Effect of Reductive Lactosamination on the Hepatic Uptake of Bovine Pancreatic Ribonuclease A Dimer*

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SUMMARY

Lactose has been coupled to the lysine residues of the cross-linked dimer of bovine pancreatic ribonuclease A by reductive amination with cyanoborohydride. Derivatives of ribonuclease dimer that contained up to 10 N'-1-(1-deoxylactitolyl)-lysine residues per molecule had greater than 75% of the enzymic activity of the unmodified enzyme toward yeast RNA. Upon intravenous injection of the IT-tivity was found in the kidneys.

Studies on the cytosstatic properties of a cross-linked dimer of ribonuclease A (1, 2) have prompted us to consider ways of tissue-directing this enzyme in an attempt to inhibit the growth of cells in a particular organ. The possibility of enhancing hepatic uptake by the coupling of α-galactopyranosyl residues has been studied. This approach stems from the findings of Ashwell, Morell, and co-workers (cf. Ref. 3) that the exposure of galactose as the terminal sugar on serum glycoproteins constitutes a specific recognition signal for receptor-mediated uptake into the liver parenchymal cells, and the demonstration by Rogers and Kornfeld (4) that the asialo-glycopeptide of fetuin (which contains terminal galactose residues), when covalently linked to either albumin or lysozyme, caused rapid and selective removal of these proteins from the circulation to the liver.

The coupling of α-galactosyl residues to RNAse dimer has been achieved by a simple reductive lactosamination of lysine residues by the method described by Gray (5, 6) and applied to asparaginase by Marsh et al. (7). This method is based on the ability of the cyanoborohydride to react selectively the Schiff's base formed between an aldehyde and an amino group (Borch et al. (8)). Lactose has been used in the coupling reaction since the presence of an intact pyranose ring in the galactose residue is essential for hepatic recognition (3).

Since RNAse dimer (M, = 27,400) is rapidly cleared by the kidney, as are other low molecular weight proteins (10), the rate of uptake by the liver must exceed that by the kidney in order to attain significant concentrations in the former organ; glycosylated derivatives of bovine serum albumin (M, = 67,000) have been prepared for control experiments with a protein that is less subject to renal excretion.

EXPERIMENTAL PROCEDURES

Materials—Bovine serum albumin (Fraction V) was obtained from Armour. Ribonuclease dimer with optimal activity toward poly(A)-poly(U) was prepared by cross-linking RNAse A (Sigma type XII-A) with dimethyl suberimidate (11). [14C]Iodoacetate acid (57 mCi/mmol) was from Amersham/Searle. Sodium cyanoborohydride (recrystallized, lot 89438) was from Alfa-Ventron. Fetuin (99% pure) was obtained from Grand Island Biological Co. Sialic acid was removed by incubating 50 mg of fetuin, in 5 ml of 0.05 M sodium acetate, pH 5.5, 0.15 M NaCl, with 100 μg of neuraminidase from Clostridium perfringens (Worthington) at 37° for 24 h. The product was dialyzed against distilled water and lyophilized. After hydrolysis of native fetuin and its derivative in 0.1 M H2SO4, for 60 min at 80°, analysis by the thiobarbituric acid method (12) showed that 87% of the sialic acid had been removed from fetuin by neuraminidase treatment.

Reductive Lactosamination Reaction—The reactants, 10 mg of protein, 40 mg of α-lactose, and 25 mg of sodium cyanoborohydride, were dissolved in a total volume of 1 ml of 0.2 M potassium phosphate buffer, pH 7.0. Phosphate buffer was used to protect Lys-41 of RNAse dimer from derivatization (cf. Ref. 13). After incubation of 37°, the solution was dialyzed (at 0-4°) twice for 12 h against 100 volumes of 0.15 M NaCl, and twice for 12 h against 100 volumes of distilled water. The protein concentration was determined by the method of Lowry et al. (14).

Determination of the Amount of Glycosylation—The amount of lactose coupled to protein was determined by the phenol/sulfuric acid method (15) calibrated against galactose; since the secondary amine formed by reductive amination is acid-stable, only galactose is produced during the hydrolysis. The number of modified lysine residues was determined by amino acid analysis after hydrolysis in 6 N HCl, at 110° for 20 h. N'-1-(1-deoxysorbitolyl)-lysine and N'-1-(1-deoxylactitolyl)-lysine were synthesized from α-t-BOC-L-lysine by reductive amination. On a Durrum D-500 analyzer, using pH 6 as the third buffer, unhydrolyzed N'-1-(1-deoxysorbitolyl)-lysine and N'-1-(1-deoxylactitolyl)-lysine were eluted as a single peak located between phenylalanine and histidine. During acid hydrolysis of this derivative, three additional ninhydrin-positive peaks were produced similar to those described by Finot et al. (16) and Marsh et al. (7), although in contrast to the results of the latter authors, we did not detect the formation of any free lysine. The same products were obtained upon hydrolysis of N'-1-(1-deoxylactitolyl)-lysine. The number of glycosylated lysine residues was determined by calculating the difference in the lysine values of the glycosylated derivatives and the unmodified proteins. In analyses of RNAse dimer, the lysine values were corrected for the additional modification that accompanied the cross-linking reaction (11).

Radioactive Labeling of Proteins—Proteins were treated with [14C]Iodoacetate acid (50 μCi/1 mg of protein), at 37° for 48 h.
Carboxymethylation of RNase derivatives was carried out in 0.1 M sodium acetate, pH 5.5 (17); the resulting derivatives are enzymically inactive. The labeling reaction with derivatives of bovine serum albumin was conducted in 0.5 M Tris/HCl, pH 8.4 (18). Labeled proteins were separated from excess reagent by gel filtration on Sephadex G-25 (10 × 1 cm columns, equilibrated with 0.15 M NaCl). The specific radioactivities of RNase derivatives were in the range 3.4 to 6.1 × 10^6 cpm/mg (i.e. the incorporation of 1 to 1.5 mol of [14C]iodoacetic acid/mol of protein); the serum albumin derivatives had specific radioactivities of 0.85 to 1.0 × 10^6 cpm/mg (i.e. the incorporation of 0.6 to 0.7 mol of [14C]iodoacetic acid/mol of protein).

Tissue Uptake Studies—Each protein derivative (100 to 200 µg in 300 µl of 0.15 M NaCl) was injected into the tail vein of male albino rats (200 to 400 g). At given times, the animals were killed by decapitation, a sample of blood was collected, and the tissues were removed and homogenized in 0.15 M NaCl. Blood (0.5 ml) and aliquots (0.5 ml) of each homogenate were dried, oxidized in a Packard Tri-Carb sample oxidizer (10°C02 was trapped in Packard Carbo-Sorb; Permafluor V was the scintillation fluid), and the radioactivity was determined. The recovery of counts after oxidation, as checked by a 14C standard, was greater than 97%. The blood volume of each rat was calculated as 64.1 ml/kg of body weight (19).

In experiments with derivatives of serum albumin, after killing the rat, the livers were perfused before being removed; 0.15 M NaCl was forced into the portal vein until the color of the liver became pale brown.

Assays for RNase Activity—The activities of RNase dimer toward cyclic 2',3'-cytidylic acid, yeast RNA, and poly(A)-poly(U) were measured as previously described (1, 11).

RESULTS AND DISCUSSION

With a large excess of lactose and cyanoborohydride in the reaction medium, at pH 7 and 37°, increasing amounts of lactose were coupled to RNase dimer as a function of time (Fig. 1). Glycosylated derivatives of serum albumin were similarly prepared; up to 20 mol of lactose/mole of protein were coupled during 5 days of reaction. Although we have routinely carried out the glycosamination reaction at pH 7, subsequent experiments have shown that the coupling reaction is 2 to 3 times as fast at pH 9, a finding which is similar to the recent results of Baus and Gray (6). Amino acid analysis of the glycosylated proteins gave the same characteristic peaks observed when N-1-(1-deoxysorbitolyl)-lysine was hydrolyzed, which shows that the derivatives contained 1-deoxylactitolyl residues linked to the ε-amino groups of lysine residues. There was generally good agreement between the results of the sugar analysis and amino acid analysis; it is, however, likely that there was some modification of the α-NH2 groups of the NH2-terminal lysine residues on the RNase dimer.

Derivatives of RNase dimer which contained up to 5 1-deoxylactitolyl-lysine residues/molecule retained complete activity toward cyclic 2',3'-cytidylic acid and yeast RNA. The coupling of additional lactose residues resulted in a gradual loss of activity toward both substrates (Table I), the drop in activity being most pronounced when greater than 10 lactose

![FIG. 1. Coupling of lactose to RNase dimer at pH 7.0, 37°. O, carbohydrate analysis by phenol/sulfuric acid; •, modified lysine residues. The slightly higher values (0.5 to 1.0 mol of lactose coupled) obtained in some of the carbohydrate analyses probably indicate modification of both the ε- and α-NH2 groups on the NH2-terminal lysine residues. Reductive lactosamination of L-lysine (5 days at 37°) resulted in greater than 80% modification of both of the NH2 groups of the amino acid.](image)

![FIG. 2. Tissue uptake of RNase dimer and its glycosylated derivative after injection into the tail vein of rats. A, liver; •, kidney; O, blood. A, 14C]RNase dimer; B, 14C]RNase dimer containing 8 N-1-(1-deoxylactitolyl)-lysine residues/molecule. The amount of radioactivity in the liver at 10 min was the average result of three separate experiments (S.E. ± 2%). The other data points were from single experiments. No significant amounts of radioactivity were detected in the lungs, spleen, testes, and brain when these tissues were removed 10 min or 24 h after injection of RNase dimer or its glycosylated derivative.](image)
plasma clearance and hepatic uptake of asialoglycoproteins. If the data for Fig. 3 are plotted on a lactose/molecule of protein are needed to give greater than (cf. Ref. 3). The present results show that 8 to 10 residues of terminal n-galactopyranosyl residues are required for efficient number of lactose residues coupled (Fig. 3). Relatively few serum albumin taken up by the liver were related to the found in the liver 10 min after injection, which demonstrated competition for the galactose-specific receptor protein of the parenchymal cells (3).

After intravenous injection of $^{14}$C RNase dimer into rats (Fig. 2A), the protein was rapidly removed from the blood stream (half-life ca. 10 min [cf. Ref. 1]) and the bulk of the radioactivity at 10 min was found in the kidneys; the liver content was only 4%. In contrast, after injection of a $^{14}$C RNase dimer derivative containing 6 1-deoxyxactotylyl-

lysine residues/molecule (Fig. 2B), the bulk of the radioactivity was found in the liver; the remaining counts were found in the kidneys. No significant amounts of radioactivity were detected in other tissues examined. Maximum hepatic uptake (69% of the total injected counts) occurred 10 min after injection; thereafter there was a gradual loss of radioactivity from the liver, which most probably reflects degradation of the protein. At 24 h after injection, less than 5% of the total radioactivity remained in the liver, while approximately 90% of the counts were found in the collected urine. When 5 mg of asialofetuin was injected with the glycosylated $^{14}$C RNase dimer derivative, only 6.5% of the total radioactivity was found in the liver 10 min after injection, which demonstrated competition for the galactose-specific receptor protein of the parenchymal cells (3).

The amounts of glycosylated RNase dimer and glycosylated serum albumin taken up by the liver were related to the number of lactose residues coupled (Fig. 3). Relatively few terminal n-galactopyranosyl residues are required for efficient plasma clearance and hepatic uptake of asialglycoproteins (cf. Ref. 3). The present results show that 8 to 10 residues of lactose/molecule of protein are needed to give greater than 50% uptake by the liver. If the data for Fig. 3 are plotted on a mole of lactose per g of protein basis, rather than per mol of protein, serum albumin is found to require fewer lactose residues to yield a given hepatic uptake than RNase dimer, probably since the albumin is not subject to the same degree of renal excretion. These results are consistent with those of Marsh et al. (7) on the effect of the number of lactose residues on the plasma clearance of asparaginase and those of Krantz et al. (21) who showed that the affinity for liver membranes of proteins that contain 1-thio-β-D-galactopyranosyl residues increased with increasing numbers of coupled thigalactoside residues. The present results show that reductive lactosamination provides a simple method for directing a protein of possible therapeutic interest to the liver even when the protein is of sufficiently low molecular weight to be subject to rapid renal clearance.

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