Significance of the Tryptophan Residue at the Low Affinity Saccharide-binding Site of Ricin E[†]

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Chemical modification of tryptophan residues in ricin E was investigated with regard to saccharide-binding. Two out of ten tryptophan residues in ricin E were modified with *N*-bromosuccinimide at pH 4.5 in the absence of specific saccharide accompanied by a marked decrease in the cytoagglutinating activity. Such a loss of the cytoagglutinating activity was found to be principally due to the oxidation of one tryptophan residue per B-chain. In the presence of lactose, one tryptophan residue/mol was protected from the modification with retention of a fairly high cytoagglutinating activity. However, GalNAc did not show such a protective effect. The binding of lactose to ricin E altered the environment of the tryptophan residue at the low affinity binding site of ricin E, leading to a blue shift of the fluorescence spectrum and an UV-difference spectrum with a maximum at 290 nm and a trough at 300 nm. The ability to generate such spectroscopic changes induced by lactose was retained in the derivative in which one tryptophan residue/mol was oxidized in the presence of lactose, but not in the derivative in which two tryptophan residues/mol were oxidized in the absence of lactose. Based on these results, it is suggested that one of the two surface-localized tryptophan residues is responsible for saccharide binding at the low affinity binding site of ricin E, which can bind lactose but lacks the ability to bind GalNAc.

Ricin E is a toxic protein found in the small-grain seeds of castor beans (*Ricinus communis*) grown in Japan.¹⁾ This is a lectin with a 63,000 mol weight and consists of two non-identical polypeptide chains (A- and B-chains) linked through a single disulfide bond. The A-chain is responsible for inhibition of protein synthesis in eucaryotic cells and the B-chain serves to combine the toxin to the carbohydrate moiety on the cell membrane,²⁾ like ricin D isolated from the large-grain seeds of *Ricinus communis*.^{3,4)} The molecular weight, toxicity for mice, and cytoagglutinating activity of ricin E is equal to those of ricin D, but the two ricins differ in toxicity for cultured cells and affinities to Sepharose 4B and Guar gum.^{2,5)} Such differences in the properties as

are found between ricin E and ricin D have been considered to be attributable to the difference in the amino acid sequences of their B-chains.⁶⁾ Physicochemical studies on the binding of saccharides to ricin E have suggested that the B-chain of ricin E carries two saccharide-binding sites with different affinities, a high affinity binding site (HA-site) and a low affinity binding site (LA-site).⁷⁾ However, the nature of the saccharide-binding sites has not been elucidated completely. To shed further light on the structure-function relationship of ricin E, tryptophan residues in this toxin were modified with *N*-bromosuccinimide and the properties of the resulting derivatives were examined. This paper describes the significance of the tryptophan residue at the

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Abbreviations: NBS, *N*-bromosuccinimide; GalNAc, *N*-acetyl-D-galactosamine; HA-site, high affinity binding site; LA-site, low affinity binding site; UV, ultraviolet.

LA-site of ricin E.

Materials and Methods

Ricin E was prepared by the method of Mise *et al.*¹⁾ from small-grain castor bean seeds (*Ricinus communis*) cultivated in a field of Kyushu University. *N*-Bromosuccinimide and *N*-acetyl-D-galactosamine were obtained from Nacalai Tesque (Kyoto), and the former was recrystallized from distilled water before use. Lactose from Wako Pure Chemicals Industries Ltd. (Osaka) was used without further purification. Other reagents used in these experiments were analytical grade.

Protein concentration. The concentration of ricin E was measured spectrophotometrically using an absorption coefficient of $A_{1\text{ cm}}^{1\%} = 15$ at 280 nm.¹⁾ The concentration of the modified ricin E was measured by the method of Lowry *et al.*,⁸⁾ using ricin E as a standard protein.

Oxidation with NBS. The oxidation of tryptophan residues with NBS was done essentially by the method of Spande and Witkop.⁹⁾ A 3 ml sample of ricin E solution (4.4 μM) in 0.1 M acetate buffer at pH 4.0 was placed in a quartz cell with a stirring bar. With stirring, 10 μl of NBS solution at a given concentration in the same buffer was added, and after 5 min the absorption spectrum was recorded on a Hitachi 200-10 spectrophotometer. A control was prepared by addition of 10 μl of the buffer solution in place of the NBS solution. To make the titration curve for NBS-oxidation of tryptophan in ricin E, the NBS solution (2.8 mM) was successively added until the decrease in the absorbance at 280 nm ceased. All reactions were done at 25°C, and the number of tryptophan residues oxidized was calculated by the method described in the literature.⁹⁾

Cytoagglutinating activity. Cytoagglutinating activities of ricin E and its derivatives were measured microscopically using Sarcoma 180 ascites tumor cells in 5 mM phosphate-buffered saline at pH 7.1, as described by Funatsu *et al.*¹⁰⁾

Fluorescence spectra. Fluorescence spectra of ricin E and its derivatives were recorded on a Hitachi 650-10S fluorescence spectrophotometer at pH 6.9 and 25°C with excitation at 290 nm. For measurements of the fluorescence spectra in the presence of lactose, 10 μl of the mixture of protein and lactose was added to the protein solution. This was done in such a manner as to keep the protein concentration the same.

UV-difference spectra. UV-difference spectra of ricin E and its derivatives induced by binding with lactose were measured with a Hitachi 200-10 spectrophotometer at pH 6.9 and 25°C. The spectra were measured by placing the

mixture of protein and lactose in the sample cell and the same concentration of protein in the reference cell, by the method described previously.⁷⁾

Isolation of the constituent polypeptide chains. Ricin E and modified ricin E were separated into their constituent polypeptide chains, essentially by the method of Olsnes and Pihl.¹¹⁾ Proteins were treated with 2% 2-mercaptoethanol in 10 mM Tris-HCl buffer at pH 8.5 containing 0.1 M lactose for 2 hr, and put on a column of DEAE-cellulose (1.5 $\times$ 15 cm) equilibrated with 10 mM Tris-HCl buffer at pH 8.5. After being washed with the buffer used for equilibration, the column was first developed with 0.5 M NaCl in the same buffer and then with 0.1 M NaOH. The unadsorbed fractions were pooled as the A-chain, and the fractions eluted with 0.5 M NaCl were collected as the B-chain of ricin E. In the case of the modified ricin E, however, the B-chain was not recovered completely in the fractions eluted with 0.5 M NaCl and was eluted with 0.1 M NaOH. The fractions eluted with 0.1 M NaOH were immediately neutralized with 50% acetic acid, combined with the fractions eluted with 0.5 M NaCl, and used as the B-chain of the modified ricin E. Each of the fractions was dialyzed against deionized water, and analyzed spectroscopically.

Results

NBS-oxidation of ricin E

As demonstrated in Fig. 1, the absorption

Fig. 1. Change in the Absorption Spectrum of Ricin E during NBS Oxidation.

To 3 ml of ricin E solution (4.4 μM) in 0.1 M acetate buffer (pH 4.0) was added 10 μl of a NBS solution in the same buffer. The spectra were recorded after 5 min of the addition of 10 μl of the buffer solution (-----), 6 mM NBS (---) and 15 mM NBS (—) at 25°C.

Fig. 2. Titration Curves for NBS Oxidation of Tryptophan in Ricin E and Its Complexes with Saccharides.

The oxidation reaction was done at pH 4.0 and 25°C in the absence of saccharide (○) and in the presence of 0.1 mM GalNAc (●) or lactose (◐). Other conditions were described under **Materials and Methods**.

Table I. CYTOAGGLUTINATING ACTIVITIES OF RICIN E AND ITS DERIVATIVES

Ricin E was modified with NBS at pH 4.5 and 25°C. Details are described under **Materials and Methods**.

Protein	Moles of Trp oxidized per mole of ricin E	Cytoagglutinating activity (%) ^a
Native ricin E	0	100
Ricin E modified in the absence of lactose	0.8	3
	1.9 ^b	0.7
Ricin E modified in the presence of 0.1 M lactose	1.2 ^b	33

^a Measured using Sarcoma 180 ascites tumor cells at pH 7.1.¹⁰ For cytoagglutination tests, samples of native and oxidized ricin E were dialyzed against 5 mM phosphate buffered saline (pH 7.1) at 4°C.

^b Number of residues eventually modified under the conditions cited.

spectrum of ricin E changed greatly on NBS-oxidation. However, the absorbance at 265 nm remained unchanged during NBS-oxidation and an isosbestic point was found at this wavelength. The titration curves for NBS-oxidation of tryptophan residues in ricin E in the absence and presence of saccharide are shown in Fig. 2. The extent of tryptophan modification increased with increasing the amount of NBS added, and 1.9 tryptophan residues/mol were eventually oxidized with a 16-fold excess of NBS at pH 4.0 in the absence

of lactose. In the presence of 0.1 M lactose, however, only 1.2 tryptophan residues/mol were modified, and 0.7 tryptophan residues/mol were protected from this modification reaction. Such a protective effect of saccharide on this modification reaction was not observed when GalNAc was used in place of lactose. Table I shows the effects of tryptophan modification on the cytoagglutinating activity of ricin E. The oxidation of tryptophan with NBS led to a marked decrease in the cytoagglutinating activity of ricin E toward Sarcoma 180 ascites tumor cells, and only 3% of the residual activity was found in the derivative, 1-oxa-ricin E, in which 0.8 tryptophan residues/mol were converted to oxindole-alanine in the absence of lactose. The cytoagglutinating activity of another derivative, 2-oxa-ricin E, in which 1.9 tryptophan residues/mol were oxidized, was less than 1% that of the native ricin E. On the contrary, when the modification reaction was done in the presence of 0.1 M lactose, the resulting derivative, 1-oxa-ricin E(lac), which contained 1.2 oxindolealanine/mol, retained fairly high cytoagglutinating activity (33%). These results showed a fairly good agreement with those obtained for the NBS-oxidation of tryptophan in ricin D isolated from the large-grain castor bean seeds.¹²⁾

Fluorescence spectra of native and modified ricin E

Figure 3 shows the fluorescence spectra of oxidized ricin E in the absence and presence of lactose compared with those of native ricin E. Upon excitation at 290 nm, ricin E had a fluorescence emission spectrum with a maximum at 335 nm. The fluorescence intensity decreased greatly upon the oxidation of tryptophan with NBS with a shift of the emission maximum to a shorter wavelength. The fluorescence intensity of the inactive derivative, 2-oxa-ricin E, was only 43% that of native ricin E. The extent of the loss of the fluorescence of ricin E by the present modification reaction was almost identical to that found in the NBS-oxidation of tryptophan in

Fig. 3. Fluorescence spectra of Ricin E and 2-Oxa-ricin E in the Absence and Presence of Lactose.

Fluorescence spectra were recorded in 4.4 mM phosphate buffer (pH 6.9) with NaCl added to give 0.1 ionic strength at 25°C. Protein concentration was 0.57 μ M and the excitation wavelength was 290 nm. a, fluorescence spectra of ricin E in the absence (—) and presence of 0.1 mM lactose (-----); b, fluorescence spectrum of 2-oxa-ricin E in the absence or presence of 0.1 mM lactose.

Fig. 4. Difference Spectra of Ricin E and Its Derivatives Induced by Binding with Lactose.

The UV-difference spectra were recorded by placing the mixture of protein and lactose in the sample cell and the same concentration of protein in the reference cell, by the method described previously.⁷⁾ Measurements were done in the same buffer as described in Fig. 3 and at 25°C. The final concentrations of protein and lactose were 0.1 mM and 11 μ M, respectively. a, ricin E; b, 1-oxa-ricin E(lac); c, 2-oxa-ricin E.

ricin D (45%).¹²⁾ Upon addition of lactose, the emission maximum of ricin E shifted from 335 nm to 332 nm. Such a change in the fluorescence spectrum, however, was not observed when lactose was added to 2-oxa-ricin E.

Fig. 5. UV-Absorption spectra of the Constituent Polypeptide Chains of Native and Modified Ricin E.

The spectra of the constituent polypeptide chain (1.6 μ M) were measured in 5 mM phosphate buffer (pH 7.0) containing 0.14 mM NaCl. Panels A and B shows the spectra of the A-chains and the B-chains, respectively.

UV-difference spectra of native and modified ricin E induced by lactose binding

The lactose-induced UV-difference spectra of ricin E and its derivatives are demonstrated in Fig. 4. As previously reported, the UV-difference spectrum of the ricin E-lactose complex *versus* ricin E had a trough at 300 nm and a small but distinct positive peak at 290 nm. An almost identical difference spectrum was obtained for the active derivative, 1-oxa-ricin E(lac). However, the UV-difference spectrum generated by the inactive derivative, 2-oxa-ricin E, was different in shape from that given by native ricin E, and neither the trough at 300 nm nor the positive peak at 290 nm was found in the difference spectrum.

UV-absorption spectra of the A- and B-chains of the modified ricin E

In Fig. 5, the UV-absorption spectra of the constituent polypeptide chains isolated from 2-oxa-ricin E are compared with those obtained from native ricin E. It is evident that the absorption spectra of the A-chains of ricin E and its derivative were almost the same. On the other hand, the shape of the absorption spectrum of the B-chain of 2-oxa-ricin E was quite different from that of native ricin E. The absorption spectrum of the B-chain of the modified ricin E was characterized by a de-

crease in the intensity at 280 nm and an increase in the intensity at 250 nm. These results indicate that in 2-oxa-ricin E the modified tryptophan residues are contained in the B-chain but not in the A-chain.

Discussion

The reactivity of tryptophan toward NBS has been used as a criterion for discrimination of the states of tryptophan residues in proteins.⁹⁾ Ricin E contains ten tryptophans per molecule: one in the A-chain and the others in the B-chain.⁶⁾ As demonstrated in this study, two out of ten tryptophan residues in ricin E are eventually modified with NBS in the absence of lactose (Fig. 2). The UV-absorption spectra of the constituent polypeptide chains isolated from the modified ricin E clearly indicate that the oxidized tryptophan residues are contained only in the B-chain (Fig. 5). These findings suggest that two of the nine tryptophan residues in the B-chain are at or near the surface of the ricin E molecule with a fairly high susceptibility to NBS oxidation. Such a view can be supported by the fluorescence spectroscopic data. The fluorescence emission maximum at 335 nm as found in the fluorescence spectrum of ricin E is characteristic of tryptophan and solely due to the exposed tryptophan.^{13,14)} Oxidation of two tryptophan residues/mol caused a 57% loss of the fluorescence of ricin E (Fig. 3). This implies that most of the fluorescence of ricin E is contributed by the two tryptophans susceptible to the NBS oxidation.

It is noteworthy that the cytoagglutinating activity of ricin E decreased greatly during NBS oxidation, and such a loss of the cytoagglutinating activity is principally due to the modification of one tryptophan residue/mol (Table I). In the presence of lactose, however, one tryptophan residue/mol escaped from this modification reaction with a retention of the cytoagglutinating activity, suggesting the involvement of one tryptophan residue in the saccharide binding of ricin E. There is a possibility that NBS attacks both tryptophan and

tyrosine in proteins. However, the modification of tyrosine in this case can be disregarded by the evidence that the isosbestic point at 263 nm in the UV-absorption spectrum was retained during the NBS-oxidation. If tyrosine residues are modified with NBS, such a isosbestic point should not be obtained.¹⁵⁾ Accordingly, the lower cytoagglutinating activity of the active derivative, 1-oxa-ricin E(lac), compared to native ricin E may be explained by some conformational changes induced by modification of an additional tryptophan residue other than the tryptophan at the LA-site.

Physicochemical studies on the interaction of ricin E with its specific saccharides have indicated that the B-chain carries two saccharide-binding sites with different affinities, the high affinity binding site (HA-site) and the low affinity binding site (LA-site).⁷⁾ As demonstrated here, the binding of lactose to ricin E induces spectroscopic changes in this lectin (Figs. 3 and 4). A trough at 300 nm in the lactose-induced UV-difference spectrum has been interpreted as being due to a change in the environment of tryptophan¹⁶⁾ and is representative of the specific binding of lactose to the LA-site of ricin E, as well as the blue shift of the fluorescence spectrum induced by lactose binding.⁶⁾ Such a trough, however, is not found in the UV-difference spectrum generated by 2-oxa-ricin E (Fig. 4), and there is no change in the fluorescence spectrum of this derivative upon the addition of lactose (Fig. 3). These observations suggest the lack of the binding ability at the LA-site in the inactive derivative, 2-oxa-ricin E. On the contrary, the active derivative, 1-oxa-ricin E(lac) has a lactose-induced UV-difference spectrum identical to that given by native ricin E. This supports the above view and suggests that in 1-oxa-ricin E(lac) the tryptophan residue at the LA-site remains unmodified and the binding ability at this site is retained. On the other hand, GalNAc does not have any effect on the NBS-oxidation of tryptophan in ricin E (Fig. 2). Spectroscopic studies on the binding of saccharide to ricin E have suggested that the

HA-site can bind both galactopyranoside and GalNAc, while the LA-site can bind galactopyranosides but lacks the ability to bind GalNAc.⁷⁾ Taking these results into account, we postulate that lactose binds to ricin E in such a manner as to prevent the access of NBS to the tryptophan residue at the LA-site, but GalNAc is bound only by the HA-site without any effect on the susceptibility of the tryptophan residue at the LA-site to NBS.

The results mentioned above provide further insight into the saccharide-binding site of ricin E. It should be noticed that the susceptibilities of tryptophan residues to NBS in ricin E (Fig 2) are almost identical to those in ricin D,¹²⁾ in spite of the difference in amino acid sequence of the B-chain between two ricins.⁷⁾ As well as in the case of ricin E, ricin D found in the large-grain castor bean seeds has two saccharide-binding sites (HA- and LA-sites) per B-chain which is composed of two domains, a N-terminal half domain and a C-terminal half domain: the HA-site containing Tyr₂₄₈ is on the C-terminal half domain and the LA-site containing Trp₃₇ on the N-terminal half domain.^{19~21)} A comparison of the amino acid sequence of the B-chain of ricin E with those of other castor bean lectins have revealed that the B-chain of ricin E is composed of the N-terminal half of ricin D and the C-terminal half of castor bean hemagglutinin,⁶⁾ another type of lectin which is relatively non-toxic but a hemagglutinating protein.⁴⁾ In a preceding paper, we suggested that the saccharide-binding ability of the HA-site of ricin E was lower than that of ricin D, but there was no appreciable difference in the binding ability at the LA-site between two ricins.⁷⁾ Assuming that the B-chain of ricin E has

a domain structure similar to that of ricin D, it can be deduced that the LA-site of ricin E has the same conformation as that of ricin D and thereby the tryptophan residue which is important for saccharide binding at this site shows the same reactivity toward NBS as the tryptophan residue present at the LA-site of ricin D.

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