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Attachment of Thioglycosides to Proteins: Enhancement of Liver Membrane Binding†

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ABSTRACT: Thioglycosides of D-galactose, D-glucose, *N*-acetyl-D-glucosamine, and D-mannose were covalently attached to *Aspergillus oryzae* α-amylase, hen's egg lysozyme, and bovine serum albumin by amidination, diazo coupling, and amide formation. The binding of the newly formed glycoproteins (neoglycoproteins) to rabbit liver membranes was measured, using asialoorosomucoid as a reference. Attachment of D-galactosides by any of the three methods enhanced binding by several orders of magnitude. Coupling of a comparable number of D-mannosides or *N*-acetyl-D-glucosaminides had little or no effect. Attachment of D-glucosides also enhanced binding but to a variable extent depending on the method of attachment. Thus, the behavior of neoglycoproteins toward rabbit liver membranes closely paralleled that of serum glycoproteins (Ashwell and Morell, 1974) with respect to sugar specificity.

Numerous hypotheses about the biological function of the carbohydrate groups in glycoconjugates have been advanced (Eyler, 1966; Roseman, 1970; Winterburn and Phelps, 1972; Roth, 1973). Although the relationship between carbohydrate structure and the biological behavior of glycoconjugates is not fully understood, there have been many examples in which subtle modifications of the carbohydrate structure of glycoconjugates result in drastic changes in their biological behavior (for examples, see Watkins, 1972; Ashwell and Morell, 1974; Moyle et al., 1975).

Since their initial observation that desialylated glycoproteins were rapidly removed from the circulation of the rabbit by the liver, Ashwell, Morell, and co-workers (see Ashwell and Morell, 1974) have extensively studied the characteristics of the clearance mechanism. They have demonstrated that the asialoglycoproteins are taken up and catabolized by liver parenchymal cells. Uptake of a glycoprotein is dependent upon the exposure of galactosyl residues by desialylation. Subsequent removal of the penultimate galactosyl residue thereby exposing *N*-acetylglucosaminide prevents its clearance. The same sugar specificity was observed for the clearance of exoglycosidase-treated antibodies (Winkelhake and Nicolson, 1976) and bovine serum albumin and lysozyme to which desialylated fetuin glycopeptides had been covalently attached (Rogers and Kornfeld, 1971). Asialoglycoproteins are bound to liver membranes in vitro by a saturable, calcium-dependent process which is sensitive to phospholipases A and C (Lunney and Ashwell, 1974) and neuraminidase. A glycoprotein which is responsible for the binding of desialylated serum glycoproteins has been isolated from liver membranes (Hudgin et al., 1974) and extensively characterized (Kawasaki and Ashwell, 1976).

We have approached the problem of the relationship between carbohydrate structure and biological behavior by attaching monosaccharides to proteins under controlled conditions. In this paper we report the results of attaching thioglycosides to three proteins by three methods including by amidination with 2-imino-2-methoxyethyl 1-thioglycosides, new reagents which we have described in the preceding paper (Lee et al., 1976). The abilities of the neoglycoproteins to bind to liver membranes were quantitatively evaluated using the inhibition binding assay of Van Lenten and Ashwell (1972), thereby enabling a comparison of the effects of incorporating varying amounts of four monosaccharides by different techniques.

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Experimental Section

Materials. 2-Imino-2-methoxyethyl 1-thioglycosides (IME[1]-thioglycosides) were generated from cyanomethyl 1-thioglycosides as described in the preceding paper (Lee et al., 1976). *p*-Aminophenyl 1-thioglycosides were prepared by the sequence of reactions described earlier (Chipowsky and Lee, 1973). 2-(6-Aminohexanamido)ethyl 1-thioglycosides were prepared as described previously (Lee and Lee, 1974).

α-Amylase from *Aspergillus oryzae* was prepared as described earlier (McKelvy and Lee, 1969). Hen's egg lysozyme (twice crystallized) and bovine serum albumin were purchased from Sigma Chemical Co.

Orosomucoid (α_1-acid glycoprotein) was kindly provided by Dr. S. Roseman and the American Red Cross National Foundation Center, Bethesda, Md. Neuraminidase from *Clostridium perfringens* was purchased from Boehringer Mannheim.

Sodium [^{125}I]iodide, carrier free, in 0.1 M NaOH was obtained from New England Nuclear. Chloramine-T was from Aldrich Chemical Co. and was used without further purification.

Plasma membranes were prepared from rabbit liver by a modification (Morell and Scheinberg, 1972) of the method of Ray (1970), which does not include the final sucrose gradient. This membrane fraction retained its ability to bind asialoglycoproteins during storage at −20 °C for at least 1 year.

^{125}I-Labeled Asialoorosomucoid. Asialoorosomucoid was prepared by neuraminidase treatment of orosomucoid. The digestion mixture contained 30 mg/ml orosomucoid and 10 μg/ml neuraminidase, in 0.1 M potassium acetate, pH 4.5. After 24 h at 37 °C, 0.38 μmol of sialic acid was released per mg of orosomucoid (102% of total), as measured by the automated thiobarbituric acid method (Krantz and Lee, 1975). Sialic acid was removed by dialysis, and neuraminidase was removed with a column of Sepharose 4B bearing *N*-(6-aminohexyl)oxamate.[2] After this treatment, no neuraminidase activity was detectable in an assay capable of measuring less than 1% of the original activity. The asialoorosomucoid was found to contain the same amounts of mannose and galactose as the intact orosomucoid.[3] Asialoorosomucoid was iodinated by the chloramine-T method (Greenwood et al., 1963), and purified by chromatography on a column (1 × 10 cm) of Sephadex G-25, in 0.02 M Hepes, pH 7.5, containing 0.9% NaCl. Fractions (0.6 ml) were collected in tubes which had been vortexed with 0.1 ml of 0.1% bovine serum albumin. Specific activity of [^{125}I]asialoorosomucoid was determined by immunoassay (Berson et al., 1964) using goat antiserum to human orosomucoid. The antibody-bound asialoorosomucoid was recovered by coprecipitation with 30 μg of rabbit γ-globulin in the presence of 50% saturated ammonium sulfate. Specific activity ranged from 20 to 70 μCi per μg, and 90 to 95% of the counts were immunoprecipitable.

Preparation of Carboxymethyl 1-Thioglycosides. Carboxymethyl 1-thio-β-D-galactopyranoside was prepared as follows: 2,3,4,6-Tetra-*O*-acetyl-α-D-galactopyranosyl bromide, 20.5 g (50 mmol), methyl thioglycolate (Evans Chemetics, Darien, Conn.), 10.6 g (100 mmol), and potassium carbonate, 13.8 g (100 mmol), were added to 25 ml of dry acetone. After stirring for 7 h at 20–22 °C, the insoluble material was filtered off and washed with chloroform. The filtrate and the washings were combined and evaporated in vacuo to a syrup which was dissolved in 250 ml of 95% ethanol and deacetylated by the addition of 275 ml of 1 M sodium hydroxide. After 10 h at 4 °C the reaction mixture was decationized with excess Dowex 50W-X8 (Hydrogen form), and the ethanolic solution was evaporated in vacuo to a syrup. The aqueous solution of the syrup was applied to a column (2 × 30 cm) of a weak anion exchange resin, CGA-316 (Hydroxide form, J. T. Baker Chemical Co.). The column was washed with water to remove neutral carbohydrate and eluted with 1 M acetic acid. The acid eluate was concentrated in vacuo and chromatographed in two batches on a column (5 × 165 cm) of Bio-Gel P-2 in 0.1 M acetic acid. The main peak fractions were concentrated in vacuo, and the product was crystallized from ethanol: yield 77%; mp 168–169 °C.

Anal. Calcd for $C_8H_{14}O_7S$ (254.26 daltons): C, 37.78; H, 5.55; S, 12.61. Found: C, 37.75; H, 5.55; S, 12.69.

Carboxymethyl 1-thio-β-D-glucopyranoside was prepared by the same sequence of reactions: yield 67%; mp 154–155 °C.

Anal. Calcd for $C_8H_{14}O_7S$ (254.26 daltons): C, 37.78; H, 5.55; S, 12.61. Found: C, 38.02; H, 5.77; S, 12.08.

Preparation and Characterization of Neoglycoproteins. Neoglycoproteins were prepared in several ways as described below.

(*a*) *Amidination.* Thioglycosylamidino derivatives of proteins were prepared by reacting IME-thioglycosides with proteins by the method described in the preceding paper (Lee et al., 1976).

(*b*) *Diazo Coupling.* In a typical diazo-coupling reaction, a solution of 0.11 M *p*-aminophenyl thioglycoside in 0.2 M HCl was cooled on ice and 0.1 volume of ice-cold, freshly prepared 1 M $NaNO_2$ was added. After 3 min at 0 °C, a portion of the diazotized solution (0.01–0.60 ml) was added to 5 ml of ice-cold protein solution (1 mg/ml in 0.2 M $NaHCO_3$, pH 10.0) and gently mixed. After 15 min at 0 °C, the reaction was terminated by adjusting the solution to pH 6.0 with 40% acetic acid. Very high levels of substitution were obtained by allowing the reaction to proceed for 3 h at room temperature.

(*c*) *Amide Formation.* Thioglycosides containing either an amino or carboxyl group at the terminal position of the aglycon were reacted with α-amylase with the aid of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDAC). Each reaction mixture contained 20 mg of protein, 32 μmol of thioglycosides, 32 μmol of *N*-hydroxysuccinimide, and 36 μmol of EDAC in 2.4 ml of dimethyl sulfoxide. After 12 h at room temperature, an equal volume of 0.1 M Tris-HCl, pH 9.0, was added to terminate the reaction.

Neoglycoproteins made by methods b and c were purified by gel filtration on a column (2 × 70 cm) of Sephadex G-25, in 0.1 M sodium acetate, pH 5.5. Protein was measured by absorbance at 280 nm, or by amino acid analysis in the case of diazo-coupled proteins. The enzyme concentration of α-amylase could also be determined by sugar analysis since the sugar composition of this protein is known (McKelvy and Lee, 1969).

The amount of protein-linked aryl or aliphatic thioglycosides was determined by automated sugar analysis (Lee, 1972) after cleavage of the thioglycoside linkage (C_1–S) with mercuric

[1] Abbreviations used are: IME, 2-imino-2-methoxyethyl; Hepes, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; RIP, relative inhibitory power; EDAC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide; Tris, tris(hydroxymethyl)aminomethane.

[2] Place, Powers, and Lee, unpublished results. About 20 μmol of ligand per ml of gel.

[3] In some commercial preparations of neuraminidases, endo-*N*-acetylglucosaminidase activity could be found (Chien et al., 1975); therefore it is imperative to ascertain that desialized glycoprotein has not been affected by the endoglycosidase.

TABLE I: Diazo Coupling of Thioglycosides to α-Amylase.

Thioglycosides[a]	Sugar/Enzyme (mol/mol) In Reaction Mixture	Bound to Protein	Enzyme Act. (%)
β-Gal	25	4.0	
	50	7.8	35
	250	25	2
	500[b]	79	3
	50[c]	4.8	88
	100[c]	9.9	80
β-Glu	50	7.6	38
	100	11.6	
	250	18.3	4
	600[b]	70	1
α-Man	50	12.0	
	100	15.7	
	250	22.0	
None[d]	50	12.5	
	250	18.9	

[a] All were the *p*-aminophenyl 1-thioglycosides of the indicated sugar, diazotized as described in the text. [b] Reaction at room temperature for 3 h. [c] Reaction in the presence of 0.1 M maltose. [d] Aniline was used in lieu of *p*-aminophenyl 1-thioglycosides.

acetate (Krantz and Lee, 1976). When aniline rather than *p*-aminophenyl thioglycosides was diazotized and reacted with the proteins, the level of incorporation was determined by measurement of absorbance at 320 nm. From the parallel experiments of coupling *p*-diazophenyl thioglycosides to proteins, molar absorbance of *p*-diazophenyl group at 320 nm was estimated to be 10 000.

Assay of Neoglycoprotein Binding by Liver Plasma Membrane. The efficiency of binding of neoglycoproteins to liver plasma membrane was determined by measurement of their ability to inhibit the binding of asialoorosomucoid to membranes. The method of Van Lenten and Ashwell (1972) was used with some modifications. Reactions were carried out in 13 × 100 mm glass test tubes, at pH 7.5, in a final volume of 0.5 ml containing 167 μmol of NaCl, 17 μmol of Hepes, 5 μmol of $CaCl_2$, 0.50 mg of bovine serum albumin, the inhibitor to be tested, and 50 μg (by dry weight) of plasma membrane. The assay mixture was incubated at 37 °C with agitation to keep the membranes suspended. After 1 h, [^{125}I]asialoorosomucoid was added and the incubation was continued for 30 min. The reaction was stopped by the addition of 3.0 ml of ice-cold Tris buffer (10 mM Tris-HCl, pH 7.5, 100 mM NaCl, 10 mM $CaCl_2$, 0.1% bovine serum albumin). The suspension was filtered through glass fiber paper (Whatman GF/C, 2.4 cm) which had been soaked in Tris buffer. The assay tube and filter were washed with three 5-ml portions of ice-cold buffer. Filters were dried under a heat lamp, wrapped in 5-cm squares of Parafilm (American Can Co.), and counted in a Nuclear Chicago γ counter.

Blanks consisted of reaction mixtures which were chilled on ice and diluted with 3.0 ml of Tris buffer before addition of membranes, and filtered immediately. To ensure saturation of the membrane binding sites, [^{125}I]asialoorosomucoid was routinely diluted with unlabeled asialoorosomucoid so that less than 5% of the radioactivity was bound in the absence of any inhibitor. Normally, 10 000 to 25 000 cpm of asialoorosomucoid was bound in the absence of inhibitor (neoglycoprotein), and about 1000 cpm in the blanks. In the absence of added $CaCl_2$, or after treatment of the membranes with neuraminidase, 96 or 94%, respectively, of the binding capacity was eliminated in agreement with the results of Ashwell and Morell (1974).

TABLE II: Coupling of Thioglycosides to α-Amylase by Amide Formation.

Thioglycosides Aglycons[a]	Sugars	Sugar/Enzyme (mol/mol) In Reaction Mixture	Bound to Protein	% Act.	RIP[b]
CM-	β-Gal	80	6.3	1	0.38
	β-Glc	80	6.7	1	0.11
AHA·AE-	β-Gal	80	8.8	1	0.91
	β-Glc	80	10.5	1	0.21

[a] CM-, carboxymethyl; AHA·AE-, 2-(6-aminohexanamido)ethyl (Lee and Lee, 1974). [b] Relative inhibitory power (see text). The RIP value of the unmodified α-amylase was 5.6×10^{-5}.

Results

Amidination. The methods and results of amidination of α-amylase, lysozyme and bovine serum albumin have been presented in detail (Lee et al., 1976).

Diazo Coupling. The results of reacting *p*-diazophenyl thioglycosides with α-amylase are shown in Table I. Increased amounts of thioglycoside in the reaction mixture resulted in increased levels of incorporation of sugar, but the extent of modification as a function of the thioglycoside:protein ratio was not highly reproducible. Although incubation of α-amylase in the reaction buffer alone caused no loss of enzyme activity, all modified preparations suffered some loss of activity, the losses being higher in the more highly modified proteins. Modification in the presence of 0.1 M maltose afforded significant protection of enzyme activity.

Amide Formation. The carbodiimide-mediated reaction of thioglycosides containing amino- or carboxyl-terminated aglycons with α-amylase in dimethyl sulfoxide is shown in Table II. Although significant incorporation was achieved, all derivatives prepared in this manner were devoid of enzyme activity and were sparingly soluble in aqueous buffers. Incubation of α-amylase in dimethyl sulfoxide in the absence of carbodiimide also resulted in complete loss of activity. All attempts to react these thioglycosides with α-amylase in aqueous buffers using only modest excesses of thioglycosides resulted in the incorporation of less than 1 mol of ligand per mol of enzyme.

Binding of Neoglycoproteins. The efficiency of binding of the neoglycoproteins to membranes was expressed with reference to asialoorosomucoid. In each set of experiments, inhibition curves were obtained for asialoorosomucoid and each of the neoglycoproteins under consideration by the technique of Van Lenten and Ashwell (1972). From these curves the amounts (in ng) of asialoorosomucoid and neoglycoprotein which inhibited the binding of ^{125}I-labeled asialoorosomucoid by 50% were determined. The amounts of inhibitors causing 50% inhibition were 2–4 ng for asialoorosomucoid, 0.01 to 10^4 ng for neoglycoproteins, and 10^4 ng to greater than 1 mg for unmodified proteins. The relative inhibitory power (RIP) of a neoglycoprotein was defined as the ratio of the amount of asialoorosomucoid producing 50% inhibition to the amount of neoglycoprotein producing 50% inhibition. Therefore the RIP values of inhibitors weaker than asialoorosomucoid are less

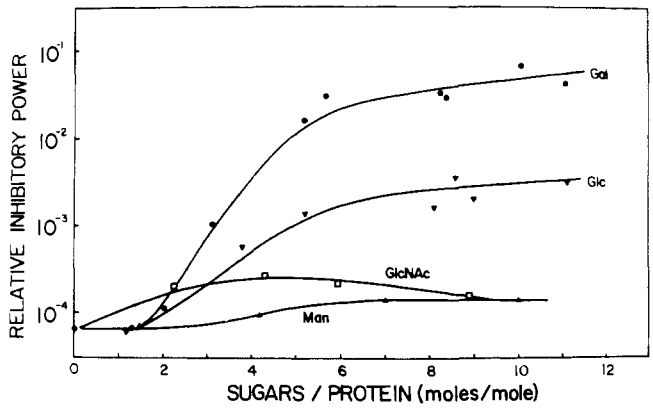

FIGURE 1: Values of relative inhibitory power (see text) of neoglycoproteins formed from α-amylase by amidination. Sugars used were: (●) 1-thio-β-D-galactopyranoside; (▼) 1-thio-β-D-glucopyranoside; (□) 2-acetamido-2-deoxyl-1-thio-β-D-glucopyranoside; and (▲) 1-thio-α-D-mannopyranoside.

TABLE III: Relative Inhibitory Power of Lysozyme Modified with IME-Thioglycosides.

Thioglycoside	Sugars/Enzyme (Molar Ratio)	RIP × 10^5
None		2.0
β-Gal	1.4	2.5
	2.1	6.0
	3.6–3.8	40–210
β-Glc	0.5	2.0
	1.2	2.3
	1.8	35–140
α-Man[a]	2.1	<4.6
	3.1	<4.0
	4.8	<4.5
β-GlcNAc[a]	1.7	<8.3
	2.4	<8.0
	3.0	<7.5

[a] These preparations showed poor binding and the available materials were insufficient for accurate determination of RIP values.

than one and the RIP values of better inhibitors are greater than one.

Binding of Amidinated Neoglycoproteins. The RIP values of the neoglycoproteins derived from α-amylase by reaction with various IME-thioglycosides are shown in Figure 1. Attaching increasing amounts of D-galactoside to α-amylase progressively increased its inhibitory power. When 10–11 D-galactosyl residues were attached to α-amylase, the RIP value was increased 10^3-fold relative to unmodified α-amylase. At this level of modification α-amylase was 1/20 as effective an inhibitor as asialorosomucoid. By contrast, the attachment of D-mannoside or *N*-acetyl-D-glucosaminide had little effect on the RIP values as compared with unmodified α-amylase. Interestingly, attaching D-glucoside increased the RIP values though not as much as D-galactoside did. The attachment of 10 D-glucosyl residues produced a 30-fold increase in the RIP value relative to unmodified α-amylase.

The neoglycoproteins obtained by reacting IME-thioglycosides with hen's egg lysozyme bound to liver membranes with the same sugar specificity as the α-amylase derivatives. As shown in Table III, the incorporation of D-galactosyl or D-glucosyl residues increased the binding by up to 100-fold relative to unmodified lysozyme. However, even at the highest levels of modification (3–4 mol of monosaccharide per mol of protein), lysozyme was a weaker (1/500) inhibitor than asialorosomucoid. Although the effect of D-mannoside and *N*-acetyl-D-glucosaminide could not be determined accurately because 50% inhibition was not achieved, the effect on the RIP, if any, was less than twofold for D-mannoside, and less than fourfold for *N*-acetyl-D-glucosaminide.

Similar results were obtained when bovine serum albumin was amidinated. The standard incubation mixture already contains 0.5 mg of bovine serum albumin; however, an additional 1 mg of bovine serum albumin did not result in measurable inhibitory activity. Attaching increasing amounts of D-galactoside to bovine serum albumin increased its ability to bind to liver membranes as shown in Figure 3. Even at the lowest level of incorporation (3 mol of D-galactoside per mol of bovine serum albumin), binding was improved relative to unmodified bovine serum albumin, although it was still a 10^4-fold weaker inhibitor than asialoorosomucoid. At the highest levels of modification (24–34 mol of D-galactoside per mol of bovine serum albumin), the neoglycoproteins are even better inhibitors than AOM. Bovine serum albumin with 24 D-galactosides is almost as good an inhibitor as asialoorosomucoid, while bovine serum albumin with 34 D-galactosides is a 20-fold better inhibitor than asialoorosomucoid. The D-glucosyl derivatives of bovine serum albumin gave similar results. As shown in Figure 3, derivatives with up to 12 D-glucosides incorporated bound as well as comparable D-galactosyl derivatives. At higher levels of incorporation, however, the D-glucosyl derivatives were even better inhibitors than the D-galactosyl derivatives. The D-mannosyl and *N*-acetyl-D-glucosaminyl derivatives were very poor inhibitors. Even at the highest levels of incorporation (33–41 mol of monosaccharide per mol of bovine serum albumin), D-mannosides and *N*-acetyl-D-glucosaminides had no measurable inhibitory activity.

Binding of Diazo-Coupled Neoglycoproteins. The binding of diazocoupled neoglycoproteins from α-amylase showed the same sugar specificity as the amidino derivatives. As shown in Figure 2, the attachment of 10 mol of D-galactoside per mol of protein increased the RIP values by 10 000 fold. On the other hand, D-mannoside was found to be rather ineffective (only threefold increase in RIP) and D-glucoside showed an intermediate effect. Diazotized aniline, coupled to the same extent as these sugars, had no effect on the RIP values.

One important difference between the effect of D-galactoside and D-glucoside was that, while the effect of D-galactoside appeared to be constant at sugar levels greater than 10 mol per mol of protein, there was a tenfold increase in the RIP as the amount of D-glucoside was increased from 10 to 70 mol per mol of protein.

Binding of Neoglycoproteins Obtained by Amide Formation. Attachment of D-galactoside and D-glucoside to α-amylase via amino and carboxyl groups of aglycons also caused a marked increase in membrane binding, as shown in Table II. The RIP values of all four neoglycoproteins of this type were at least 1000-fold greater than that of the native protein. D-galactosyl derivatives were about fourfold more effective than the D-glucosyl analogues. In a control experiment, incubation of α-amylase in the same reaction mixture but without carbodiimide and subsequent treatment as the other neoglycoproteins did not increase its RIP value.

Discussion

In the present studies, thioglycosides of D-galactose, D-glucose, D-mannose, and *N*-acetyl-D-glucosamine were attached to α-amylase, lysozyme, and bovine serum albumin by

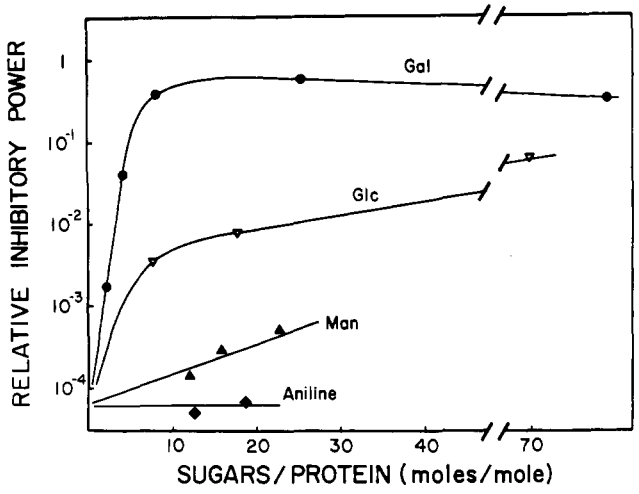

FIGURE 2: Values of relative inhibitory power (see text) of neoglycoproteins formed from α-amylase by diazo coupling. Sugars used were: (●) 1-thio-β-D-galactopyranoside; (▼) 1-thio-β-D-glucopyranoside; (▲) 1-thio-α-D-mannopyranoside; and (♦) aniline in lieu of aminophenyl 1-thioglycosides.

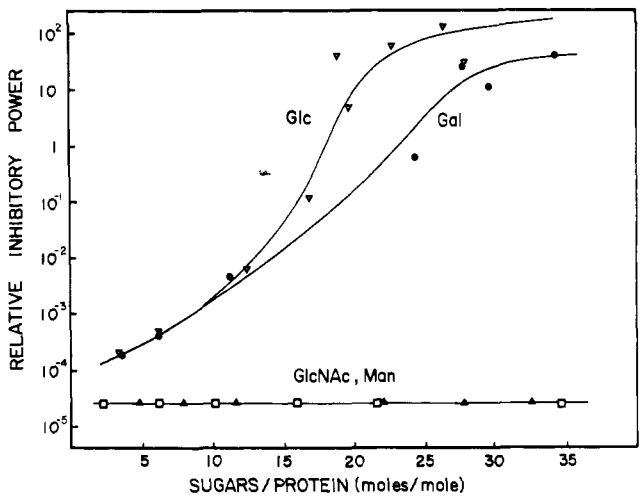

FIGURE 3: Values of relative inhibitory power (see text) of neoglycoproteins formed from bovine serum albumin by amidination. Sugars used were: (●) 1-thio-β-D-galactopyranoside; (▼) 1-thio-β-D-glucopyranoside; (□) 2-acetamido-2-deoxy-1-thio-β-D-glucopyranoside; and (▲) 1-thio-α-D-mannopyranoside.

three different methods, and the ability of the resultant derivatives to bind to liver membranes was examined. Analogous derivatives prepared by different methods had similar abilities to bind to membranes, but exhibited very different levels of enzymatic activity. Amidination proved to be a superior method for a number of reasons. As shown in the preceding paper (Lee et al., 1976), extensive modification of protein amino groups with imidates can be achieved under mild conditions. The extent of reaction is easily controlled, and all derivatives retain a high degree of enzyme activity, implying that there is no gross change in their conformation.

Incorporation of ligands by diazo coupling occurs readily only in strong alkaline solution, under which conditions the extent of the reaction is difficult to control. Even with a protein which is stable at high pH (e.g., α-amylase), substantial enzyme activity was lost during modification. In addition, introduction of thioglycosides into proteins via the aryl diazonium salt may not be desirable, because of the accompanying increase in hydrophobicity and the instability of diazo linkages.

Although water soluble carbodiimide has been used to modify proteins extensively in aqueous solution (Carraway and Koshland, 1972), success of the reaction demands huge excesses of ligand and carbodiimide. Although we were able to accomplish the reaction with limited amounts of IME-thioglycosides using nonaqueous solvent, all enzyme activity was lost. In addition, ionic groups on the protein were eliminated, thus reducing solubility in aqueous solution.

Neoglycoproteins bound to rabbit liver membranes with the same sugar specificity as the serum glycoproteins treated with exoglycosidases to expose different residues (Ashwell and Morell, 1974). Attachment of D-galactosyl residues to proteins by any of the methods described here resulted in a dramatic increase in their binding to membranes. On the other hand, attachment of D-mannosyl or *N*-acetyl-D-glucosaminyl residues had little or no effect. Derivatives of α-amylase made by different methods but with equal amounts of D-galactoside incorporated bound to membranes with similar efficiency. The RIP values for α-amylase derivatives with 10–11 D-galactosides ranged from 0.08 (the amidino derivative) to 0.9 (the diazo-coupled and amidated derivatives). The small improvement in binding of the diazo-coupled and amidated derivatives relative to the amidino derivative may reflect changes in hydrophobicity or surface charge produced by the former methods.

It is interesting to note that α-amylase from *A. oryzae*, although known to contain 1–2 mol of D-galactose per mol of enzyme (McKelvy and Lee, 1969), was a poor inhibitor of asialoorosomucoid binding. Apparently the D-galactosyl residues in the α-amylase do not fulfill the requirements for efficient binding to membranes. Except for the most extensively modified derivatives of bovine serum albumin none of the neoglycoproteins bound as efficiently to membranes as asialoorosomucoid. Although it has been reported that relatively few exposed D-galactosyl residues are needed for effective binding of natural glycoproteins (Ashwell and Morell, 1974), a considerably higher number of D-galactosyl residues appear to be needed for binding of neoglycoproteins. This difference may be a reflection of the relatively greater availability of D-galactosyl residues on the oligosaccharide chains of orosomucoid to the binding site. Alternatively, more stringent structural requirements may have to be met for effective binding.

Unexpectedly, the attachment of D-glucosides to all three proteins enhanced their binding to liver membranes. The D-glucosyl derivatives of α-amylase did not bind as well as the D-galactosyl derivatives but were still nearly 100-fold more effective than unmodified α-amylase. The ability of the D-glucosyl derivatives to bind was quite different depending on the linkage type. The derivatives made by amide bond formation or diazo coupling were better inhibitors than the amidino derivatives. The linkage type seemed to affect the binding of the D-galactosyl and D-glucosyl derivatives differently since with the diazo linkage, the D-galactosyl derivative (10–11 mol of monosaccharide per mol of α-amylase) was 100-fold more effective than the comparable D-glucosyl derivative, while with the amidino linkage the difference was 15-fold, and with the amide linkage, 4-fold. By contrast, the D-glucosyl derivatives of lysozyme and bovine serum albumin bound as well as or better than the D-galactosyl derivatives. Since animal glycoproteins except collagen rarely contain D-glucose, there is no obvious physiological explanation for the binding of D-glucosyl neoglycoproteins. Because D-glucose is not a typical component

of glycoproteins, hepatic glycoprotein recognition sites may not require the ability to discriminate accurately between D-*galacto*- and D-*gluco*-configurations. Several enzymes lacking this specificity are known, for example, almond emulsion β-D-glucosidase and human β-*N*-acetylhexosaminidase. It cannot be determined at this point whether the binding site for D-glucosyl neoglycoproteins is the same as the site that binds D-galactosyl neoglycoproteins and asialoorosomucoid.

We have demonstrated that the incorporation of simple thioglycosides by three different methods had a dramatic effect on the binding of proteins to liver membranes, which was consistent with the behavior of aglycosyl serum glycoproteins. Although extensive incorporation of thioglycosides could be achieved by all three methods, the amidino neoglycoproteins retained activity much better than other derivatives. The IME-thioglycosides will be useful reagents for exploring the role of carbohydrate in biologically active glycoconjugates as well as in such clinical studies as specific, target-organ-directed, replacement therapy.

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