells in the same manner as native ricin D, but was not degraded by incubation with the lysate of rabbit reticulocytes. From these results, it was inferred that ricin D is split into its constituent polypeptide chains by reduction of the inter-chain disulfide bond in cytoplasm and thus its toxic action is elicited.

Ricin D, a highly purified toxic lectin present in castor bean seeds (Ricinus communis), consists of an Ile-chain, which inhibits eukaryotic cell-free protein synthesis, and an Ala-chain, which agglutinates mammalian cells. In a previous paper, we have shown that these two constituent polypeptide chains are linked with a single disulfide bond cleavable with reducing reagents such as 2-mercaptoethanol without denaturation of ricin D and suggested that this inter-chain disulfide bond plays an important role in the elicitation of the toxic action on the basis of the fact that the toxicity of ricin D is destroyed by splitting this inter-chain disulfide bond.

In order to investigate how the inter-chain disulfide bond participates in the toxic action of ricin D, we prepared a cross-linked ricin D, uncleavable by reduction, using N,N’-o-phenylenedimaleimide, a bifunctional reagent for neighboring sulfhydryl groups.

The present paper describes the preparation procedure and some properties of the cross-linked ricin D.

MATERIALS AND METHODS

Materials. Ricin D and its Ala-chain were prepared as described earlier. N,N’-o-Phenylenedimaleimide, abbreviated as o-Ph(Mal)$_2$, was obtained from Aldrich Chemical Co. (U.S.A.). Trypsin, chymotrypsin and nagarse were purchased from Sigma Co., Worthington Biochemical Co. and Nagase Co., respectively.

Preparation of cross-linked ricin D. 2-Mercaptoethanol (0.1 ml) was added to 10 ml of ricin solution (25 mg/ml of 5 mM Tris-HCl buffer, pH 8.0). After 2 hr at room temperature, the reaction mixture was applied to a Sephadex G-25 column (3 x 40 cm) which was previously washed with 5 mM phosphate buffer, pH 5.5, and eluted with the same buffer. By this procedure, 2-mercaptoethanol used for reduction was removed and 5 mM Tris-HCl buffer of pH 8.0, in which ricin D was dissolved, was replaced with 5 mM phosphate buffer, pH 5.5. Two hundred milligrams of crystal o-Ph(Mal)$_2$ (approximately 200 times mole of ricin D) was added to the reduced ricin D solution and the reaction mixture was allowed to stand for 2 hr with stirring in an ice-bath. After removal of unreacted crystal o-Ph(Mal)$_2$ on a glass filter, the protein was precipitated with ammonium sulfate.

Preparation of cross-linked cysteine. Cross-linked cysteine was prepared as above. Thirty-six mg of o-Ph(Mal)$_2$ was added to the cysteine solution (0.6 mg/ml of 5 mM phosphate buffer, pH 5.5), and stood with stirring for 2 hr in an ice-bath.

DEAE-cellulose column chromatography. The protein precipitate was dissolved in deionized water, dialyzed first against deionized water and then against 5 mM
Tris-HCl buffer, pH 8.5. The protein solution was applied to a DEAE-cellulose column (2 x 40 cm) previously equilibrated with 5 mM Tris-HCl buffer, pH 8.5. After washing the column with the same buffer containing 18 mM NaCl, the adsorbed protein was eluted by a linear gradient of NaCl from 18 mM to 100 mM in the same buffer.

Disc-electrophoresis. Disc-electrophoresis was performed at pH 8.3 using 7.5% gel by the method of Orstcin. The protein in the gel was detected by staining with amide black 10 B.

SDS-polyacrylamide gel electrophoresis. The molecular size of the protein was determined by SDS-polyacrylamide gel electrophoresis according to Weber and Osborn. Ten microliters of 20 mM phosphate buffer, pH 7.2, containing 50% glycerol and 2% SDS was added to 10 µl of the protein solution. After incubation at room temperature overnight, the protein solution was subjected to electrophoresis.

Amino acid analysis. The protein was hydrolyzed with 5.7 N HCl containing 0.05% 2-mercaptoethanol in an evacuated sealed tube at 108°C for 24 hr. The amino acid was analyzed with a JEOl amino acid analyzer (Type JLC-6AH). Cross-linked cysteine in the cross-linked ricin D was determined as 2-amino-2-carboxyethylmercapto-succinic acid after hydrolysis with 5.7 N HCl.

Determination of pH and heat stabilities. One ml of protein solution (0.03% in 5 mM phosphate buffer, pH 7.0) was dialyzed against 100 ml of 0.1 M KCl-HCl buffer, pH 11.0, at 4°C for 24 hr. After dialysis against a large amount of 5 mM phosphate buffer, pH 7.0, containing 0.9% NaCl, cytoagglutinating activity was determined. For determination of heat stability, on the other hand, the protein solution (0.03% in 5 mM phosphate buffer, pH 7.0, containing 0.9% NaCl) was heated at 50°C for 1 hr. After cooling in an ice-bath, cytoagglutinating activity was determined.

Proteolytic digestion. Digestion of the protein was performed using trypsin, chymotrypsin and nagarse. Ten microliters of the protease solution (1% in 5 mM Tris-HCl buffer, pH 8.0) was added to 250 µl of the protein solution (2% in 50 mM Tris-HCl buffer, pH 8.0). After incubation at room temperature for 1 hr, the digest was lyophilized.

Cytoagglutinating activity. Cytoagglutinating activity was determined with sarcoma 180 ascites (SA) tumor cells as previously described. To 0.2 ml of SA cell suspension (1 x 10⁶ cells/ml) were added 0.2 ml portions of the protein solution at different concentrations. After incubation for 30 min at room temperature, the agglutinating activity was measured microscopically.

Toxicity. Toxicity of the protein toward mice was determined with pure-bred male mice (ddN) as previously described. Increasing amounts of the protein were injected intraperitoneally into groups of five mice, weighing 25 ± 2 g, and the LD₅₀ dose was calculated for deaths occurring within 48 hr.

Inhibitory activity of the protein toward the growth of the cultured cells was determined with XC and NIH3T3 cells as described previously. Tryptsin treated cells (3.5 x 10⁶) were plated on to 35 mm Falcon plastic dishes in 1 ml of Eagle's MEM supplemented with 10% fetal calf serum and 0.1 µg or 1 µg of ricin D, cross-linked ricin D or the isolated Ala-chain was added. After incubation for 2 hr at 37°C, the medium was removed and replaced with fresh medium. After incubation for 24 hr at 37°C, the cells were removed from the dishes by treatment with 0.1% trypsin solution and counted after staining with trypan blue dye with a hemacytometer.

Labeling with ¹²⁵I. Labeling of the protein with ¹²⁵I was carried out according to Hunter. Fifty microliters of 5 mM phosphate buffer, pH 7.0, containing 50 µg of the protein, was added to 110 µl of 0.5 M phosphate buffer, pH 7.0, containing 1 mCi of carrier-free Na¹²⁵I (NEN) and then 50 µg of chloramine-T solution (100 µg) was added. After 1 min at room temperature with stirring, 100 µl (500 µg) of sodium metabisulfite was added to stop the reaction. For the purification of ¹²⁵I-labeled protein, the reaction mixture was applied to a Sephadex G-200 column (1.5 x 22 cm) previously washed with 5 mM phosphate buffer-0.9% NaCl, pH 7.2 (PBS solution), containing 1% bovine serum albumin and eluted with PBS solution.

Irreversible binding of ¹²⁵I-labeled protein to cells. 0.3 ml of ¹²⁵I-labeled native ricin D solution (7 µg, specific activity: 2.4 x 10⁶ cpm/µg) or the cross-linked ricin D solution (8.8 µg, specific activity: 1.4 x 10⁶ cpm/µg) was added to 1.5 ml of SA cell suspension (1 x 10⁶ cells/ml of PBS). After incubation for various times at 0°C or 37°C, 0.3 ml of the reaction mixture was poured into 3 ml of ice-cold PBS solution containing 0.1 M lactose and stood for 10 min at 0°C. The cells were washed three times with the same solution by centrifugation (3000 rpm, 1 min). The radioactivity of final cell pellet was counted in a Aloka auto-well gamma system (JDC-751).

Preparation of crude rabbit reticulocyte lysate. Crude rabbit reticulocyte lysate was prepared according to the method of Woodward et al. The reticulocytes obtained from an anematized rabbit were lysed by suspension in an equal volume of distilled water and allowed to stand at 0°C for 5 min. After removal of
unlysed cells by centrifugation, the lysate was stored in aliquots of 3 ml in dry-ice acetone.

In order to remove the materials of low molecular weight from the crude rabbit reticulocyte lysate, the lysate (1 ml) was gel-filtered on a column (1 x 15 cm) of Sephadex G-25 in PBS, and the fraction having hemoglobin color eluted in the void volume was collected.

**Polyacrylamide gel electrophoresis of \( ^{125}\text{I}-\text{labeled protein} \).** After incubation of \( ^{125}\text{I}-\text{labeled protein} \) in the crude rabbit reticulocyte lysate at 37°C for 30 min or 2 hr, 10 µl of the reaction mixture was subjected to SDS-polyacrylamide gel electrophoresis. After electrophoresis, the gel was quickly removed from the tube, frozen with dry-ice and uniformly sliced into pieces of 1 mm thickness. The radioactivity of each gel-slice was measured with a Aloka auto-well gamma system (JDC-751).

**RESULTS**

**Purification of cross-linked ricin D**

The elution profile of the reaction mixture of cross-linked ricin D on DEAE-cellulose column chromatography is shown in Fig. 1; three fractions—F-1, F-2 and F-3—were obtained. As shown in Fig. 2, fraction F-1 gave a single band identical to that of native ricin D on disc-electrophoresis and gave two bands after treatment with 2-mercaptoethanol on SDS-polyacrylamide gel electrophoresis. On the other hand, fraction F-2 gave a single band of faster mobility than native ricin D on disc-electrophoresis and was not split into two chains by treatment with 2-mercaptoethanol. Fraction F-3 was not split into two chains by treatment with 2-mercaptoethanol, but gave two bands on disc-electrophoresis. From these results, it was revealed that fraction F-1 is native ricin D and fractions F-2 and F-3 are cross-linked ricin D. Yields of fractions F-1, F-2 and F-3 were approximately 6%, 80% and 11%, respectively.

**Amino acid composition of fraction F-2**

The amino acid composition of fraction F-2 is given in Table I compared with that of native ricin D. Their compositions are very close to each other and no decrease of lysine was found in fraction F-2. As shown in Fig.3, 2-amino-2-carboxyethylmercaptosuccinic acid was found in the acid hydrolysate of fraction F-2 and its amount was estimated to be approximately two moles per one mole of ricin D. This result clearly indicates that the cross-linking reaction occurred only with the cysteine...
After hydrolysis of the cross-linked ricin D with HCl for 24hr at 108°C, the hydrolysate was analyzed with a JEOL amino acid analyzer.

Table I. Comparison of Amino Acid Compositions of Native Ricin D and Fraction F-2, See Fig. 1.

<table>
<thead>
<tr>
<th>Amino acids</th>
<th>Ricin D</th>
<th>Fraction F-2</th>
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<tr>
<td>Asp</td>
<td>64.0a</td>
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<tr>
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</tr>
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</tr>
<tr>
<td>Arg</td>
<td>34.5</td>
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</table>

2-Amino-2-carboxymethylmercaptoproline acid 0 2.2

Digestibility of cross-linked ricin D by proteolytic enzymes

The digests of the cross-linked ricin D with trypsin, chymotrypsin and nagarse were subjected to SDS-polyacrylamide gel electrophoresis and compared with those of native ricin D. As shown in Fig. 4, the tryptic or chymotryptic digest of ricin D gave a new small band, which migrated faster than that of native ricin D, in addition to that of native ricin D, whereas those of the cross-linked ricin D only gave one band at the same position as ricin D. Both native and cross-linked ricin D were hydrolyzed more easily with nagarse than with trypsin or chymotrypsin, although cross-linked ricin D was slightly more resistant than native ricin D.

From these results, it was concluded that the cross-linked ricin D is as resistant as native ricin D to proteolytic hydrolysis.

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Cytoagglutinating activity and toxicity of cross-linked ricin D

Table II shows the cytoagglutinating activity...
and toxicity of the cross-linked ricin D toward mice in comparison with those of native ricin D. Cytoagglutinating activity of the cross-linked ricin D was identical, within experimental error, to that of native ricin D and was not destroyed even after treatment at pHs 1.0 and 11.0, or at 50°C. On the contrary, the toxicity of the cross-linked ricin D toward mice was approximately 1.6% of that of native ricin D.

The effect of the cross-linked ricin D on the *in vitro* growth of two cell lines after 24 hr incubation with cross-linked ricin D (0.1 or 1 µg/ml) was investigated and compared with those of native ricin D or its isolated Ala-chain. At a concentration of 0.1 µg/ml, as shown in Fig. 5, native ricin D inhibited 49% and 24% of the growth of XC and NIH3T3 cells, respectively, whereas cross-linked ricin D and the isolated Ala-chain had no effect. On the other hand, at a concentration of 1 µg/ml, native ricin D, cross-linked ricin D and the isolated Ala-chain inhibited approximately 87%, 45% and 60% of the growth of XC cells, and 51%, 32% and 39% of that of NIH3T3 cells, respectively.

**Incorporation of cross-linked ricin D into SA cells**

In order to compare the incorporation kinetics of native and cross-linked ricin D into cells, irreversible binding of the two ricins to the cells was examined using 125I-labeled ricin D and SA cells. As shown in Fig. 6, ricin D bound...
After incubation with the rabbit reticulocyte lysate, \(^{125}\)I-labeled ricin D was submitted to SDS-polyacrylamide gel electrophoresis. Details are in the text. (A), \(^{125}\)I-labeled native ricin D incubated with crude lysate; (B), \(^{125}\)I-labeled cross-linked ricin D incubated with crude lysate; (C), \(^{125}\)I-labeled native ricin D incubated with gel-filtered lysate. •—•, before incubation; x—x, after 30 min incubation; O—O, after 2 hr incubation.

to the cells at 0°C was almost completely removed by washing with lactose, whereas ricin D incubated with SA cells at 37°C for 30 min was irreversibly bound to the cells and the amounts of native and cross-linked ricin D bound to the cells after 2 hr incubation were calculated to 1.05 µg and 1.25 µg, respectively. The irreversible binding kinetics of the cross-linked ricin D were not distinguishable from those of native ricin D, suggesting that the cross-linked ricin D is incorporated into the cells in the same manner as native ricin D.

Integrity of cross-linked ricin D in the rabbit reticulocyte lysate

Fifty microliters of \(^{125}\)I-labeled native ricin D (0.204 µg, specific activity: \(6.2 \times 10^6\) cpm/µg) and \(^{125}\)I-labeled cross-linked ricin D (0.21 µg, specific activity: \(9.1 \times 10^6\) cpm/µg) were added to 200 µl of the crude lysate. After incubation at 30°C for 1 hr, 10 µl of the reaction mixture was submitted to SDS-polyacrylamide gel electrophoresis. As shown in Fig. 7A, native ricin D incubated with the crude lysate gave four peaks—P-1, P-2, P-3 and P-4. The position of P-1 was the same as that of native ricin D, and the positions of P-2 and P-3 were identical to those of the Ala- and Ile-chains of ricin D which can be obtained by treatment of native ricin D with 2-mercaptoethanol. Peak P-4 was slightly smaller than the Ile-chain. Radioactivities of peaks P-1, P-2, P-3 and P-4 after 30 min incubation were approximately 65%, 11%, 17% and 4% of that of peak P-1 obtained without incubation, and 33%, 28%, 22% and 16% after 2 hr incubation, respectively. Total activities of these four peaks obtained from 30 min and 2 hr incubations were approximately 97% and 99%, respectively.

On the other hand, the cross-linked ricin D incubated with the crude lysate and the native ricin D incubated with gel-filtered lysate were not degraded into smaller molecules and gave a single peak at the position of native ricin D (Figs. 7B and 7C).

From these results, it was suggested that native ricin D is first split into Ala- and Ile-chains by reduction of the inter-chain disulfide bond and then the chains are limitedly degraded into smaller molecules in the crude lysate.

**DISCUSSION**

N,N'-o-Phenylenedimaleimide is an efficient cross-linking reagent for joining two constituent polypeptide chains of ricin D at the inter-
Cross-linking of Ricin D

chain disulfide bridge. Since it is known that this reagent reacts with amino groups, the reaction was performed at pH 5.5 in the present experiment. Amino acid analysis of the cross-linked ricin D revealed that ε-amino groups of lysine residues in ricin D were not modified under the conditions used. This cross-linking reaction was successful only when o-Ph(Mal)$_2$ was added to the reduced ricin D in which both Ile- and Ala-chains coexisted.

Cross-linked ricin D behaved as a more acidic protein than native ricin D on DEAE-cellulose column chromatography at pH 8.5 and easily separated from native ricin D. Since amino groups in ricin D were not modified in this reaction as mentioned above, the different behavior in the chromatography may be due to a conformational alternation. Details of the difference in the conformation between native and the cross-linked ricin D are under investigation.

Cytoagglutinating activity of the cross-linked ricin D thus obtained was almost identical to that of native ricin D, indicating that the Ala-chain in the cross-linked ricin D is intact, whereas the toxicity toward mice was remarkably reduced. Since the cross-linking reaction is mild and restricted to the inter-chain disulfide bridge, and the Ile-chain is very stable during physical treatments, it seems unlikely that the decrease of toxicity could be caused by the modification and/or denaturation of the Ile-chain. The results on the stability and digestibility of the cross-linked ricin D also show that the low toxicity of the cross-linked ricin D is not due to inactivation and/or breakdown of the injected cross-linked ricin D on the way to the cells in which the toxic action is exerted.

Inhibitory activity of the cross-linked ricin D on the growth of cultured cells was much lower than that of native ricin D, suggesting that the decrease of toxicity occurs at cell level.

From biological and electronmicroscopic studies, it has been shown that the action of ricin is elicited by following sequence of events: (a) binding of ricin to receptors on cell surface; (b) clustering of ricin receptors; (c) endocytosis of the ricin molecule; (d) release of ricin from endocytotic vesicles into the cell cytoplasm and activation of ricin; (e) interaction of activated ricin with ribosomes.

To see the differences in these events between native and cross-linked ricin D, we used $^{125}$I-labeled ricin D and studied the kinetics of irreversible binding to the intact cells and the degradation in the rabbit reticulocyte lysate.

The cross-linked ricin D was bound irreversibly to the cells in a slightly smaller amount than native ricin D and its binding kinetics were almost identical to those of native ricin D. This result suggests that the cross-linked ricin D interacts with the ricin receptor and penetrates into the cells in the same manner as native ricin D.

With regard to the activation of ricin by the cell-free system during protein synthesis, Lugnier et al. have shown by polyacrylamide gel electrophoretic analysis that native ricin is split into its A (Ile)- and B (Ala)-chains in the incubation with reticulocyte lysate. We obtained a similar result for native ricin D incubated with crude lysate, but the cross-linked ricin D incubated with crude lysate and the native ricin D incubated with the gel-filtered lysate remained intact. These results suggest that the splitting of native ricin D into two chains could be caused by reduction of the inter-chain disulfide bond, but not by proteolytic hydrolysis, and that the smaller component appearing as a shoulder (peak P-4 in Fig. 7A) in the peak of the Ile-chain may be produced by proteolytic hydrolysis after the splitting into two chains. Low toxicity of the cross-linked ricin D is due to unsplitting of the inter-chain disulfide bond, and this is consistent with the result that the toxicity of the cross-linked ricin D toward cultured cells was similar to that of the isolated Ala-chain when the Ile-chain is not involved in the toxic action. With regard to the susceptibility of ricin D to protease, recently we found that native ricin D is not susceptible to lysosomal protease, while the isolated chains are attacked and the degraded Ile-chain has a higher inhibitory activity for cell-free protein synthesis than native ricin D.
From these results and others,\textsuperscript{13,14} it was inferred that ricin D released from pinocytotic vesicles into the cell cytoplasm is split into its constituent polypeptide chains by reduction of the inter-chain disulfide bridge and the resulting polypeptide chains are degraded by protease in the cytoplasm. Of course, we cannot rule out other possibilities and further investigation is required.

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