Hybridization between the Heterologous Chains of Native Ricin D and Iodinated or Maleyl Ricin D[†]

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Two constituent polypeptide chains of native or iodinated ricin D were separated by affinity chromatography on Sepharose 4B after reduction with β -mercaptoethanol, and purified by DEAE-cellulose column chromatography.

The hybrid molecule between the native Ile chain and the iodinated Ala chain was easily formed by reduction-reoxidation of a mixture of both isolated chains. Its toxicity to mice was about 20% of that of ricin D and about ten fold that of the iodinated ricin D.

On the other hand, the hybrid molecule between native Ala chain and maleyl Ile chain was obtained by reduction-reoxidation of a mixture of native and maleyl ricin D, and its toxicity to mice was about 15% of that of ricin D and about four fold that of maleyl ricin D.

These results suggest that tyrosine and lysine residues in each chain of ricin D are involved in the toxic action of ricin D.

In recent years, it has been shown that ricin, a toxic protein from the castor bean (*Ricinus communis*), consists of two different polypeptide chains¹⁾—Ile and Ala chains—and its toxicity is elicited by their cooperative action.^{2,3)} In addition, the chemical modification of ricin with iodine⁴⁾ or anhydrous acetic acid⁵⁾ suggests that both tyrosine and lysine residues are probably involved in the toxic action of ricin. However, it was not certain whether the detoxification of ricin resulted from the modification of tyrosine or lysine residues either in the Ile chain, which inhibits protein synthesis, or in the Ala chain, which binds to cell membrane and may probably mediate a *trans*-membrane of the Ile chain.⁶⁾ It was necessary, therefore, to prepare ricin derivatives, in which only one chain is modified, and determine their toxicities.

Here, we shall describe the preparation procedures and some properties of two ricin-derivatives in which only the Ala chain was iodinated or only the Ile chain was maleylated.

MATERIALS AND METHODS

Materials. Ricin D was isolated as previously described.⁷⁾

Isolation of constituent polypeptide chains of ricin D. Each constituent polypeptide chain of native or iodinated ricin D was isolated by affinity chromatography on Sepharose 4B principally by the method of Nicolson and Blaustein⁸⁾ followed by DEAE-cellulose column chromatography at pH 8.5. To a 1% solution of native or iodinated ricin D in 0.2 M NaCl-5 mM phosphate buffer, pH 8.0, β -mercaptoethanol was added to a final concentration of 1% and the solution was adjusted to 8.0 with NaOH. After the solution was let stand for one hour at room temperature with stirring, solid NaCl was added to it to a final concentration of 0.2 M and the reaction mixture was dialyzed against 0.2 M NaCl-5 mM phosphate buffer, pH 8.0, containing 0.1% β -mercaptoethanol, for 2.5 hr at 7°C. The resulting insoluble material was centrifuged, and the supernatant was applied to a column of Sepharose 4B (Pharmacia) previously equilibrated with the same buffer. After washing the column with the same buffer until the absorbance at 280 nm almost disappeared, the adsorbed proteins were eluted with a solution of 0.1 M lactose in the same buffer.

DEAE-cellulose column chromatography. The protein solution was applied to a column of DEAE-cellulose (Brown) equilibrated with 5 mM Tris-HCl

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buffer, pH 8.5, and eluted with a linear gradient of NaCl from 0 to 0.2 M in the same buffer.

Iodination of ricin D. Iodinated ricin D was prepared as previously described.⁴⁾ To 150 ml of 0.5% ricin D solution in 0.1 M phosphate buffer, pH 7.0, 10 ml of 25 mM KI₃ solution (the molar ratio between ricin D and I₂ was 1:20) was added dropwise over a period of 20 min at 0°C under vigorous stirring. After the reaction mixture was let stand for 100 min to complete iodination, 0.1 M sodium thiosulfate solution was added to it until the colour of I₂ disappeared. The iodinated ricin D was exhaustively dialyzed against 5 mM acetic acid solution at 7°C to remove the used reagents. The number of diiodotyrosine residues in the iodinated ricin D was spectrophotometrically determined at 312 nm by the molar extinction, 5.815×10^3 , of diiodotyrosine.

Maleylation of ricin D. Ricin D was maleylated by the method of Butler *et al.*⁹⁾ To 25 ml of 1% ricin D solution in borate buffer, pH 9.0, 3.75 ml of 1 M maleic anhydride in dioxane (approximately 90 mole per free amino group of ricin D) was added in several portions at 2°C and the pH of the mixture was maintained at 9.0 by adding 1 N NaOH. After the reaction was complete, the reaction mixture was treated with 0.8 M hydroxylamine at pH 9.5 for 20 min at 25°C and then dialyzed against deionized water. The number of amino-bound maleyl groups was determined from the optical density of ricin D at 280 nm and 250 nm before and after maleylation, and as the molar extinction coefficients for the maleylamido group, 310 at 280 nm and 3,360 at 250 nm were used.¹⁰⁾

Preparation of hybrid molecule between heterologous chains. Approximately equal amounts of the native Ile chain and the iodinated Ala chain as a suspension in saturated ammonium sulfate solution (measured by turbidity at 660 nm) were mixed and centrifuged at 9,000 rpm for 40 min. The resulting precipitate was dissolved in 0.1 M Tris-HCl buffer, pH 8.5, containing 1% β -mercaptoethanol and 0.2% EDTA, and then dialyzed against the same buffer for 1~2 hr at room temperature. After removal of the insoluble material by centrifugation, the supernatant was applied to Sephadex G-25 prewashed with 5 mM Tris-HCl buffer, pH 8.5, to remove β -mercaptoethanol. The protein solution was gently stirred overnight at 7°C in an Erlenmeyer flask after dialysis against 5 mM Tris-HCl buffer, pH 8.5, or without dialysis. A hybrid molecule between native Ala chain and maleyl Ile chain was prepared from a mixture of ricin D and maleyl ricin D by the above procedure. The resulting hybrid molecule was separated by affinity chromatography on Sepharose 4B with 0.2 M NaCl-5 mM phosphate buffer, pH 7.1, by the method of Nicolson and Blaustein⁸⁾ after adjustment to pH 7.1 with acetic acid and addition of NaCl

to 0.2 M, and purified by DEAE-cellulose column chromatography at pH 8.5.

Polyacrylamide gel electrophoresis. Electrophoresis in polyacrylamide gel was performed in Tris-glycine buffer, pH 8.3, with a gel concentration of 7.5% by the method of Orstein.¹¹⁾

Determination of molecular weight. Molecular weight of the protein was determined by SDS-polyacrylamide gel electrophoresis by the method of Weber and Osborn.¹²⁾

N-Terminal amino acid. N-Terminal amino acid of the protein was analyzed directly by Edman degradation as modified by Iwanaga *et al.*¹³⁾

Amino acid analysis. Amino acid composition of the protein was analyzed with an automatic amino acid analyzer (JEOL Type JLC-6AH) after hydrolysis in evacuated and sealed tubes with constantly boiling HCl at 108°C for 24 hr.

Determination of toxicity and cytoagglutinating activity. Toxicity of the protein was determined with pure-bred male mice (ddN) and cytoagglutinating activity, with sarcoma 180 ascites tumor cells or human red blood cells (type 0) as previously described.⁸⁾ Increasing amounts of the protein were injected intraperitoneally into groups of three or more mice, weighing 24 ± 3 g, and the LD₅₀ dose was calculated for deaths occurring within 48 hr. To 0.2 ml of tumor cell suspension (1×10^7 /ml) or 4% red cell suspension, 0.2 ml portions of the protein solution at different concentrations were added. After incubation with gentle mixing for 30 min at room temperature, the agglutinating activity was measured macroscopically.

RESULTS

Isolation of two constituent polypeptide chains from native ricin D

Two polypeptide chains of ricin D were isolated in a reduced form by taking advantage of the high affinity of the Ala chain for Sepharose 4B. As shown in Fig. 1, affinity chromatography of the β -mercaptoethanol-treated ricin D on Sepharose 4B gave two fractions—F-1 and F-2. From their N-terminal amino acids and behaviours on polyacrylamide gel electrophoresis, it was found that fraction F-1 which possessed only a slight affinity for Sepharose was Ile chain and fraction F-2 eluted with lactose was a mixture of Ala chain and native ricin D.

FIG. 1. Affinity Chromatography of β -Mercaptoethanol-treated Ricin D on a Sepharose 4B Column. Reduced ricin D was applied to a column (4.8×44 cm) of Sepharose 4B equilibrated with 0.2 M NaCl-5 mM phosphate buffer (pH 8.0) containing 0.1% β -mercaptoethanol. The column was washed with the same buffer, and the adsorbed protein was eluted with 0.1 M lactose in the same buffer at a rate of 62 ml/hr.

FIG. 2. DEAE-cellulose Column Chromatography of Fraction F-2 Obtained by Affinity Chromatography on Sepharose 4B and Polyacrylamide Gel Electrophoresis of Each Fraction.

Fraction F-2 (Fig. 1) was applied to the column (3.2×29 cm) equilibrated with 5 mM Tris-HCl buffer (pH 8.5) and eluted with a linear gradient from 0.01 M to 0.20 M of NaCl in the same buffer at a flow rate of 40 ml/hr. Different protein fractions were analyzed by electrophoresis in 7.5% polyacrylamide gels at pH 8.3. (a) ricin D, (b) fraction F-1, (c) fraction F-2 in Fig. 1, (d) fraction F-2a, (e) fraction F-2b and (f) fraction F-2c.

Separation of Ala chain from the native ricin D was achieved by DEAE-cellulose column chromatography at pH 8.5. As shown in Fig. 2, fraction F-2 was separated into three fractions—F-2a, F-2b and F-2c—of which

fraction F-2a was native ricin D and the other two were Ala chain. Fraction F-2c moved faster than fraction F-2b in polyacrylamide gel electrophoresis at pH 8.3 and gave a main band corresponding to a molecular weight of about 60,000 in SDS-polyacrylamide gel electrophoresis, suggesting that fraction F-2c might be a dimer of the Ala chain. The hemagglutinating activity of this fraction was about fourfold that of fraction F-2b which is a monomer of Ala chain. Fractions F-2b and F-2c were combined and employed for the hybridization experiment.

Isolation of two constituent polypeptide chains from iodinated ricin D

After reduction with β -mercaptoethanol, the iodinated ricin D, in which 5.28 moles of tyrosine residues were iodinated, was separated into three fractions—IR-1, IR-2 and IR-3—by affinity chromatography on a Sepharose 4B column as shown in Fig. 3. Analyses of the N-terminal amino acid revealed that fractions IR-1 and IR-2 were Ile and Ala chains, respectively. The number of diiodotyrosine residues in fraction IR-2 was determined to be 3.01 from the difference spectrum at pH 10 referred to that in 50 mM acetic acid solution (Fig. 4b), and the number in fraction IR-1, to be 2.38 from the difference spectrum be-

FIG. 3. Affinity Chromatography of β -Mercaptoethanol-treated Iodinated Ricin D on a Sepharose 4B Column.

Chromatographic conditions were the same as in Fig. 1.

FIG. 4. Difference Spectra of Iodinated Ricin D (a), and Iodinated Ala Chain (b) at pH 10 Referred to That in 0.05 N Acetic Acid.

The concentrations of iodinated ricin D and iodinated Ala chain were 0.069% and 0.048%, respectively.

FIG. 5. Ultraviolet Absorption Spectra of Native and Iodinated Ile Chains in 0.2 M Carbonate Buffer (pH 10.0).

The concentrations of native Ile chain (a) and iodinated Ile chain (b) were 11.7 μ M and 4.68 μ M, respectively.

tween the native Ile chain and fraction IR-1 at pH 10 (Fig. 5).

From these results, it was found that fraction IR-1 was the iodinated Ile chain in which two tyrosine residues were iodinated and fraction IR-2 was the iodinated Ala chain in which three tyrosine residues were iodinated.

Each chain was stored in a saturated solution of ammonium sulfate at 7°C.

Isolation of hybrid molecule between native Ile chain and the iodinated Ala chain

The product obtained by reduction-reoxidation of a mixture of the native Ile chain and the iodinated Ala chain was applied to Sepharose 4B column and washed with 0.2 M NaCl-5 mM phosphate buffer, pH 7.1. As shown in Fig. 6, the adsorbed protein was eluted with 0.1 M lactose in the same buffer. The molecular weight of the adsorbed protein was determined to be 63,000 by SDS-polyacrylamide gel electrophoresis, and the treatment with β -mercaptoethanol broke the protein into two bands of 33,000 and 31,000 in molecular weights, indicating that this fraction was a hybrid molecule from the native Ile chain and the iodinated Ala chain (Ile chain/I₂-Ala chain). Toxicity of the hybrid molecule Ile chain/I₂-Ala chain to mice was about 20% of that of ricin D and about tenfold that of the iodinated ricin D (Table I). On the other hand, its cytoagglutinating activity on sarcoma 180 ascites tumor cells was about 4% of that of ricin D.

FIG. 6. Separation of the Hybrid Molecule between Native Ile Chain and Iodinated Ala Chain by Affinity Chromatography on Sepharose 4B.

The product obtained by reduction-reoxidation of a mixture of native Ile chain and iodinated Ala chain was applied to a column (2 $\times$ 20 cm) of Sepharose 4B equilibrated with 0.2 M NaCl-5 mM phosphate buffer (pH 7.1) and washed with the same buffer. The adsorbed protein was eluted with 0.1 M lactose in the same buffer. Temperature was 4°C.

TABLE I. TOXICITY AND CYTOAGGLUTINATING ACTIVITY OF IODINATED RICIN D AND THE HYBRID MOLECULE Ile CHAIN/I₂-Ala CHAIN

Proteins	Toxicity		Cytoagglutinating activity	
	MLD ₄₈ (OD ₂₈₀)	%	min. conc. (OD ₂₈₀)	%
Ricin D	0.001	100	0.006	100
Iodinated-ricin D	0.050	2	0.100	6
Hybrid Ile chain/ I ₂ -Ala chain	0.005	20	0.145	4

TABLE II. TOXICITY AND CYTOAGGLUTINATING ACTIVITY OF MALEYL RICIN D AND THE HYBRID MOLECULE Ala CHAIN/*m*-Ile CHAIN

Proteins	Toxicity		Cytoagglutinating activity	
	MLD ₄₈ (OD ₂₈₀)	%	min. conc. (OD ₂₈₀)	%
Ricin D	0.001	100	0.006	100
Maleyl ricin D	0.025	4	0.073	8
Hybrid Ala chain/ <i>m</i> -Ile chain	0.007	15	0.006	100

Preparation of maleyl ricin D

The maleylated product of ricin D was fractionated into three components—MR-1, MR-2 and MR-3—by affinity chromatography on Sepharose 4B in 0.2 M NaCl-5 mM phosphate buffer, pH 7.1 (Fig. 7). The extent of maleylation in fraction MR-2, which was the major product, was spectrophotometrically determined to be 11.6, indicating that all the 11 amino groups of ricin D were maleylated. Fraction MR-2 was referred to as maleyl

ricin D. The toxicity and cytoagglutinating activity of the maleyl ricin D were about 4% and about 8% of those of ricin D, respectively (Table II).

Isolation of the hybrid molecule between native Ala chain and maleyl Ile chain

The product obtained by reduction-reoxidation of a mixture of the maleyl ricin D and native ricin D was separated by affinity chromatography on Sepharose 4B into two fractions—P-1 and P-2 (Fig. 8). Since the maleyl ricin D

FIG. 7. Affinity Chromatography of Maleylated Ricin D on a Sepharose 4B Column and Polyacrylamide Gel Electrophoresis of the Fractions Obtained. Chromatographic conditions were the same as in Fig. 6. Electrophoresis was carried out at pH 8.3 with 7.5% gel concentration. (a) ricin D, (b) fraction MR-1, (c) fraction MR-2 and (d) fraction MR-3.

FIG. 8. Affinity Chromatography of the Hybrid Molecule from Native Ricin D and Maleyl Ricin D on a Sepharose 4B Column and Polyacrylamide Gel Electrophoresis of the Fractions Obtained.

The conditions of chromatography and electrophoresis were the same as in Fig. 7. (a) ricin D, (b) maleyl ricin D, (c) fraction P-1 and (d) fraction P-2.

FIG. 9. DEAE-cellulose Column Chromatography of Fraction P-2 Referred to in Fig. 8 and Polyacrylamide Gel Electrophoresis of the Fractions Obtained.

Fraction P-2 (in Fig. 8) was applied to the column of DEAE-cellulose equilibrated with 5 mM Tris-HCl buffer (pH 8.5) and eluted in a linear gradient from 0 to 0.2 M NaCl in the same buffer. Electrophoretic conditions were the same as in Fig. 7. (a) ricin D, (b) fraction P-2 in Fig. 8, (c) fraction P-2a and (d) fraction P-2b.

was not adsorbed on Sepharose 4B, it is apparent that fraction P-1 contains maleylated-Ala chain, whereas fraction P-2 contains the native Ala chain in its components. Fraction P-2 was further fractionated into two components—P-2a and P-2b—by DEAE-cellulose column chromatography at pH 8.5 (Fig. 9). Polyacrylamide gel electrophoresis (Fig. 9) and an ultraviolet-spectrum showed that fraction P-2a was native ricin D and fraction P-2b was maleylated ricin D.

The number of amino-bound maleyl groups in fraction P-2b was found to be three corresponding to the number of free amino groups in the Ile chain. From these results, it was concluded that fraction P-2b was a hybrid molecule (Ala chain/*m*-Ile chain) consisting of the native Ala chain and the maleylated Ile chain. The hybrid molecule Ala chain/*m*-Ile chain restored the full cytoagglutinating activity, whereas its toxicity was about 15% of that of ricin D and about fourfold that of maleyl ricin D, clearly showing the involvement of the free amino groups of the Ile chain and Ala chain in the toxic action of ricin D (Table II).

DISCUSSION

Affinity chromatography on Sepharose 4B

was very useful for the isolation of the constituent polypeptide chains of native as well as iodinated ricin D and also for the preparation of the hybrid molecule. When reduction with β -mercaptoethanol and subsequent affinity chromatography are performed at a low concentration of ricin D, for example less than 0.5%, ricin D is almost completely reduced and is not detected in the fraction eluted with lactose. Long tailing of the peak of Ile chain in affinity chromatography (Fig. 1) indicates a gradual reduction of the ricin D adsorbed on Sepharose with β -mercaptoethanol in the buffer during chromatography. The isolated chains have a tendency to precipitate during long dialysis against deionized water, and the iodinated chains are especially ready to precipitate.

Determination of diiodotyrosine in each chain of the iodinated ricin D clearly endorsed the previous results⁴⁾ that the two tyrosine residues in the Ile chain and three tyrosine residues in the Ala chain were iodinated.

The findings of the present study on the hybridization between native Ile chain and the iodinated Ala chain suggest not only the contribution of the tyrosine residues in Ala chain to the cytoagglutinating activity as well as the toxic action of ricin D, but also that of the tyrosine residues in the Ile chain to the toxic action of ricin D. Although we have failed to purify the hybrid molecule between native Ala chain and iodinated Ile chain because of its small amount, the preliminary finding that the partially purified hybrid possessed the full agglutinating activity, whereas its toxicity was about 10% of that of ricin D, indicates that the tyrosine residues in Ile chain have an important role in the toxic action of ricin D.

The differences in affinity to Sepharose and of the charges in the native and the maleyl Ala chain facilitated isolation of the hybrid molecule from the native Ala chain and the maleyl Ile chain. Losses in affinity for Sepharose and in cytoagglutinating activity of the maleyl ricin D probably resulted from the inability of the maleyl Ala chain to bind to the cell membrane. In addition to these results,

the remarkable increase in toxicity when the maleyl Ala chain of maleyl ricin D was replaced with the native Ala chain suggests the importance of the free amino groups of the Ala chain to the toxic action of ricin D. However, we must consider the effects of changes in the conformation and the charge caused by the introduction of many carboxyl groups.

Low toxicity of the hybrid molecule Ala chain/*m*-Ile chain indicates the contribution of free amino groups of the Ile chain to the toxic action of ricin D. The Ile chain contains three free amino groups of which two are present in the N-terminal region of the Ile chain and are not necessary to the toxic action.¹⁴⁾ Therefore, another lysine residue may be essential for the full toxic action of ricin D. Localization of this lysine residue is now under study.

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