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Identification of a tyrosine residue in the saccharide binding site of ricin B-chain using *N*-[^{14}C]acetylimidazole

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The binding of ricin B-chain to Sepharose, a galactose-based adsorbent, was reversibly inactivated by acetylation of tyrosine residues in the absence of lactose. In the presence of lactose, two tyrosine residues were protected against modification and the B-chain retained its binding ability. Analyses of tryptic peptides from B-chain modified with *N*-[^{14}C]acetylimidazole in the presence and absence of lactose showed that Tyr-248 is present in one of the galactose-binding sites.

Ricin; B-chain; Saccharide binding; Tyrosine residue; *N*-[^{14}C]Acetylimidazole

1. INTRODUCTION

Ricin is a highly toxic glycoprotein isolated from the castor bean, *Ricinus communis*. It consists of two different subunits which are linked by a disulphide bond: the A-chain, which catalytically inactivates the 60 S ribosomal subunit in eukaryotes [1,2], and the B-chain, which contains two lactose-binding sites [3–5]. The toxin binds by means of the B-chain to galactose-terminating oligosaccharides present on the surface of nearly all cell types in higher animals. In subsequent steps of the intoxication process, the toxin is internalised by endocytosis and the A-chain is then translocated, probably across the membrane of the endocytic vesicle, into the cytosol where it inhibits protein synthesis (reviews [6,7]). Isolated ricin A-chain is practically non-toxic since it lacks the lectin property of the B-chain and so cannot bind to and enter cells.

Tyrosine residues were first implicated in the saccharide binding sites of ricin by chemical modification. The ability of ricin to bind to Sepharose, a copolymer of galactosyl and 3,6-anhydrogalactosyl residues, was found to be diminished reversibly by acetylation of tyrosine residues with *N*-acetylimidazole but binding ability was retained if acetylation was performed in the presence of a saturating concentration of lactose [8,9]. In order to locate the binding site residue(s), we synthesized *N*-[^{14}C]acetylimidazole and used it to label tyrosine residues in isolated B-chain. We chose to work with the B-chain rather than native ricin to facilitate the isolation of peptides containing *O*-[^{14}C]acetyltyrosine residues which are unstable in the presence of thiol reagents needed to separate the A- and B-chains in the intact toxin.

In common with ricin, the binding of ricin B-chain to Sepharose 4B was reversibly inactivated

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Abbreviations: RCA, *Ricinus communis* agglutinin; N-AcIm, *N*-acetylimidazole; PBS, phosphate-buffered saline, pH 7.2; HPLC, high-pressure liquid chromatography; DMF, dimethylformamide; TPCK, L-(tosylamido-2-phenyl)ethylchloromethylketone

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by acetylation of tyrosine residues in a lactose-protectable manner. Using N-[^{14}C]AcIm we were able to demonstrate that Tyr-248 was protected against modification by the presence of lactose suggesting that this tyrosine residue is involved in saccharide binding.

2. MATERIALS AND METHODS

2.1. *Chemical modification*

Ricin B-chain was isolated as described [10] and RCA B-chain was isolated by a similar procedure. B-chains were modified by adding aliquots of a freshly prepared solution of N-AcIm (Sigma, Dorset, England) at 1 M in dry DMF to the protein solution in 1 ml PBS or PBS containing 0.2 M lactose at 20°C. Reaction was terminated by rapidly desalting the protein solution on a column (10 cm × 1.6 cm diameter) of Sephadex G25(M) (Pharmacia) equilibrated with PBS. The level of tyrosine acetylation was determined spectrophotometrically from the increase in absorbance at 278 nm resulting from the deacetylation of *O*-acetyltyrosine residues on treatment with 0.5 M NH_2OH [11]. 1/10 vol. of 5 M NH_2OH solution adjusted to pH 7.0 with NaOH was added to the protein solution and to the solution in the reference cuvette; deacetylation of modified tyrosine residues was complete after 30 min at 25°C. The concentration of protein solutions was calculated after deacetylation assuming $E^{1\%}_{280\,nm}$, 1 cm = 14.9 [12].

2.2. *Radioacetylation*

N-[1-^{14}C]AcIm was prepared by adding 10 mg imidazole (BDH, Poole, England), dissolved in 1 ml dry pyridine, to 0.5 mCi of [1-^{14}C]acetic anhydride (112 mCi/mmol) obtained from Amersham, England. After 20 min at 20°C, one drop of acetic anhydride was added to acetylate the imidazole completely. The solvent and by-products of the reaction were removed by evaporation under vacuum. N-[^{14}C]AcIm was isolated by subliming the solid residue at 50°C into a pre-weighed glass tube. The product had a specific activity of 0.8–1.0 mCi/mmol. The number of [^{14}C]acetyl groups incorporated into protein was determined from the specific activity of the protein after desalting. Labelled samples were dissolved in Optiphase MP (LKB) and counted on an LKB Rackbeta liquid scintillation counter.

2.3. *Saccharide binding*

A measure of the extent of inactivation of saccharide binding by ricin B-chain due to acetylation was obtained by determining the proportion of acetylated protein (estimated from the absorbance at 280 nm) which retained affinity for Sepharose 4B equilibrated with PBS at 4°C. Bound protein was eluted from the column with 0.1 M galactose in PBS.

2.4. *Peptide isolation and characterisation*

Samples of unmodified and radioacetylated ricin B-chains in PBS were denatured by the addition of an equal volume of 20 mM sodium phosphate buffer, pH 6.8, containing 8 M urea. The protein was digested in this mixture by adding an aliquot of a 1 mg/ml solution of TPCK-treated trypsin (Worthington Enzymes, NJ, USA) to give a 1:30 ratio by weight of proteinase to substrate. After 1 h at 28°C, the proteinase was inactivated by adding a 1.2-fold molar excess of soybean trypsin inhibitor (Sigma). Tryptic peptides were separated by HPLC on a 5 μm particle reversed-phase column (25 cm × 0.4 cm diameter) of Lichrosorb RP18 (LKB Instruments, South Croydon, England) protected by an LKB Ultropac precolumn (3 cm × 0.4 cm diameter). Samples (0.5 ml) of the tryptic digests were subjected to a linear gradient of 0–50% (v/v) CH_3CN HPLC grade S (Rathburn Chemicals, Walkerburn, Scotland) in 0.1 M aqueous ammonium acetate, pH 7.0, with a flow rate of 1 ml/min at ambient temperature. Peptide fractions for amino acid analysis were freeze-dried, hydrolysed in 6 M HCl for 24 h at 110°C and analysed using a Beckman 6300 amino acid analyser. N-terminal amino acid sequencing was performed on an Applied Biosystems 470A sequenator; PTH-amino acids were identified by the Applied Biosystems 120A analyser.

3. RESULTS

In the absence of lactose, 4.2 (± 0.1) tyrosine residues of ricin B-chain were acetylated when the B-chain was reacted with 0.1 M N-AcIm at 20°C for 15 min or longer. After treatment of acetylated ricin B-chain with NH_2OH at pH 7 to regenerate tyrosine from *O*-acetyltyrosine residues [13],

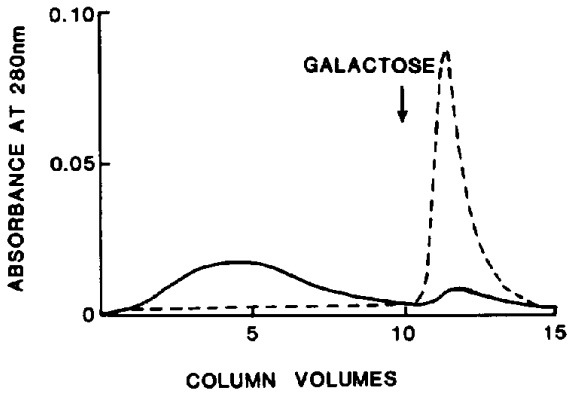

Fig.1. Reversal of saccharide-binding blockade by NH_2OH. Ricin B-chain containing 4.2 *O*-acetyltyrosine residues (——) was eluted from Sepharose 4B without galactose. After treatment with NH_2OH at pH 7.0, the deacetylated B-chain (– – –) was only eluted with 0.1 M galactose.

binding to Sepharose 4B was restored (fig.1). This demonstrated that loss of binding was due to the modification of tyrosine and not of other amino acids because residues of ϵ-*N*-acetyllysine and *O*-acetylserine or *O*-acetylthreonine are resistant to this treatment [13]. Lactose protected 2.0 tyrosine residues per B-chain against modification with 0.1 M N-AcIm for 15 min. More than 85% ricin B-chain modified in the presence of 0.2 M lactose retained the ability to bind to Sepharose 4B as compared with only 9% modified in the absence of lactose. Thus, one or more of the tyrosine residues that are protected against acetylation appear to be involved with saccharide binding. In contrast with ricin B-chain, acetylation of RCA B-chain under similar conditions led to the modification of 1.5 (± 0.1) tyrosine residues per B-chain subunit and inactivation of Sepharose-binding regardless of the presence or absence of lactose.

Amino acids other than tyrosine were also found to be modified when B-chain was reacted with N-[^{14}C]AcIm. In order to minimise this stable radioacetylation of non-tyrosine residues, the B-chain was reacted with a reduced concentration of N-[^{14}C]AcIm (5 mM) for 30 min at 20°C. In the absence of lactose, 1.7 of the 4.2 acetylatable tyrosine residues were modified. Lactose (0.2 M) protected 0.9 tyrosine residues against acetylation and also reduced the extent of radioacetylation of other amino acid residues stable to NH_2OH (table 1). Tryptic digests of unmodified ricin B-chain and B-chains radioacetylated in the presence or absence of 0.2 M lactose were analysed by HPLC. A com-

Table 1

Radioacetylation of tyrosine residues in ricin B-chain[a]

Modified residues	No lactose	0.1 M lactose
Total [^{14}C]acetyl	2.7	1.3
O-[^{14}C]Acetyl-Tyr[b]	1.7	0.8
Other [^{14}C]acetyl[c]	1.0	0.5

[a] Ricin B-chain at 1.50 mg/ml modified with 5 mM N-[^{14}C]AcIm

[b] Determined spectrophotometrically

[c] Determined by difference

Results expressed as mol/mol B-chain

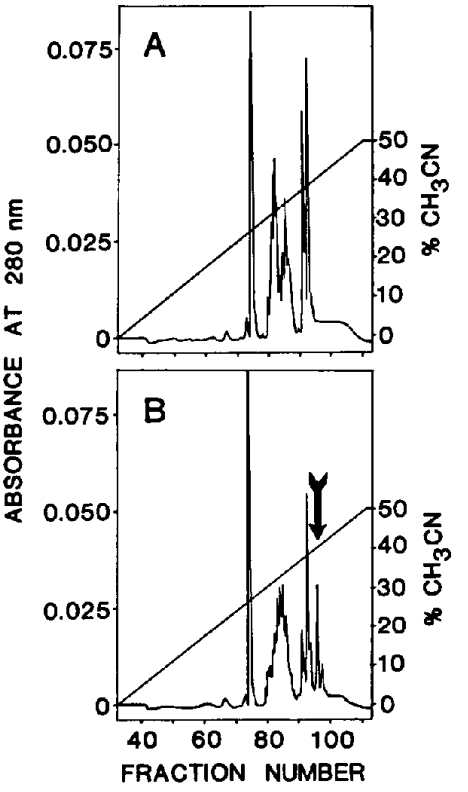

Fig.2. Reversed-phase HPLC chromatography of a tryptic digest of *O*-[^{14}C]acetyl ricin B-chain. (A) Unmodified ricin B-chain, (B) B-chain radioacetylated in the absence of 0.2 M lactose. Fraction 96 is denoted by the solid arrow.

parison of HPLC-chromatograms (fig.2) revealed the presence of a peptide (fraction 96) in the digest of B-chain which had been radioacetylated in the absence of lactose that was not present in the digest of unmodified B-chain. This peptide was highly radiolabelled and was virtually absent from the digest of B-chain radioacetylated in the presence of 0.2 M lactose (fig.3). The specific activity of the peptide was consistent with the incorporation of a single [^{14}C]acetyl group and its recovery indicated that about half of the radiolabel incorporated into all tyrosine residues of the B-chain was present in this peptide.

The radiolabelled peptide contained no lysine or arginine by amino acid analysis and had a composition which identified it as the C-terminal tryptic peptide of ricin B-chain, Gln-244–Phe-262 (table 2). N-terminal amino acid sequencing of the complete peptide gave a single detectable sequence (not shown) which matched precisely the primary structure of the C-terminal region of ricin B-chain as determined by direct amino acid sequencing of the protein [14] and as deduced from the nucleotide sequence of the ricin gene [15,16]. After treatment of the [^{14}C]acetyl peptide with NH_2OH, the peptide was eluted from the reversed-phase column at a slightly lower CH_3CN concentration, consistent with the removal of the hydrophobic acetyl group, and was no longer radiolabelled. This confirmed that the peptide, which contained no acetylatable cysteine, lysine, serine or threonine residues, was radioacetylated only on Tyr-248.

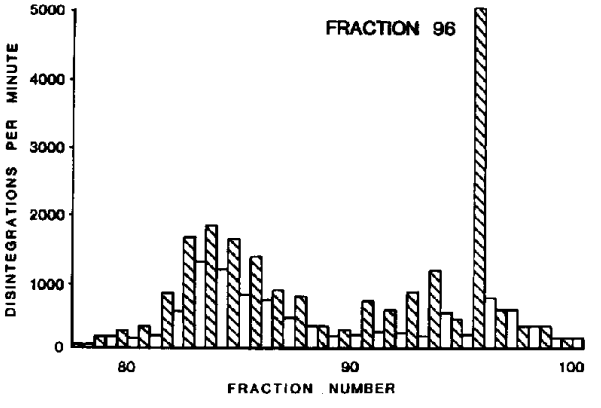

Fig.3. Radioactivity of tryptic peptide fractions of *O*-[^{14}C]acetyl ricin B-chains. Ricin B-chain radioacetylated in the presence of 0.2 M lactose (open bars) or in the absence of lactose (hatched bars).

Table 2

Amino acid analysis of ricin B-chain *O*-[^{14}C]acetyl peptide

Residue	Predicted[a]	Found
Asx	2	2.1
Thr	0	<0.1
Ser	0	<0.1
Glx	2	2.3
Pro	3	3.4
Gly	1	1.1
Ala	0	<0.1
Val	0	<0.1
Met	0	<0.1
Ile	3	2.8
Leu	4	4.0
Tyr	1	0.9
Phe	1	1.0
His	1	1.0
Lys	0	<0.1
Arg	0	<0.1

[a] Sequence of ricin B-chain residues 244–262

4. DISCUSSION

We synthesized N-[^{14}C]AcIm in order to label and identify tyrosine residues in the lactose-binding sites of ricin. *O*-Acetyltyrosine residues are labile in the presence of low concentrations of nucleophiles and above pH 7 [13] and so the successful application of N-[^{14}C]AcIm to identify modified tyrosine residues depended upon the use of mild procedures for generating and isolating peptides which avoided loss of *O*-[^{14}C]acetyl groups. For this reason we radioacetylated the isolated B-chain which, unlike intact ricin, is susceptible to trypsinolysis in the presence of urea at pH 7. In common with native ricin, the ability of ricin B-chain to bind to Sepharose was inactivated reversibly by the acetylation of tyrosine residues. A maximum of 4 of the 9 tyrosine residues present in the B-chain could be acetylated using N-AcIm. The presence of 0.2 M lactose protected an average of 2 of these acetylatable tyrosines against acetylation. As with the intact toxin [8,9], B-chain acetylated in the presence of lactose retained binding to Sepharose suggesting that one or more tyrosine residues are present in the saccharide binding sites.

By radioacetylating B-chain with N-[^{14}C]AcIm in the presence and absence of lactose and comparing tryptic digests, we were able to identify a tyrosine residue, Tyr-248, that was protected by lactose against *O*-acetylation with this reagent. In contrast with ricin B-chain, the number of tyrosine residues of RCA B-chain that could be modified with N-AcIm was unaffected by the presence of lactose in agreement with similar studies on intact RCA [17]. The absence of Tyr-248 in the primary structure of the RCA B-chain deduced from the nucleotide sequence of the RCA gene [18] and by direct amino acid sequencing of the protein [19] is consistent with this finding. Funatsu and his coworkers [20] recently showed that Tyr-248 was protected against modification by iodination of intact ricin. Iodination of ricin also modified B-chain tyrosine residues at positions 67, 78 (or 125), 148 and 176 although these modifications did not correlate simply with the presence or absence of lactose [20]. This may explain why we were able to identify only a single lactose-protectable tyrosine residue in the present study. Alternatively, it may be that the second lactose-protected residue was either Tyr-125 or Tyr-148 which are both expected to be present in a single large tryptic peptide which we did not isolate in this study.

Although Tyr-248 was reported to be present in the high-affinity lactose-binding site of ricin, its iodination apparently did not affect the binding of lactose to this site [20] and ricin iodinated in the absence of lactose was still able to bind to Sepharose [21] in contrast with the present results. One explanation for this apparent paradox is that the introduction of iodine atoms adjacent to the phenolic hydroxyl group of Tyr-248 and the concomitant reduction in pK_a of this group does not significantly influence the interaction with lactose whereas substitution of the hydroxyl group itself actually weakens binding. This suggests firstly, that Tyr-248 may be located at the periphery of the high-affinity binding site, and secondly, that bulkier substituents of the hydroxyl group may be more effective at inhibiting saccharide binding.

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