Identification of Three Oligosaccharide Binding Sites in Ricin

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ABSTRACT: The galactoside-binding sites of ricin B chain can be blocked by affinity-directed chemical modification using a reactive ligand derived from asialoglycopeptides containing triantennary N-linked oligosaccharides. The terminal galactosyl residue of one branch of the triantennary oligosaccharide is modified to contain a reactive dichlorotriazine moiety. Two separate galactoside-binding sites have been clearly established in the ricin B chain by X-ray crystallography [Rutenber, E., and Robertus, J. D. (1991) *Proteins 10*, 260−269], and it is necessary to covalently attach two such reactive ligands to the B chain to block its binding to galactoside affinity matrixes. A method was developed using thiol-specific labeling of the ligand combined with subsequent immunoaffinity chromatography which allowed the isolation of ricin B chain peptides covalently linked to the ligand from proteolytic digests of purified blocked ricin. The sites of covalent attachment of the two ligands in blocked ricin were inferred from sequence analysis to be Lys 62 in domain 1 of the B chain and Tyr 148 in domain 2. A minor species of blocked ricin contains a third covalently attached ligand. From the analysis of peptides derived from blocked ricin enriched in this species, it is inferred that Tyr 67 in domain 1 is the specific site on the ricin B chain where a third reactive ligand becomes covalently linked to the protein. These results are interpreted as providing support for the notion that the ricin B chain has three oligosaccharide binding sites.

Ricin, an extremely cytotoxic protein extracted from the castor bean, *Ricinus communis*, is the most well-characterized example of the large class of ribosome-inactivating proteins found in plants (*1*, *2*). Ricin is a heterodimer comprised of two disulfide-linked polypeptide chains (*3*). The A chain (M_r = 30 500) is a specific *N*-glycosidase that cleaves adenine A_{4324} from the rRNA of the large ribosomal subunit (*4*), while the B chain (M_r = 32 000) is a galactoside-specific lectin (*3*). Through the B chain, ricin binds to terminal galactosyl moieties of glycoproteins and/or glycolipids found on the surface of virtually all eukaryotic cells (*5*). Intoxication of cells is thought to require internalization of the ricin (*6*−*8*) and, ultimately, the transport of (at least) the A chain across an intracellular membrane into the cytosol (*9*, *10*) where the free A chain inactivates ribosomes causing cell death.

The ricin B chain binds simple sugars, such as galactose or lactose, at two independent sites with K_a values in the range of 10^3−10^4 M^{-1} (*11*−*13*). Analysis of the amino acid sequence of ricin and the determination of its three-dimensional structure by X-ray crystallography showed that the B chain folds into two homologous, topologically similar domains (domains 1 and 2), each of which is comprised of three homologous subdomains (α, *β*, and *γ*) and a "linker" peptide (*λ*) (*1*, *14*−*16*). It has been proposed that the three homologous subdomains in each domain arose through duplication of an ancestral 40-amino acid galactoside-binding peptide (*1*, *16*, *17*), although only two of the subdomains, one from each domain, bound galactose in the X-ray crystal. The galactoside-binding pockets are bounded on one side by the aromatic side chains of Trp 37 and Tyr 248 in subdomains 1α and 2*γ*, respectively, and on the other side by three-residue kinks in the polypeptide chain at residues 24−26 in subdomain 1α and 236−238 in subdomain 2*γ*. These two galactoside-binding sites are located at the opposite ends of the B chain and are separated by about 75 Å (*16*).

Ricin binds to galactosyl moieties on cell surfaces with a much higher affinity than to simple mono- or disaccharides (*18*). Most cell surface galactosyl residues are components of complex oligosaccharides with structures similar to those that can be isolated from the glycoprotein fetuin (*19*). The asialotriantennary N-linked oligosaccharide from asialofetuin is reported to bind to ricin with a K_a value of $>10^7$ M^{-1} (*19*). Little is known about the interactions between the ricin B chain and such complex oligosaccharides that can explain the 1000-fold higher affinity relative to galactose or lactose; attempts at analysis by X-ray crystallography of such lectin−ligand complexes have not been revealing (*1*). Mutational analysis using recombinant ricin B chain (*20*−*22*) has confirmed that residues identified by X-ray crystallography as being involved in galactoside binding are also involved in binding the complex oligosaccharides of asialofetuin, while analysis using single binding domains of the ricin B chain has shown that each domain of the ricin B chain binds independently with high affinity to asialofetuin (*23*, *24*). Presumably, in each domain of the ricin B chain, a terminal galactosyl residue of one branch of a complex oligosaccharide can bind to the galactoside-binding pocket of the domain, while other structural features of the branched oligosaccharide interact with other parts of the protein surface, contributing additional binding energy.

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The galactoside-binding sites of ricin can be blocked by affinity-directed chemical modification using a reagent derived from glycopeptides containing triantennary N-linked oligosaccharides. One branch of the triantennary structure is modified to contain a reactive dichlorotriazine moiety, while the other two branches contain terminal galactosyl residues to allow specific binding to the ricin B chain (*25*). Ricin that has both of its galactoside-binding sites blocked by covalent attachment of two such reactive ligands to the B chain (blocked ricin) is not able to bind galactoside affinity matrixes, and has its cytotoxicity reduced about 1000-fold compared to that of native ricin. Blockade of only one galactoside-binding site on the B chain (modified ricin containing only one covalently linked ligand) did not abrogate the binding of ricin to galactoside affinity columns and only reduced the cytotoxicity by about 10-fold relative to that of native ricin (*25*).

In this paper, we describe the location of the sites of covalent modification in blocked ricin. Furthermore, we have identified a specific site on ricin B chain where a third reactive oligosaccharide ligand becomes covalently linked to the protein. These data thus provide direct evidence showing that the ricin B chain can bind three oligosaccharide ligands at each of three specific sites on the B chain. A technique in which a combination of thiol-specific labeling of the ligand with subsequent immunoaffinity chromatography was used was developed (*26*) which allowed us to isolate and identify the modified residues.

EXPERIMENTAL PROCEDURES

Materials

Ricin [ricin D according to the nomenclature of Wei and Koh (*27*)] was from Inland Laboratories (Austin, TX). 2-(4′-Maleimidylanilino)naphthalene-6-sulfonic acid, sodium salt (MIANS)[1] was purchased from Molecular Probes, Inc. (Eugene, OR). Endoproteinase Lys C (endo Lys C) from *Achromobacter* was purchased from Wako Pure Chemical Industries Co., Ltd. (Osaka, Japan), and sequencing grade chymotrypsin was purchased from Boehringer Mannheim (Indianapolis, IN). Protein A−Sepharose CL-4B, Protein G−Sepharose CL-4B, and *N*-succinimidyl-3-(2-pyridyldithio) propionate (SPDP) were purchased from Pierce Chemical Co. (Rockford, IL). DE-52 cellulose was purchased from Whatman (Fairfield, NJ), while Sephadex G25 resin and the Superdex Peptide HR 10/30 column (separation range of 100−7000 Da) were from Pharmacia (Uppsala, Sweden). Keyhole limpet hemocyanin (KLH) and dimethyl pimelimidate (DMP) were from Sigma Chemical Co. (St. Louis, MO). A Zorbax SB300 C18 column (4.6 mm × 250 mm) was purchased from MacMod (Chadds Ford, PA). Other chemicals were purchased commercially at the highest available grade.

Preparation of Blocked Ricin. Blocked ricin was prepared from ricin D by affinity-directed chemical modification of the galactoside-binding sites using a reactive affinity ligand derived from glycopeptides containing triantennary N-linked oligosaccharides, as described previously (*25*, *28*). One branch of the triantennary oligosaccharide is modified to contain a terminal residue of 6-(*N*-methylamino)-6-deoxy-β-D-galactoside which can be activated by reaction with cyanuric chloride. The dichlorotriazine derivative of the ligand so formed will, upon binding of the ligand to ricin, react covalently with an accessible nucleophile of the protein, thereby stabilizing the ligand−binding site interaction (see the first reaction shown schematically in Figure 1).

The reaction conditions were optimized (0.5 mg/mL ricin, a 3-fold molar excess of activated ligand, pH 8.0, and incubation for 48 h at 25 °C) so that the major species that formed was blocked ricin containing two covalently linked ligands (*25*, *29*). The blocked ricin was purified from residual native ricin and partially blocked ricin (species having only one covalently linked ligand) by cation exchange chromatography followed by galactoside affinity chromatography using conditions described previously (*29*). Using these conditions, a small amount of ricin (about 10%) was obtained that had three ligands attached to its B chain [modification with higher concentrations of ligand increased the proportion of this species (*29*)]. This species could be enriched by a fractionation scheme utilizing cation exchange chromatography as described by Grossbard et al. (*29*). The resulting preparation of this fraction of blocked ricin had about 80% of its B chain covalently linked to three oligosaccharide ligands, as judged by polyacrylamide−sodium dodecyl sulfate gel electrophoresis (see the Results).

Preparation of an Immunoaffinity Column That Binds MIANS-Modified Proteins and Peptides. A hybridoma expressing a murine IgG_1 monoclonal antibody that recognizes MIANS-modified proteins and peptides was developed from mice immunized with MIANS−KLH, an immunogen prepared by modification of KLH [5 mg in 0.5 mL of 0.1 M sodium borate buffer (pH 8.5)] with SPDP (0.5 mM), followed by treatment with dithiothreitol (2 mM) and then purification by gel filtration prior to incubating the thiolated KLH (1.6 mg/mL) with MIANS (0.3 mM) at pH 7.4. Hybridoma clones were screened for binding to MIANS−BSA, a preparation of bovine serum albumin where the endogenous thiol of the protein was modified with MIANS. The selected monoclonal antibody, LG85, was produced as ascites fluid, and the antibody was purified by affinity chromatography on a column of Protein A−Sepharose CL-4B, using 0.1 M Tris-HCl buffer (pH 8.9) containing 3.0 M NaCl, for binding to the column and subsequent column washing, and 0.1 M HOAc containing 0.15 M NaCl (pH 2.8) for the elution. Purified LG85 was stored frozen at −80 °C after dialysis into 10 mM potassium phosphate buffer (pH 7.2) containing 0.145 M NaCl (PBS).

An immunoaffinity resin was prepared by binding the LG85 antibody to a column of Protein G−Sepharose CL-4B (2 mg of LG85 per milliliter of resin) in PBS and then protein−protein cross-linking by treating the column with 1 column volume of DMP (a 20 mM freshly prepared solution) in 0.1 M sodium borate buffer (pH 9.0) for 30 min at ambient temperature. Unreacted cross-linker was blocked by treating the column with ethanolamine (0.1 M) in the pH 9.0 buffer for an additional 30 min. The column was then washed with 0.1 M glycine-HCl buffer (pH 2.7) to remove any non-cross-linked LG85. MIANS-labeled blocked ricin (see below) binds

[1] Abbreviations: MIANS, 2-(4′maleimidylanilino)naphthalene-6-sulfonic acid; endo Lys C, endoproteinase Lys C from *Achromobacter*; SPDP, *N*-succinimidyl-3-(2-pyridyldithio)propionate; KLH, keyhole limpet hemocyanin; PBS, 10 mM potassium phosphate and 145 mM NaCl (pH 7.2); TFA, trifluoroacetic acid; DMP, dimethyl pimelimidate.

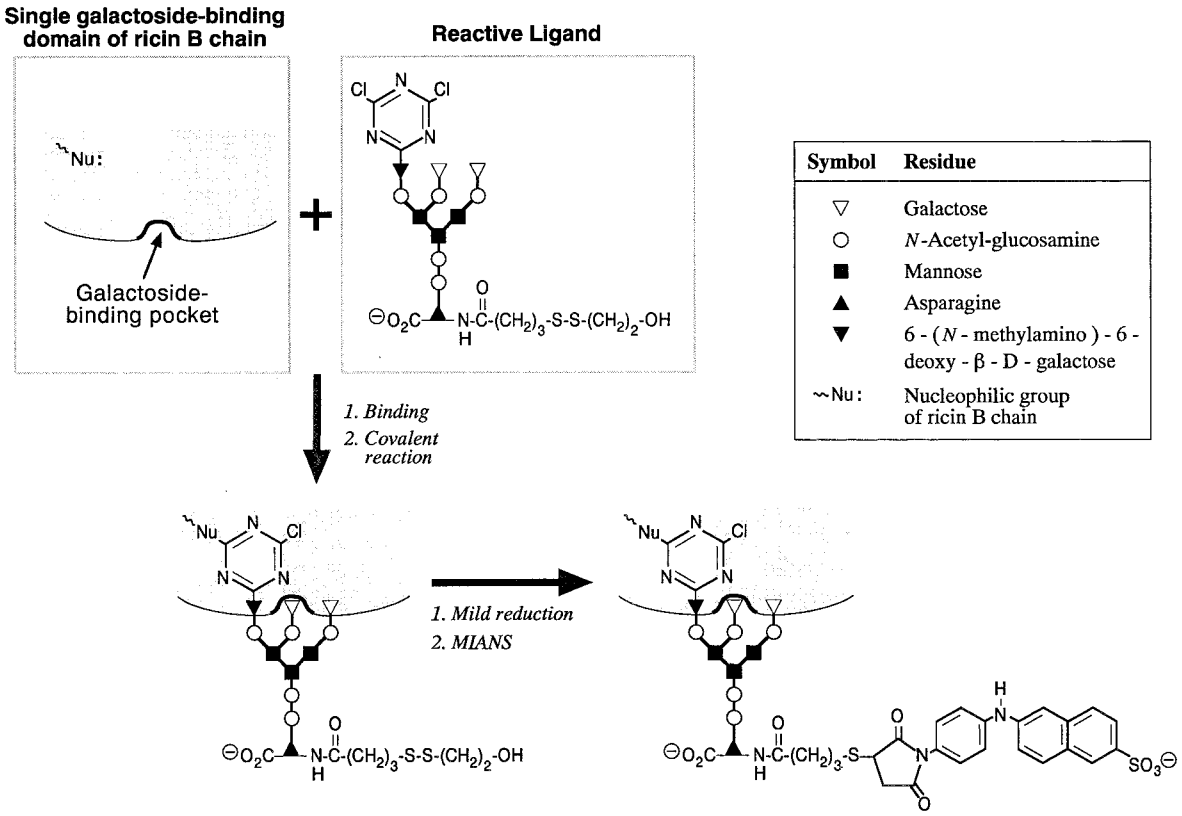

FIGURE 1: Schematic representation of the reaction between a single galactoside-binding domain of the ricin B chain and a reactive affinity ligand, and the subsequent tagging of the covalently bound affinity ligand by MIANS.

to this resin in PBS containing up to 1.0 M guanidine-HCl. Elution required 4.0 M guanidine-HCl in 0.1 M sodium citrate/phosphate buffer (pH 2.9). Even under this elution condition, the immunoaffinity resin could be used for several cycles.

Methods

Polyacrylamide Gel Electrophoresis. Samples of protein were analyzed by gel electrophoresis in the presence of sodium dodecyl sulfate in 12% (w/v) polyacrylamide gel slabs (80 mm × 80 mm × 1.5 mm) purchased from Novex (San Diego, CA). Samples run under reducing conditions were prepared in buffers containing 2% (v/v) 2-mercaptoethanol. Gels were stained with Coomassie brilliant blue R-250.

Measurement of the Protein Concentration and the Extent of MIANS Labeling by Spectrophotometry. Concentrations of solutions of ricin (M_r = 62 500) and the B chain (M_r = 32 000) were determined from their absorbance at 280 nm using ϵ_{280} values of 73 750 and 47 360 M^{-1} cm^{-1}, respectively (*30*). Modification of the ricin B chain with reactive ligand (linked ligand M_r of 2422) has a negligible effect on A_{280} (*25*). The degree of modification of ricin or the ricin B chain with MIANS was assessed using an ϵ_{320} value of 20 000 M^{-1} cm^{-1} (*31*), and an experimentally determined value of 0.9 for the A_{280}/A_{320} ratio for MIANS to deduce the A_{280} due to protein.

Specific Labeling of Ligands Covalently Bound to the Ricin B Chain by Reaction with MIANS. The N-terminus of the peptide portion of the reactive affinity ligand was linked via an amide bond to a disulfide group (*25*, *32*) which, upon selective reduction, generates a free thiol for conjugation or for labeling as shown schematically in Figure 1 (second reaction sequence). Samples of blocked ricin (3 mg/mL), including samples enriched in species having a third covalently linked ligand, were reduced by incubation with dithiothreitol (4.5 mM) in PBS, adjusted to pH 6.8, for 17 h on ice. Excess dithiothreitol was removed by gel filtration at 4 °C through a column (sample load of no more than 15% of the column volume) of Sephadex G25 (fine), equilibrated with 5 mM NaOAc buffer (pH 4.7) containing 50 mM NaCl and 0.5 mM EDTA. Under these conditions, only the disulfide bond of the ligand (see Figure 1) was reduced, while the disulfide bond between the A and B chains of ricin remained intact, as shown by polyacrylamide−sodium dodecyl sulfate gel analysis under nonreducing conditions (*28*). The four intrachain disulfide bonds of the B chain are not susceptible to reduction except under denaturing conditions (*33*), and the cysteine residue, Cys 171, of the A chain is inaccessible to modification except under denaturing conditions (*34*).

The protein concentration was then adjusted to 1 mg/mL, and the concentration of sulfhydryl groups was measured using the Ellman's assay (*35*). MIANS, from a freshly prepared stock solution (10 mM) in 0.1 M Tris-HCl buffer (pH 7.4), was added, and the pH of the solution was adjusted to pH 7.4 using 1.0 M Tris base. The molar ratio of MIANS to sulfhydryl groups ranged from 0.9 to 20, in different experiments. After incubation for 1 h at 37 °C, any unreacted MIANS was blocked by incubation with an equimolar concentration of cysteine for 1 h. Excess sulfhydryl groups were finally alkylated by incubation with a 5-fold molar excess of iodoacetamide for 30 min. Excess reagents were removed by dialysis into 0.1 M Tris-HCl buffer (pH 7.7).

Separation of the A and B Chains of MIANS-Labeled Blocked Ricin Samples. The disulfide bond between the A and B chains of samples of blocked ricin was reduced, and the chains were separated by using a modification of the method of Olsnes and Pihl (*3*). 2-Mercaptoethanol (4.5% v/v) was added to 10 mg of each MIANS-labeled sample (1 mg/mL in pH 7.7 buffer), and the samples were then incubated at ambient temperature for 20 h. The pH was raised to 8.5 with 0.1 M Tris base, and the protein (10 mg) was applied to a column of DE-52 anion exchange resin (1.5 cm × 6.5 cm) equilibrated in 0.1 M Tris-HCl buffer (pH 8.5) containing 0.1% (v/v) 2-mercaptoethanol. Under these conditions, the A chain does not bind to the resin. The column was then washed with equilibration buffer (20 mL) followed by 0.1 M Tris-HCl buffer (pH 8.5) without 2-mercaptoethanol (40 mL). The blocked ricin B chain was eluted with 0.1 M Tris-HCl buffer (pH 8.5) containing 1.0 M NaCl, and concentrated to approximately 2 mg/mL by ultrafiltration.

Carboxymethylation and Enzymatic Digestion of the MIANS-Labeled Blocked Ricin B Chain. Solid guanidine-HCl was added to a final concentration of 6.0 M to each preparation of the MIANS-labeled blocked ricin B chain in pH 8.5 buffer, and each solution was then treated with dithiothreitol (20 mM) for 16 h at 37 °C to effect complete reduction of protein disulfide bonds. The reduced samples were carboxymethylated by treatment with iodoacetic acid (100 mM) for 1 h at ambient temperature. 2-Mercaptoethanol was added to quench the reaction, and the samples were then dialyzed against 0.1 M Tris-HCl buffer (pH 8.5).

Guanidine-HCl (to a final concentration of 6.0 M) was again added to the reduced, carboxymethylated protein samples, which were then incubated at 50 °C for 30 min to ensure complete denaturation. Samples (about 1 mg/mL protein) were diluted by adding 2 volumes of 0.1 M Tris-HCl buffer (pH 8.5) and digested for 5 h at 37 °C with endo Lys C (1:20 enzyme:substrate, by weight). Chymotrypsin (1:20 by weight) was then added, and digestion continued at ambient temperature for an additional 20 h. The digestion was stopped, and the enzymes were inactivated by adding the reaction mixture to 3 volumes of boiling methanol (78 °C) and incubating at 78 °C for 3 min. Methanol was removed by rotary evaporation.

The extent of the digestion was evaluated using a Superdex Peptide HR 10/30 column equilibrated with 0.1 M Tris-HCl buffer (pH 8.5) containing 2.0 M guanidine-HCl, connected to a Hitachi L-6200 Intelligent Pump and L-4000 UV/vis detector. Reverse phase HPLC cannot be used to evaluate the digestion of the blocked ricin B chain because the ligand by itself gives a complex profile, making it difficult to assess the peptide map (*26*). Elution positions were calibrated by reference to a sample of the affinity ligand (nonactivated; see ref *25*) that had been reduced and then modified with MIANS, using the same reaction conditions as described above for blocked ricin.

Affinity Purification of Ligand-Containing Peptides. Samples of digested MIANS-labeled blocked B chain preparations (2 mg) were diluted to about 0.5 M guanidine-HCl by adding 3 volumes of 0.1 M Tris-HCl buffer (pH 8.5) and then applied to an immunoaffinity column (1.5 mL) of LG85 (described above) equilibrated in 0.1 M Tris-HCl buffer (pH 8.5). The resin was washed with the pH 8.5 buffer until the absorbance of the column outflow was zero, and then further washed with about 4 column volumes of 0.1 M sodium citrate/phosphate buffer (pH 2.7). MIANS-labeled peptides were finally eluted from the column using the pH 2.7 buffer, containing 4.0 M guanidine-HCl. The absorbance of fractions was measured at both 320 and 280 nm, and the yield of MIANS-labeled peptides was determined from the A_{320}.

Sequencing of Peptides. Immunoaffinity-purified peptides were desalted using a Zorbax SB300 C18 RP-HPLC column (4.6 mm × 250 mm). The column was equilibrated in 0.1% (v/v) trifluoroacetic acid (TFA) in water at a rate of 0.5 mL/min. After application of each sample (approximately 2−4 nmol/sample), the column was washed for 15 min with the equilibration solution, and then the bound peptides were eluted with 0.1% TFA (v/v) in 10% water/90% acetonitrile (v/v). The peptide solutions were concentrated on a Savant Speed Vac instrument and submitted to J. Leszyk at the Core Laboratory for Protein Microsequencing and Mass Spectrometry at the University of Massachusetts Medical School (Worcester, MA) for sequencing. Modified amino acids (sites of covalent coupling to the reactive group of the affinity ligand) were inferred during the sequencing by the absence of an identifiable peak (a blank cycle at the position in the sequence).

RESULTS

MIANS Labeling of the Covalently Linked Ligands of the Ricin B Chain. Treatment of blocked ricin with dithiothreitol under mild conditions generated about 1.2−1.5 free sulfhydryl groups per molecule of blocked ricin, which were then modified by reaction with the maleimido group of the reagent, MIANS. Analysis by polyacrylamide−sodium dodecyl sulfate gel electrophoresis under nonreducing conditions showed that the disulfide bond between the A and B chains remained untouched during this reaction sequence (Figure 2a).

The MIANS-modified blocked B chain was separated from the A chain by an anion exchange step using columns of DE-52 cellulose. Analysis of fractions by polyacrylamide−sodium dodecyl sulfate gel electrophoresis (Figure 2b) shows that the A chain was not retained by the column (lane 2) while the blocked B chain was retained and subsequently eluted with 1.0 M NaCl (lane 3). The A chain exhibited little absorbance at 320 nm, equivalent to the absorbance for 0.1 equiv of MIANS per A chain. The purified blocked B chain (about 74% yield) exhibited absorbance at 320 nm equivalent to 1.0−1.1 equiv of MIANS per sulfhydryl group originally measured in blocked ricin (5 equiv of MIANS per sulfhydryl group was used for labeling). When 0.9 equiv of MIANS was used to modify sulfhydryl groups of reduced blocked ricin, the resulting purified blocked ricin B chain contained 0.9 equiv of MIANS per original sulfhydryl group.

In the case of blocked ricin containing three covalently linked ligands, the yield of the purified blocked B chain was relatively low (63%) and analysis by polyacrylamide−sodium dodecyl sulfate gel electrophoresis (Figure 2c) shows that the proportion of blocked B chain with three ligands falls from about 80% in the starting material (lane 1) to about 50% in the purified blocked B chain preparation (lane 3). It is likely that such selective loss is due to poor elution of those molecules modified with MIANS at more than one site, due both to hydrophobic interactions and to charge

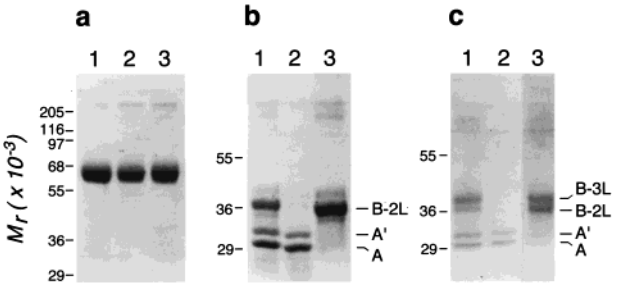

FIGURE 2: Polyacrylamide−sodium dodecyl sulfate gel analysis of MIANS-labeled blocked ricin and the purified blocked ricin B chain. (a) Electrophoresis under nonreducing conditions: lane 1, blocked ricin; lane 2, blocked ricin following reduction with dithiothreitol, followed by gel filtration to remove dithiothreitol; and lane 3, blocked ricin following reduction (as for the sample in lane 2) and subsequent labeling with MIANS. (b) Electrophoresis under reducing conditions: lane 1, MIANS-labeled blocked ricin; lane 2, nonretained protein fraction following anion exchange chromatography; and lane 3, protein fraction eluted from anion exchange resin by 1.0 M NaCl. (c) Same as panel b except that the samples were from MIANS-labeled blocked ricin containing three covalently linked ligands. The gels were calibrated with the following marker proteins (M_r): carbonic anhydrase (29 000), lactate dehydrogenase (36 500), glutamate dehydrogenase (55 000), bovine serum albumin (68 000), phosphorylase *b* (97 000), β-galactosidase (116 000), and myosin (205 000). The bands corresponding to the monoglycosylated ricin A chain (M_r = 30 000), the diglycosylated ricin A chain (M_r = 32 000), and the ricin B chain covalently linked to two (M_r = 37 500) or three (M_r = 40 000) affinity ligands are denoted by A, A′, B-2L, and B-3L, respectively, in panels b and c.

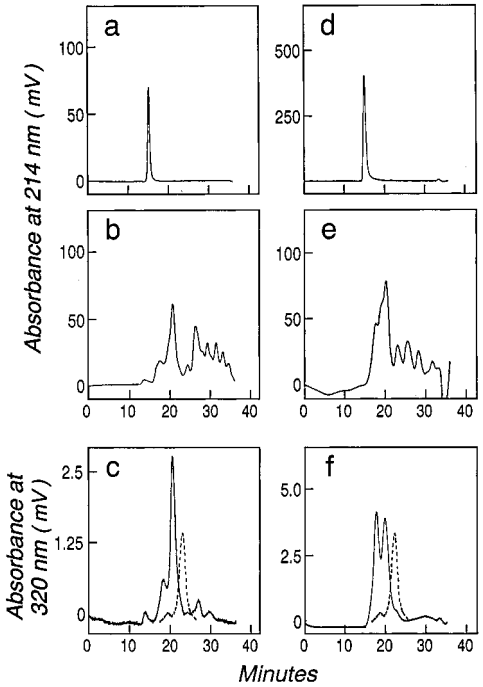

FIGURE 3: Analysis of proteolytic digests of samples of MIANS-modified blocked ricin by gel filtration. Samples were run on a Superdex Peptide HR 10/30 column at a rate of 0.5 mL/min using 0.1 M Tris-HCl buffer (pH 8.5) containing 2.0 M guanidine-HCl. Elution profiles were generated by monitoring the column effluent at 214 (detects peptide) and 320 nm (detects the anilinonaphthalene-6-sulfonic acid tag on the affinity ligand): (a) analysis of undigested blocked ricin, (b) analysis of peptides generated by treatment of blocked ricin with endo Lys C and chymotrypsin, (c) the same as panel b, except that detection was at 320 nm, (d) analysis of undigested blocked ricin containing three covalently linked ligands, (e) analysis of peptides generated by treatment of blocked ricin having three covalently linked ligands with endo Lys C and chymotrypsin, and (f) the same as panel e, except that detection was at 320 nm. The dotted lines drawn in panels c and f represent the elution profiles of a standard sample of MIANS-modified ligand ($M_r \sim 2500$).

interactions between the sulfonate groups and the DE-52 matrix. Nevertheless, the recovery of modified protein was sufficient for subsequent analysis.

Proteolytic Digestion of the MIANS-Modified Blocked B Chain. The blocked B chain proved to be extraordinarily resistant to proteolysis. Complete digestion was only possible after denaturation in 6.0 M guanidine-HCl together with complete reduction and carboxymethylation of all cysteine residues. Furthermore, the two-step digestion with endo Lys C and chymotrypsin needed to be performed in the presence of 2.0 M guanidine-HCl. The digests were evaluated by gel filtration using a Superdex Peptide HR 10/30 column as shown in Figure 3. The elution profiles were monitored at both 214 and 320 nm, the latter for locating MIANS-modified ligand−peptide complexes. The analysis shows that there was no undigested material remaining following proteolysis of either the blocked B chain or the preparation containing the blocked B chain having three ligands (Figure 3; compare panels b and e to panels a and d). Reincubation with the proteases did not result in any detectable change in the elution profiles shown in panels b and e, suggesting again that the digestion was complete.

In the case of the blocked B chain, the gel filtration elution profile at 320 nm exhibits one major peak of MIANS-modified material (Figure 3c), eluting significantly earlier (at 20.5 min) than a standard sample of MIANS-modified ligand (22.5 min). Since the latter has an M_r of about 2500, an apparent M_r in the range of 3500−4500 can be estimated for the MIANS-modified material derived from the blocked B chain.

The profile of the MIANS-labeled material from a digest of the preparation of the blocked B chain which comprised about 50% of the B chain having three covalently linked ligands exhibits two major peaks in roughly equivalent amounts (Figure 3f). The lower-molecular weight species has an elution time (20.5 min) that is identical to that of the major peak found in the digest of the blocked B chain. The higher-molecular weight peak (17.5 min) has an elution time corresponding to an apparent M_r in the range of 7000−8000. These data are consistent with the peaks measured at 320 nm being MIANS-labeled ligands covalently linked to different peptides from the ricin B chain.

Immunoaffinity Purification of B Chain Peptides Modified with MIANS-Labeled Ligand. The MIANS-labeled peptides were purified from the digests by immunoaffinity chromatography using an immobilized monoclonal antibody selected for high-affinity binding to MIANS-modified protein. Analysis of the nonretained fractions and bound fractions using the Superdex Peptide HR 10/30 column showed that virtually all the material having absorbance at 214 nm was not retained by the column (Figure 4a); the bound material that was subsequently eluted with guanidine-HCl at low pH exhibited very little absorbance at 214 nm (panel b). In contrast, analysis of the column profiles at 320 nm demonstrated that most of the MIANS-modified peptide was bound by the column since there was almost no absorbance at 320 nm in the nonretained material (Figure 4c). The shape of the

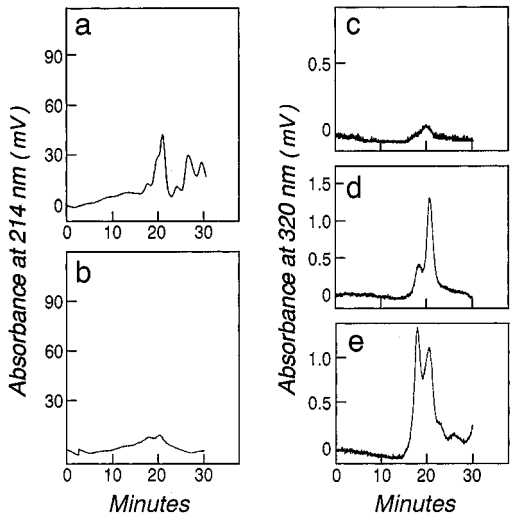

FIGURE 4: Analysis by gel filtration of B chain peptides fractionated by immunoaffinity purification selective for MIANS-labeled peptides. Samples were run on a Superdex Peptide HR 10/30 column at a rate of 0.5 mL/min using 0.1 M Tris-HCl buffer (pH 8.5) containing 2.0 M guanidine-HCl: (a) elution profile at 214 nm of peptides (derived by digestion of MIANS-labeled blocked ricin) that were not retained by the immunoaffinity column, (b) elution profile at 214 nm of bound peptides (derived by digestion of MIANS-labeled blocked ricin) that were eluted from the immunoaffinity column using guanidine-HCl at low pH, (c) same as panel a, except that the elution profile was at 320 nm, (d) same as panel b, except that the elution profile was at 320 nm, and (e) same as panel d, except that the peptides bound to the immunoaffinity column were derived by digestion of MIANS-labeled blocked ricin having three covalently linked affinity ligands.

analytical gel filtration profile at 320 nm of the material eluted with guanidine-HCl at low pH (Figure 4d) was very similar to that of the unfractionated digest (compare with Figure 3c), indicating that there was no selective loss of MIANS-labeled material.

Similarly, for the MIANS-labeled peptides from the B chain having three covalently linked ligands, the elution profile of the immunoaffinity-purified material at 320 nm (Figure 4e) looked comparable to that before purification (compare with Figure 3f). Approximately 15 nmol of MIANS-labeled peptide was recovered from each column run, as determined from the absorbance measurement taken at 320 nm.

Sequence of Ricin B Chain Peptides Modified with MIANS-Labeled Ligand. The complete sequences of the immunoaffinity-purified peptides from the digestion of the blocked B chain (Figure 4d) were obtained with a Procise sequencer (Perkin-Elmer) and are shown in Table 1. Only two major peptide sequences were present. One peptide corresponded to residues 53−74 of domain 1 of the ricin B chain, except that Lys 62 was absent, while the second peptide corresponded to residues 141−152 of domain 2 of the B chain, except that Tyr 148 was absent. Cysteine residues in these peptides were identified as carboxymethylcysteine and thus were not sites of modification with MIANS. We infer that the blank cycles in the sequencing, Lys 62 and Tyr 148, correspond to the sites of covalent reaction of the ricin B chain with the reactive dichlorotriazine group of the oligosaccharide ligand (see Figure 1). The apparent M_r of the single major peak obtained via gel filtration (see Figure 3c), estimated to be about 3500−4500, is consistent with the peak containing a mixture of oligosaccharide ligand ($M_r \sim 2500$) covalently linked to either residues 53−74 ($M_r = 2465$) or residues 141−151 ($M_r = 1251$).

Surprisingly, when the immunoaffinity-purified peptides from the blocked B chain that contained three covalently linked ligands (about 50% of the purified blocked B chain preparation) were sequenced, again only two major peptide sequences were present. Despite the fact that the MIANS-labeled material contained an additional major peak with a higher molecular weight (see Figure 4e), these peptides corresponded to the same two sequences of the ricin B chain previously found for blocked ricin having only two covalently linked ligands (Table 1).

A portion of the immunoaffinity-purified peptides from the blocked B chain containing three covalently linked ligands was then further submitted to gel filtration on the Superdex Peptide HR 10/30 column, and the first peak with a higher molecular weight (absorbing at 320 nm) was collected separately and sequenced. Only a single peptide was found, corresponding again to residues 53−74 of domain 1 of the ricin B chain. However, there were two cycles in which no amino acid was detected, corresponding to Lys 62 and Tyr 67. The apparent M_r of this peak, estimated to be about 7000−8000, is consistent with residues 53−74 ($M_r = 2465$) being covalently linked to two oligosaccharide ligands (each with an M_r of ∼2500). Close examination of the initial sequence data for the nonfractionated peptides showed that the yield of Tyr at position 67 was about half of that of the latter residue, Tyr 69, consistent with about half of this peptide being covalently linked to one ligand (at Lys 62) and about half being linked to two ligands (at Lys 62 and Tyr 67).

DISCUSSION

The amino acid residues of the ricin B chain covalently linked to reactive affinity ligands derived from glycopeptides containing triantennary N-linked oligosaccharides (*25*, *29*) were identified using an approach that exploited the disulfide moiety of the affinity ligand (the reactive affinity ligand was designed to be a heterobifunctional reagent) as a site for labeling with a purification tag. Free sulfhydryl groups, generated by specific reduction of the ligand disulfide, were modified with MIANS which allowed subsequent isolation of MIANS-labeled peptides using MIANS-specific immunoaffinity chromatography (*26*). Furthermore, reaction with MIANS provided a convenient method for following the purification of tagged peptides via absorbance (or fluorescence). This approach was developed after repeated attempts to purify ligand-modified peptides by standard chromatographic techniques had failed due to the highly complex elution profiles caused by the heterogeneous nature of the ligand itself (*26*). Model studies with the ricin A chain showed that the reaction of MIANS was specific for sulfhydryl groups under the conditions described here (*26*).

Our results suggest that the two ligands of blocked ricin were covalently linked to the B chain at Lys 62 and Tyr 148. The positions of these residues, and of aromatic residues Trp 37 and Tyr 248 which interact with the hydrophobic face of the galactosyl ring in each of the galactoside-binding pockets, are denoted in the diagram shown in Figure 5a of a model of the ricin B chain based on the X-ray crystal-

Table 1: Sequence of Ricin B Chain Peptides Covalently Linked to the Affinity Ligand[a]

sample[b]	peptide 1	peptide 2
1	R^{53}DNTIRSNG**X**^{62}CLTTYGYSPGVY^{74}	V^{141}TTIVGL**X**^{148}GLCL^{152}
2[c]	R^{53}DNTIRSNG**X**^{62}CLTT**Y**^{67}GYSPGVY^{74}	V^{141}TTIVGL**X**^{148}GLCL^{152}
3	R^{53}DNTIRSNG**X**^{62}CLTT**X**^{67}GYSPGVY^{74}	not detected

[a] **X** represents blank cycles in the sequencing. All cysteine residues (C) were identified as carboxymethylcysteine. [b] The samples analyzed are as follows: (1) MIANS-labeled peptides from the blocked B chain (two covalently linked ligands), (2) MIANS-labeled peptides from the blocked B chain containing three covalently linked ligands, and (3) MIANS-labeled peptide from the first-eluting peak from gel filtration of sample 2 (see Figure 4e). [c] **Y**^{67} was recovered at yields lower than that of Y^{69} in sample 2.

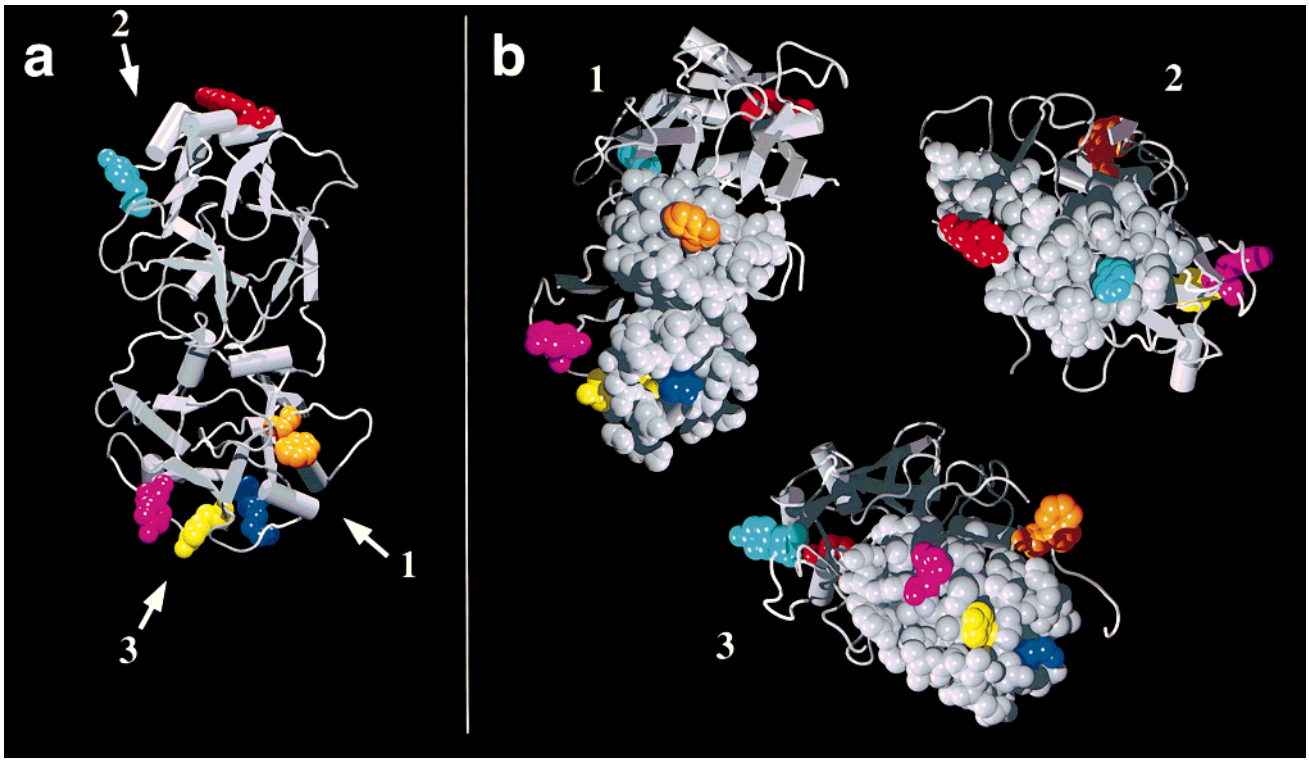

FIGURE 5: Models of the ricin B chain. (a) A model of the ricin B chain (262 amino acids) based on information available in the Brookaven PDB database (J. D. Robertus and M. P. Ready, 1991). The backbone of the B chain is shown as a cartoon representation based on the secondary structure of the residues with β strands shown as arrows and α helices as cylinders. Key amino acid residues that are illustrated in the test are shown with colored space-filled atoms: Trp 37, orange; Lys 62, blue; Tyr 67, magenta; Tyr 78, yellow; Tyr 148, red; and Tyr 248, cyan. The approximate locations of galactoside-binding sites 1 and 2 in domains 1 and 2, respectively, are denoted by the arrows (labeled 1 and 2); the third site described in the text is denoted by the arrow labeled 3. (b) Representations of each of the galactoside-binding sites labeled 1−3, showing with space-filled atoms all residues within 9 Å of the residues of interest. The rest of the B chain in each representation is shown as a cartoon with selected colored space-filled atoms as described for panel a. From the top left, clockwise are 1, a representation of binding site 1 in domain 1 showing with space-filled atoms (gray) all residues within 9 Å of either Trp 37 (orange) or Lys 62 (blue); 2, a representation of binding site 2 in domain 2 showing with space-filled atoms (gray) all residues within 9 Å of either Tyr 148 (red) or Tyr 248 (cyan); and 3, a representation of the site of binding of a third oligosaccharide ligand in domain 1 showing with space-filled atoms (mid gray) all residues within 9 Å of either Tyr 67 (magenta) or Tyr 78 (yellow).

lographic data (*1*). Also shown in Figure 5 are different views of the B chain which show representations of binding subdomain 1α (representation 1 in panel b) and binding subdomain 2γ (representation 2 in panel b) where all the residues within 9 Å of Trp 37, Lys 62, Tyr 248, and Tyr 148 are shown as space-filled atoms. From the crystallographic data, it can be calculated that the distance between the side chains of Trp 37 in site 1 and Lys 62 is about 19 Å, while the distance between Tyr 248 in site 2 and Tyr 148 is about 14 Å. The distances between galactosyl residues in an asialotriantennary N-linked oligosaccharide are in the range of 15−40 Å (*36*) so that two branches of the ligand (one bearing a dichlorotriazine derivative of 6-methylaminogalactoside; see Figure 1) could span the distance between the galactoside-binding pockets in each domain and the sites of modification at Lys 62 and Tyr 148 in domains 1 and 2, respectively (Figure 5b, representations 1 and 2, respectively).

The reaction of the dichlorotriazine moiety of the activated ligand with the phenolic hydroxyl group of tyrosine was unexpected, since dichlorotriazine likely reacts more readily with the primary amino groups of lysine (*37*). The results suggest that the triantennary ligand structure interacts with each binding site at many points over the surface of the binding subdomain in a specific manner, and not only with the shallow galactoside-binding pocket. The complex ligands thus bind in specific conformations which bring the dichlorotriazine groups into proximity with specific amino acids, a tyrosine residue in the case of site 2γ. Multiple weak interactions as the complex ligands lie on the surface of the protein can account for the fact that they bind to the B chain

with a much higher affinity (about 1000-fold) than galactose or lactose (*19*).

Our study of blocked ricin also included an analysis of the blocked ricin species having an additional ligand covalently attached to the B chain. We infer that Tyr 67 is the additional site of modification of the B chain by the third activated ligand in this molecule. The analysis of the X-ray crystallographic data of ricin by Robertus and colleagues suggested that the ricin B chain contained six homologous subdomains, each with about 40 amino acids, that were also homologous to a galactoside-binding peptide of a similar size found in *Dictyostelium discoideum* (*16*, *17*). X-ray analysis showed that two subdomains, 1α and 2γ, were able to bind lactose (*15*, *16*). However, further analysis suggested that subdomain 1β retained some of the essential features for galactose binding. A potential galactosyl-binding pocket formed by the aromatic side chain of Tyr 78 and a three-residue "kink" in the polypeptide backbone structure in subdomain 1β was postulated (*1*), although no binding was actually demonstrated by the X-ray analysis. Rutenber and Robertus (*1*) also reported that none of the other three homologous subdomains (1γ, 2α, and 2β) contained accessible binding pockets that could accommodate galactose.

Our results suggest that subdomain 1β can indeed bind galactosyl moieties. In the model of the ricin B chain shown in Figure 5a, tyrosine residues 78 (forming the hydrophobic face of the putative galactoside-binding pocket) and 67 (the site of modification in the B chain having three covalently linked ligands) are shown as space-filled atoms. Figure 5 also shows a different view of the ricin B chain featuring subdomain 1β (representation 3 in panel b) where all the residues within 9 Å of Tyr 78 and Tyr 67 are shown as space-filled atoms. The distance between the side chains of Tyr 78 and Tyr 67 is about 10 Å, again well within the range that can be spanned by two branches of an asialotriantennary ligand (*36*).

What other evidence is there to support the notion that the ricin B chain contains three functional binding sites? Blocked ricin having two covalently linked oligosaccharide ligands still does have a residual capacity to bind galactoside, albeit weakly (*25*, *28*). We have shown, in fact, that the target-specific potency of immunotoxins containing blocked ricin (the antibody component of such conjugates providing a means of high-affinity binding to selected cells) is dependent on the residual galactoside-binding capacity (*38*). Furthermore, covalent linkage of a third affinity ligand to the ricin B chain completely abrogates the residual galactoside binding of the blocked ricin and reduces its potency as an effector moiety of an immunotoxin by 5 orders of magnitude (*29*). These data have been interpreted as suggesting that a third galactoside-binding site may be important for the correct intracellular routing of ricin to a cellular compartment that allows for the efficient transport of the ricin A chain into the cytosol (*38*, *39*).

A recent mutational analysis of the ricin B chain has also provided evidence of three galactoside-binding sites in domains 1α, 1β, and 2γ (*22*), and specifically implicated Tyr 78 as being an important residue for galactoside binding to the 1β domain, as suggested by the X-ray crystallographic analysis (*1*). Ricin made with the "triple-mutant" B chain also exhibits little residual cytotoxicity.

Indirect evidence for a third galactoside-binding site in ricin B chain also comes from a study of the pH-dependent and concentration-dependent reversible self-association exhibited by blocked ricin (*40*). Galactose, or those sugars having the same orientation of hydroxyl groups as galactose at C2 and C4, have been shown to stabilize a noncovalent dimeric form of blocked ricin at low pH, a property that has also been attributed to the presence of a third galactoside-binding site on the B chain (*39*). The available evidence from X-ray analysis, mutational analysis, and the work described herein strongly indicates that such a site is located in subdomain 1β. However, the putative galactoside-binding site of subdomain 1β lacks residues equivalent to Asp 22 and Asp 46 in site 1α, and Asp 234 and Asp 255 in site 2γ, that form hydrogen bonds with the C3 and C4 hydroxyls of galactose (*1*). However, our previous studies suggest that the polar interactions stabilizing galactose binding to this site may involve bonding to the C2 and C4 hydroxyls (*39*), and thus, one would not expect the amino acids occupying equivalent structural positions to be conserved between putative site 1β and the well-characterized sites 1α and 2γ.

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