Influence of Glycopeptide Structure on the Regulation of Galactosyltransferase Activity†

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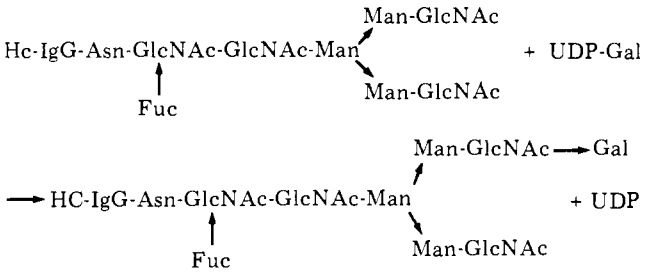
ABSTRACT: A β1,4-galactosyltransferase which has been isolated from swine mesentary lymph node as a homogeneous preparation catalyzes the transfer of galactose from UDP-Gal to the terminal *N*-acetylglucosaminyl residues of the heavy chains of IgG immunoglobulin, as shown in the following reaction (where Hc means heavy chain):



The specificity of the interaction of this enzyme with different glycosyl intermediates of porcine IgG heavy-chain glycopeptides was examined by kinetic analysis. Evidence was obtained for a mechanism which allows for the transfer of only a single galactosyl residue to one of the terminal *N*-acetylglucosaminyl units in the incomplete oligosaccharide chain. The rate of transfer of galactose to the second terminal *N*-acetylglucosaminyl unit decreased when galactose was already present on the other chain. The sialylation of this galactosyl moiety essentially stopped the transfer of galactose to the remaining terminal *N*-acetylglucosaminyl unit on the other chain. The apparent K_m of the enzyme for a glycopeptide prepared from the heavy chain of porcine IgG which contains no galactose was 0.25 mM. The apparent K_m increased to 2 mM when a single galactose residue was present, and sialylation of the galactosyl residue increased the K_m to 10 mM. The apparent K_m of the enzyme for UDP-Gal was also significantly influenced by the presence of a galactosyl residue on the glycosyl acceptor. The apparent K_m for UDP-Gal with the ungalactosylated derivative was 12 μM. The apparent K_m increased to 40 μM when one galactosyl residue was present in the glycosyl acceptor. Sialylation of this galactosyl unit increased the apparent K_m to 160 μM. A large amount of IgG immunoglobulin is present as a monogalactosylated, monosialylated derivative in plasma. The results obtained in the present study provide an explanation for this observation. These experiments showed that the rate of transfer of galactose to terminal *N*-acetylglucosaminyl residues in incomplete branched oligosaccharide chains of glycopeptides was dependent on whether a galactosyl residue was already present on one of the chains and on the rate of transfer of sialic acid to this galactosyl residue. The transfer of sialic acid to growing branched oligosaccharide chains could control the degree of completion of adjacent chains during synthesis. These findings further suggest that the amount of heterogeneity in the carbohydrate chains of secreted glycoproteins may be dependent on the relative activities of sialyltransferase and galactosyltransferase.

Galactosyltransferases (UDP-galactose:glycoprotein-(β1→4)galactosyltransferase, EC 2.4.1.38) catalyze the transfer of galactose from UDP-Gal to proteins with terminal GlcNAc residues, free GlcNAc, and glycosyl derivatives of GlcNAc (Roseman, 1970). These transferases are usually bound to membranes in the particulate fractions of most tissues. A number of these enzymes have been purified; however, very little is known about the regulation of the activity of galactosyltransferases which function in the synthesis of the oligosaccharide chains of glycoproteins.

In previous studies, we reported the isolation of a (β1→4)galactosyltransferase from the soluble fraction of mesentary lymph node homogenates by affinity chromatography on Sepharose 4B containing covalently bound *p*-aminophenyl-β-D-*N*-acetylglucosamine. The molecular and kinetic properties of this homogeneous galactosyltransferase were examined. The enzyme contains a single polypeptide chain with a molecular weight of 57 000. The enzyme catalyzed the formation of β1→4 bonds between galactose and terminal *N*-acetylglucosaminyl residues of solubilized preparations of the heavy chain of porcine IgG (Rao et al., 1976). Endogenous glycoprotein present in the microsomal subcellular fraction was also a very good substrate for the enzyme, and it was subsequently shown to be the IgG heavy chain with an incomplete carbohydrate structure. Labeled glycopeptides prepared from the endogenous IgG heavy-chain acceptor and those prepared from the heavy chain of porcine IgG isolated from serum had nearly identical structures. The structure of the carbohydrate chains of porcine IgG was determined by sequential enzymatic hydrolysis with specific glycosidases and methylation analysis. The outer chains of the glycopeptides were heterogeneous, and the major component, >80%, contained an incomplete oligosaccharide chain lacking galactose and sialic acid. In these previous investigations, it was observed that a solubilized preparation of the heavy chain of porcine IgG, molecular weight 35 000, was a good glycosyl acceptor for the purified enzyme. However, the removal of the remaining sialic acid and galactose residues with specific glycosidases significantly increased the rate of transfer of galactose.

The present studies were undertaken in order to examine the effects of the structure of completely characterized glycosyl acceptors on the activity of purified lymph node galactosyltransferase. The complete understanding of the specific requirements and mechanisms for the regulation of glycosyl-

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transferases will ultimately depend on studies with homogeneous enzymes and definitive structural studies of their substrates. In this report, we present the results of a detailed study using modified glycopeptides prepared from the heavy chain of porcine IgG as glycosyl acceptors. The results suggest a role for sialyltransferase in the termination of the synthesis of oligosaccharide chains and provide an explanation for the marked heterogeneity in the outer portion of the oligosaccharide chains of porcine IgG. Preliminary studies on the kinetic regulation of galactosyltransferases have recently been reported (Reddy et al., 1978).

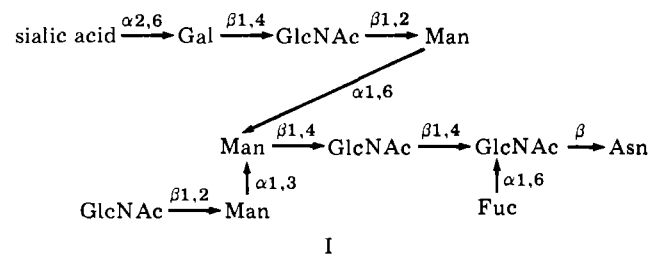
Experimental Procedures

Purification of Galactosyltransferase. A homogeneous preparation of β1,4-galactosyltransferase was isolated from the soluble fraction of swine mesentary lymph node extracts as described in a previous report (Rao et al., 1976). The principal steps in the purification of the enzyme involved affinity chromatography on Sepharose 4B columns containing covalently bound *p*-aminophenyl-GlcNAc or UDP-glucuronic acid. UDP-substituted Sepharose 4B was prepared by coupling the carboxyl group of UDP-glucuronic acid to 6-aminohexyl-Sepharose 4B. 6-Aminohexyl-Sepharose 4B was prepared by reacting 1,6-hexanediamine with cyanogen bromide activated Sepharose 4B (Cuatrecasas, 1970). Coupling of UDP-glucuronic acid to 6-aminohexyl-Sepharose 4B was carried out using *N*-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (EEDO) in 70% aqueous ethanol at room temperature for 18 h. The extent of coupling was estimated by absorption of UDP at 262 nm. The gel contained about 1.7 μmol/mL UDP-glucuronic acid. The affinity column was equilibrated with 0.05 M Tris-HCl[1] (pH 7.5), 10 mM $MnCl_2$, and 5 mM GlcNAc, and the enzyme preparation from the DEAE-Bio-Gel A step (Rao et al., 1976) was adjusted to 0.05 M Tris-HCl (pH 7.5), 10 mM $MnCl_2$, and 5 mM GlcNAc and applied to the column. The column was washed until the absorbance at 280 nm was below 0.1, and the enzyme was then eluted with 0.05 M Tris-HCl (pH 7.5), 0.05 M EDTA. This fraction was then further purified as described by Rao et al. (1976). The isolated enzyme showed only a single band on disc gel electrophoresis and had a specific activity of 35 nmol min^{-1} (mg of protein)$^{-1}$ at 37 °C in the standard assay system. Microsomes and other subcellular fractions were isolated from mesentary lymph nodes as described previously (Rao et al., 1976).

Assay of Galactosyltransferase Activity. All the kinetic experiments were carried out with the homogeneous soluble galactosyltransferase. The activity of the enzyme was determined by measuring the rate of transfer of [U-^{14}C]galactose from UDP-[U-^{14}C]galactose to glycoprotein or glycopeptide acceptors. The standard reaction mixture was incubated at 37 °C for 30 min and contained 0.1 M 2-(*N*-morpholino) ethanesulfonic acid (Mes) (pH 6.5), 10 mM $MnCl_2$, 0.4 mM UDP-[U-^{14}C]galactose (2.2 cpm/pmol), 1 mg of ovomucoid or modified fetuin, and galactosyltransferase in a total volume of 0.5 mL. The reaction was terminated by the addition of acid, and the labeled glycoprotein was isolated by repeated precipitation with 2.8 N perchloric acid containing 2% phosphotungstic acid, as described in our previous studies (Rao et al., 1976).

The transfer of galactose to glycopeptides and other low-molecular-weight glycosyl acceptors was measured under the standard incubation conditions with 20 mM $MnCl_2$ in a total volume of 0.1 mL. After incubation at 37 °C for 30 min, the reaction mixture was diluted to 0.5 mL with 0.001 N HCl and applied to a Dowex 1-Cl column (0.5 × 4 cm) which was previously equilibrated with 0.001 N HCl. The column was washed three times with 0.5 mL of 0.001 N HCl, and the filtrates were collected in a scintillation vial and counted. Controls which were incubated in the absence of glycopeptide acceptors contained very little radioactivity. The rates obtained in both assays were directly proportional to the time of incubation and enzyme concentration. In the presence of 0.5 mM glycopeptide III and 1 mM UDP-Gal, 2, 4, and 8 μg of purified enzyme catalyzed the transfer of 1.1, 2.1, and 3.9 nmol of galactose in 15 min, respectively. After 30 min of incubation under the same conditions, 2, 4, and 8 μg of enzyme transferred 2.0, 3.8, and 7.7 nmol of galactose to the glycopeptide acceptor. One unit of activity is defined as the amount of enzyme required to transfer 1 nmol of galactose per min.

Preparation of Glycopeptides from the Heavy Chain of Porcine IgG. In order to use well-characterized galactosyl acceptors in kinetic studies, various glycopeptides were prepared from porcine IgG. The immunoglobulin (Pentex, Kankakee, Ill.) was purified by chromatography on Sephadex G-200 and DEAE-cellulose columns (Small and Lamm, 1966). The purified porcine IgG showed only a single component when examined by gel electrophoresis and immunoelectrophoresis (Scheidigger, 1955). The sample, 100 g, was denatured by heating, and it was incubated with 1 g of Pronase in 2 L of 0.05 M Tris-HCl (pH 8.0) and 2 mM $CaCl_2$ for 20 h at 37 °C under toluene. The digest was passed through a Dowex 50-NH_4^+-X16 column (5 × 12 cm), and the column was washed with distilled water. The filtrate and wash which contained more than 90% of the carbohydrate were combined, concentrated under vacuum, and treated again with 1 g of Pronase for 20 h. The second digest was passed through a Dowex 50-H^+-X16 (5 × 12 cm), and the filtrate was neutralized with 1 N NH_4OH and concentrated under reduced pressure. The sample, in several batches, was chromatographed on Sephadex G-50 columns (2.5 × 80 cm) with water, and a major glycopeptide fraction, molecular weight about 2200, was collected (Spragg and Clamp, 1969). The desalted crude glycopeptide fraction was concentrated, and aliquots of 75 μmol were applied to DEAE-cellulose columns (5 × 75 cm) previously equilibrated with 1 mM potassium phosphate (pH 6.7). Glycopeptides were eluted with increasing concentration of potassium phosphate according to the procedure of Kornfeld and Kornfeld (1970). A major glycopeptide peak, 55 μmol, was eluted with 4 mM potassium phosphate. This glycopeptide was concentrated and desalted on a Bio-Gel P-2 column (2.5 × 30 cm). Paper electrophoresis at pH 6.5 showed the presence of only a single major acidic component which stained with ninhydrin and periodate-rosaniline sprays. Carbohydrate and amino acid analyses showed that this glycopeptide fraction contained 3.0 μmol of mannose, 3.9 μmol of GlcNAc, 1.2 μmol of galactose, 0.8 μmol of fucose, 1.0 μmol of sialic acid, 1.1 μmol of aspartic acid, and traces of other amino acids. The major glycopeptide present had the following structure:



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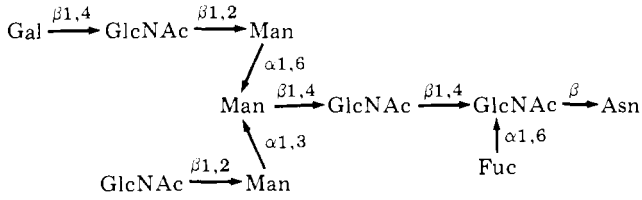
[1] Abbreviations used: DEAE, diethylaminoethyl; Tris, 2-amino-2-hydroxymethyl-1,3-propanediol; EDTA, (ethylenedinitrilo)tetraacetic acid.

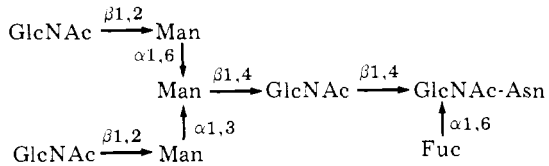
About 20% of a digalalactosylated glycopeptide was also present. The preparation was treated with a highly purified β-galactosidase in order to convert this glycopeptide to the monogalactosylated derivative. The mixture, 100 μmol, was incubated at 37 °C for 3 days with 50 units of *Escherichia coli* β-galactosidase in 0.05 M sodium phosphate (pH 7.5), 10 mM 2-mercaptoethanol, and 1 mM $MgCl_2$. This enzyme was completely free of β-*N*-acetylglucosaminidase activity under these conditions (Rao et al., 1976; Kornfeld and Kornfeld, 1970). About 24 μmol of galactose was released by this treatment. The glycopeptide was reisolated on a Bio-Gel P-2 column (2.5 × 30 cm). Carbohydrate analysis showed that the preparation now contained 3.0 μmol of mannose, 3.95 μmol of GlcNAc, 0.98 μmol of galactose, 0.85 μmol of fucose, and 1.0 μmol of sialic acid based on the amount of mannose present. The glycosidic linkages in this glycopeptide were determined by permethylation and analysis of the methylated alditol acetates by gas chromatography (Rao et al., 1976).

Sialic acid was removed by incubating 66 μmol of glycopeptide I with 5 units of neuraminidase in 0.05 M Tris–maleate, (pH 6.5) for 2 days at 37 °C under chloroform. The resulting glycopeptide was reisolated by chromatography on a Bio-Gel P-2 column (2.5 × 30 cm). The structure of the resulting glycopeptide II was established by carbohydrate and methylation analysis.



II

The galactose was removed from this glycopeptide by incubating 30 μmol with β-galactosidase as described previously. The resulting glycopeptide was reisolated on a Bio-Gel P-2 column and concentrated. About 28 μmol of galactose was released by this treatment. Carbohydrate analysis showed that glycopeptide III was devoid of sialic acid and galactose.



III

Materials and Analytical Methods. UDP-[1-^{14}C]galactose and UDP-Gal were prepared from [1-^{14}C]galactose and UTP as described previously (Mendicino and Rao, 1975; Mendicino and Hanna, 1970). Paper chromatography was performed with several different solvent systems (Hanna and Mendicino, 1970). Paper electrophoresis was carried out at pH 6.5 with pyridine–acetic acid–water (100:4:900) and at pH 8.5 with 0.1 M sodium tetraborate (Mendicino and Hanna, 1970). Glycopeptides were detected with ninhydrin and periodate–rosaniline sprays (Bonner, 1960). The phenol–sulfuric acid method was used to detect carbohydrate in intact glycoproteins and glycopeptides. The compositions of all of the glycopeptides used in the present studies were determined by specific colorimetric and enzymatic methods following acid hydrolysis. The positions of the glycosidic linkages were determined by permethylation and gas chromatographic analysis of methylated alditol acetate derivatives, as described in our previous studies (Rao et al., 1976; Garver, et al., 1975). The highly purified glycosidases used in these studies included viral and bacterial neuraminidase and β-galactosidase. The purities of these enzymes were examined with *p*-nitrophenylglycosyl derivatives of mannose, GlcNAc, and galactose. The neuraminidase was completely free of other glycosidase activities, and after purification (Rao et al., 1976; Kornfeld and Kornfeld, 1970) the β-galactosidase was also free of β-*N*-acetylglucosaminidase and α-mannosidase activity.

A large soluble glycoprotein fragment, molecular weight 35 000, of the heavy chain of porcine IgG was prepared by treating the intact immunoglobulin with pepsin as described by Utsummi and Karush (1965). Galactose and sialic acid were removed from this glycoprotein with β-galactosidase and neuraminidase as described in our previous studies (Rao et al., 1976). Modified fetuin devoid of sialic acid and galactose was prepared by the procedure of Spiro (1964).

Results

The subcellular distribution of galactosyltransferases in different subcellular fractions isolated from swine mesentary lymph node homogenates was examined. The activity was measured with the standard assay in the presence of excess fetuin, devoid of sialic acid and galactose, as the galactosyl acceptor. Under these conditions, 0.21, 0.20, 0.26, and 0.18 nmol per min per g wet weight of tissue was found in the plasma membrane, Golgi membrane, microsome, and soluble fractions, respectively. In contrast to the results obtained with many other tissues, about 20% of the total galactosyltransferase activity was consistently found in the 100 000*g* fraction of lymph node extracts. The possibility that this activity represented enzyme which was derived from the membrane fractions during disruption of the tissue was investigated. Treating particulate preparations with 0.5 M NaCl or 0.1% Triton X-100, sonication at 3 °C for 30 min, or incubation in the standard assay reaction mixture did not solubilize significant amounts of membrane-bound galactosyltransferase. Homogenization of the tissue in different buffers and in the presence of 0.3 M NaCl or 0.1% Triton X-100 did not increase the amount of galactosyltransferase found in the soluble high-speed supernatant fraction. This evidence strongly suggested that galactosyltransferase is present in both the soluble and particulate fractions of lymph node tissue. The enzymatic properties of the membrane-bound and purified soluble galactosyltransferase were similar. The effect of the concentration of UDP-Gal on the activity of the homogeneous soluble galactosyltransferase and the enzyme bound to microsomes with saturating concentrations of fetuin devoid of sialic acid and galactose, as the galactosyl acceptor, is shown in Figure 1A. The apparent K_m of the soluble enzyme for UDP-Gal was 22 μM, compared to a K_m of 17 μM for the membrane-bound enzyme.

Influence of the Structure and Concentration of Galactosyl Acceptors on the Activity of Galactosyltransferase. The subcellular distribution of endogenous galactosylglycoprotein acceptors in subcellular fractions prepared from swine mesentary lymph node was also studied. In the absence of added glycosyl acceptor, only the microsome and Golgi membrane preparations incorporated galactose into acid-precipitable protein. The high-speed supernatant fraction did not contain endogenous galactosyl acceptor, and no galactose was transferred to acid-precipitable protein in this fraction in the absence of added acceptor. The addition of soluble galactosyltransferase increased the rate of transfer of galactose into microsome membrane-bound acceptor by threefold. Thus, the membrane-bound substrate is accessable to the soluble

transferase under the standard assay conditions. The activity of all of the subcellular fractions was significantly increased upon the addition of exogenous glycoprotein substrates. The results obtained in our previous studies showed that the principal endogenous substrate in these membrane preparations was the heavy chain of IgG and that the oligosaccharide chains in this galactosyl acceptor were still incomplete and lacked appreciable amounts of galactose and sialic acid (Rao et al., 1976). It is interesting to note that the light chains of IgG are found almost exclusively in the soluble fraction (Rao et al., 1976; D'Amico and Kern, 1968; Swenson and Kern, 1967) and that very little intact IgG containing both heavy and light chains is found inside the cell. Taken collectively, these data indicate that the tetrameric structure of IgG is not formed until glycosylation of the bound heavy chain is nearly complete and ready to be secreted.

The effect of different glycoprotein acceptors on the activity of galactosyltransferases was examined. Purified intact porcine IgG was inactive as a galactosyl acceptor for both the soluble and bound galactosyltransferases. When this preparation was extensively reduced and alkylated and the light and heavy chains were separated by chromatography on Sephadex G-200 in 5 M guanidine hydrochloride or 1 M propionic acid (Small and Lamm, 1966), an active preparation of the heavy chain was obtained. However, when the sample was concentrated and dialyzed to reduce the concentration of salt, most of the glycoprotein precipitated from solution. Only the effects of very low concentrations of the isolated heavy chains could be examined because this preparation was very insoluble in the standard assay mixture. Moreover, UDP-Gal with a high specific activity (88 cpm/pmol) and long incubation times (2 h) were required to obtain appreciable incorporation. In order to overcome these difficulties, a large soluble glycoprotein fragment of the heavy chain with a molecular weight of 35 000 was prepared by treating intact porcine IgG with pepsin as described by Utsumi and Karush (1965). This solubilized preparation was an excellent substrate for lymph node galactosyltransferases, and it was used to compare the activity of galactosyltransferases with different glycoprotein substrates.

The influence of increasing concentrations of ovalbumin, fetuin, and the solubilized heavy chain of IgG devoid of sialic acid and galactose on the activity of soluble galactosyltransferase in the presence of saturating amounts of UDP-Gal (0.2 mM) was examined, and the results are shown in Figure 1B. An apparent K_m of 20 μM was obtained for the solubilized heavy chain of IgG, 29 μM was obtained with fetuin devoid of sialic acid and galactose, and an apparent K_m of 54 μM was found with ovalbumin. Similar values were obtained with galactosyltransferase bound to microsomes in the presence of 1% Triton X-100 under the same assay conditions. It was clear from these results that differences in the amino acid sequence of glycoprotein substrates and the influence of the concentration of UDP-Gal and terminal GlcNAc residues in the galactosyl acceptor on the activity of soluble and bound galactosyltransferases would not be responsible for the observed heterogeneity of the oligosaccharide chains in porcine IgG immunoglobulin.

Influence of the Structure and Concentration of the Oligosaccharide Chains of Glycopeptides Prepared from the Heavy Chain of IgG on the Activity of Galactosyltransferase. Although the apparent K_m values for low-molecular-weight galactosyl acceptors were significantly higher than those of macromolecular acceptors, the dependence of the activity of the enzyme on the structure of the branched oligosaccharide chain of IgG could be more conveniently studied with well-

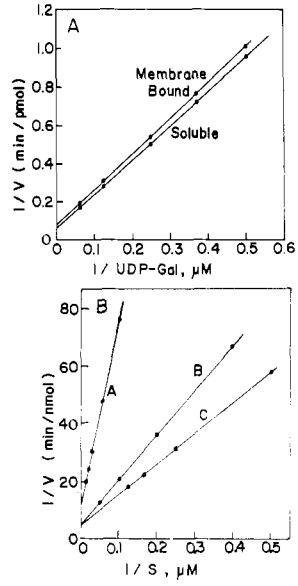


FIGURE 1: Influence of the concentration of UDP-Gal and glycoprotein substrates on the activity of lymph node galactosyltransferases. (A) The effect of UDP-Gal on the activity of the purified soluble enzyme and the particulate microsomal transferase were measured with the standard assay. Incubations were carried out in a total volume of 0.5 mL at 37 °C for 30 min with the amounts of UDP-Gal (44 cpm/pmol) shown in the figure and 2 mg of fetuin depleted of sialic acid and galactose. (B) The effects of glycoprotein substrates on the activity of the purified galactosyltransferase were measured with the standard assay. Reaction mixtures in a total volume of 0.5 mL were incubated at 37 °C for 30 min and contained 0.2 mM UDP-Gal (2.2 cpm/pmol) and the amounts of glycosyl acceptors shown in the figure. Curve A was obtained with ovalbumin, curve B with fetuin devoid of sialic acid and galactose, and curve C with solubilized heavy chain of porcine IgG devoid of sialic acid and galactose. The concentrations were based on molecular weights of 48 400, 45 000, and 35 000 for fetuin, ovalbumin, and the solubilized heavy chain of porcine IgG, respectively.

characterized glycopeptides prepared from the heavy chain of porcine IgG. These experiments were prompted by an observation made in our previous studies (Rao et al., 1976) which indicated that the rate of transfer of galactose to the terminal GlcNAc units of solubilized preparation of the heavy chain of IgG increased by at least one order of magnitude when all of the galactose was removed from the glycoprotein with β-galactosidase. The influence of the concentration of glycopeptide III, which contains two free terminal GlcNAc residues, on the activity of purified galactosyltransferase as a function of the concentration of 2.5, 5.0, and 10 μM UDP-Gal is shown in Figure 2A. Only a partial structure of the terminal end of this glycopeptide, which is described in the text, is shown in the figure. The reducing end of the oligosaccharide chain is attached to asparagine, and the nonreducing chains are completely devoid of galactose and sialic acid. As seen in Figure 2A, a series of intersecting lines was observed with this glycopeptide, and an apparent K_m of 0.25 mM was calculated from primary and secondary reciprocal plots of the data.

The effect of increasing concentrations of glycopeptide II on the activity of galactosyltransferase under identical conditions and at the same concentrations of UDP-Gal is shown in Figure 2B. An apparent K_m of 2 mM was obtained with this glycopeptide, which contains branched oligosaccharide chains terminating in a GlcNAc and a galactose residue. The presence of a galactose residue on one of the branched oligosaccharide chains increased the K_m of the enzyme for the glycopeptide by eightfold. Clearly, the transfer of the first galactose unit from UDP-Gal to glycopeptide III, which contains two terminal GlcNAc residues, would greatly suppress the transfer of a

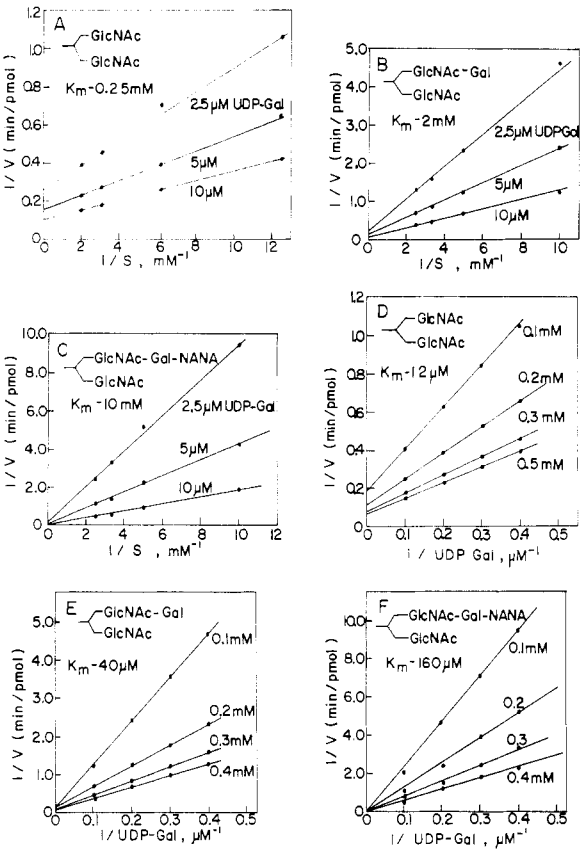


FIGURE 2: Influence of the concentration of different porcine IgG glycopeptides on the activity of galactosyltransferase. The complete structures of the glycopeptides are given in the text. In each case, the standard reaction mixture in a total volume of 0.1 mL was incubated at 37 °C for 30 min with the amounts of UDP-Gal and glycopeptide shown in the figure. (A) Effect of glycopeptide III on the activity of the enzyme at 2.5, 5, and 10 μM UDP-Gal (44 cpm/pmol). (B) Effect of glycopeptide II on the activity at 2.5, 5, and 10 μM UDP-Gal (88 cpm/pmol). (C) Effect of glycopeptide I on the activity at 2.5, 5, and 10 μM UDP-Gal (88 cpm/pmol). (D) Effect of UDP-Gal (44 cpm/pmol) on the activity of the enzyme at 0.1, 0.2, 0.3, and 0.5 mM glycopeptide III. (E) Effect of UDP-Gal (88 cpm/pmol) on the activity at 0.1, 0.2, 0.3, and 0.4 mM glycopeptide II. (F) Effect of UDP-Gal (88 cpm/pmol) on the activity at 0.1, 0.2, 0.3, and 0.4 mM glycopeptide I.

second galactose unit to the remaining terminal GlcNAc residue on the other chain.

The influence of increasing concentrations of glycopeptide I on the activity of galactosyltransferase under these conditions at 2.5, 5, and 10 μM UDP-Gal is shown in Figure 2C. An apparent K_m of 10 mM was found with this glycopeptide, which contains a galactose and sialic acid residue on one of the branched oligosaccharide chains. The addition of a sialyl residue to the galactose present on one of the chains in glycopeptide II increased the K_m of the enzyme for the glycopeptide by fivefold (2 mM compared to 10 mM). These results suggest that the addition of sialic acid to the first galactosyl residue transferred to one of the terminal GlcNAc units in the branched oligosaccharide chain would tend to further decrease the rate of transfer of galactose to the terminal GlcNAc residue on the other chain. The addition of both galactose and sialic acid to one of the branched chains results in a 40-fold increase in the apparent K_m of the enzyme for the glycopeptide (0.25 mM for glycopeptide III compared to 10 mM for glycopeptide I).

Influence of the Structure and Concentration of Various Glycopeptides on the Affinity of Galactosyltransferase for UDP-Gal. Cooperative interactions between the galactosyl acceptor and UDP-Gal binding sites can influence the activity of galactosyltransferase. Therefore, it was of some interest to determine whether the structure of the oligosaccharide chain could also influence the affinity of the enzyme for UDP-Gal. The effect of increasing concentrations of UDP-Gal on the activity of the enzyme as a function of the concentration of glycopeptide III was studied, and the results are shown in Figure 2D. The activity of the transferase increased with increasing concentrations of UDP-Gal at glycopeptide concentrations ranging from 0.1 to 0.5 mM. An apparent K_m of 12 μM for UDP-Gal was calculated from replots of the data in Figure 2D. This value compares favorably with the apparent K_m values of the soluble enzyme (22 μM) and the membrane-bound enzyme (17 μM) for UDP-Gal using a glycoprotein as the high-molecular-weight galactosyl acceptor.

The effect of the concentration of UDP-Gal on the activity of galactosyltransferase in the presence of 0.1, 0.2, 0.3, and 0.4 mM glycopeptide II was measured under identical conditions, and the results are shown in Figure 2E. An apparent K_m of 40 μM for UDP-Gal was obtained with this monogalactosylated glycopeptide. The presence of a galactose residue on one chain of the glycopeptide increased the K_m of the transferase for UDP-Gal by about fourfold (12 μM for glycopeptide I compared to 40 μM for glycopeptide II).

As seen in Figure 2F, a similar increase in the K_m for UDP-Gal was observed when the effect of the concentration of UDP-Gal on the activity of the enzyme was measured as a function of the concentration of glycopeptide I. An apparent K_m of 160 μM for UDP-Gal was obtained when both galactose and sialic acid were present on one of the oligosaccharide chains. This represented a fourfold increase over the monogalactosylated derivative (40 μM for glycopeptide II compared to 160 μM for glycopeptide I) and an overall increase of 13-fold over the glycopeptide containing two terminal GlcNAc residues (12 μM for glycopeptide III compared to 160 μM for glycopeptide I). In each case, a family of straight lines which intersected on the abscissa was obtained, which indicated that only the affinity of the enzyme for UDP-Gal and the galactosyl acceptor were altered by modification of an adjacent oligosaccharide chain and not the basic mechanism of the transferase reaction. These dramatic kinetic effects of the structure of one of the branched oligosaccharide chains on the rate of transfer of galactose to the other chain could explain the results reported in our previous studies, which showed that the oligosaccharide chains in endogenous IgG heavy chain solubilized from microsomal membranes and IgG isolated from porcine serum were incomplete. The predominant glycopeptide isolated from both of these sources lacked one galactose and one sialic acid residue. This type of heterogeneity would be consistent with the properties of the galactosyltransferase described in this report. The accumulation of glycoproteins in the endoplasmic reticulum and Golgi membranes with incomplete oligosaccharide chains resembling the structures in glycopeptides II and III would be expected based on the effect of these branched oligosaccharide chains on the transfer of galactose to the remaining terminal GlcNAc on one of the chains.

Discussion

The (β1→4)galactosyltransferase isolated from mesentary lymph node in our previous studies is probably involved in the synthesis of β1→4 linked galactosyl residues in the heavy chain of IgG immunoglobulin. The evidence presently available suggests that the transfer occurs while the heavy chain of IgG is still attached to membranes in the endoplasmic reticulum before they combine with light chains of IgG which are located almost exclusively in the cytosol. A number of studies with

lymph node tissue (Rao et al., 1976) and isolated cells (D'Amico and Kern, 1968) have shown that the principal galactosyl acceptor in this tissue is the heavy chain of IgG and that it is tightly bound to membranes. Labeling experiments with myelomas that produce normal immunoglobulins have shown that the light chains are synthesized on 150–200S polysomes, whereas the heavy chains are formed on 250–300S polysomes (Scharff and Uhr, 1965; Williamson and Askonas, 1967). Pulse-labeling studies have further shown that the light chains are released into the cytosol (Askonas and Williamson, 1967), whereas labeled free heavy chains could not be found in the soluble fraction of the cytoplasm (Shapiro et al., 1966). Askonas and Williamson (1967) treated membrane preparations with detergents and released bound heavy-chain intermediates from this fraction. All of these observations are consistent with the results obtained in our present studies, which showed that endogenous IgG heavy chain bound to membranes in the microsome fraction (Rao et al., 1967). Heavy chain separated by reduction and alkylation, and a soluble fragment of the heavy chain prepared by treating intact IgG with pepsin acted as galactosyl acceptors. However, intact tetrameric IgG from which these samples were prepared was inactive. The biosynthesis of the carbohydrate unit of the heavy chain of IgG occurs in the microsome and Golgi membrane fractions, since most of the glycosyl transferases and essentially all of the endogenous glycosyl acceptors are firmly bound to membranes in these subcellular fractions. The soluble subcellular fraction, which contains light chains of IgG, is essentially devoid of endogenous galactosyl acceptors.

Ovalbumin, ovomucoid, fetuin, and a solubilized preparation of porcine IgG are excellent galactosyl acceptors for both soluble and bound mesentary lymph node galactosyltransferases. Sialic acid and galactose are removed from the latter two substrates to expose terminal GlcNAc residues. They all contain branched oligosaccharide chains which terminate in GlcNAc. The structure of the protein moiety of galactosyl acceptors does not significantly influence the kinetic properties of the bound or soluble purified galactosyltransferase. However, a macromolecular structure is undoubtedly required, since glycopeptides prepared from these glycoproteins and low-molecular-weight galactosyl acceptors have very high apparent K_m values. The apparent K_m for fetuin (29 μM), solubilized heavy chain of IgG (20 μM), and ovalbumin (54 μM) were much lower than those of free GlcNAc (5.8 mM), *p*-nitrophenyl-GlcNAc (660 μM), and a glycopeptide prepared from the heavy chain of IgG (250 μM).

In previous studies, it was observed that the rate of transfer of galactose to the terminal GlcNAc units of glycoproteins decreased appreciably after 1 mol of galactose was incorporated. It was decided to examine this kinetic effect with glycopeptides prepared from porcine IgG immunoglobulin. The data obtained in the present study show that low-molecular-weight well-characterized glycopeptides prepared from porcine IgG immunoglobulin can be used to examine the effects of changes in the oligosaccharide chains on the activity of purified galactosyltransferase. These results further demonstrate that kinetic regulation of glycoprotein synthesis can occur in branched oligosaccharide chains because the introduction of one sugar residue to one branch of the chain decreases the transfer of the same sugar to other chains in the oligosaccharide. This effect is particularly pronounced after sialic acid has been transferred. The mechanism of this inhibition appears to involve an increase in the K_m of the enzyme for both the glycosyl acceptor and UDP-Gal. The K_m of purified soluble galactosyltransferase for IgG glycopeptide acceptors increased 40-fold, and the K_m for UDP-Gal increased 13-fold when both galactose and sialic acid were present on an adjacent chain.

These findings provide an explanation for the observation that porcine IgG immunoglobulin is present as a monogalactosylated, monosialylated derivative in plasma. The rate of transfer of galactose to terminal *N*-acetylglucosaminyl residues in oligosaccharide chains of the heavy chain of IgG is dependent on whether a galactosyl residue is already present on one of the chains and on the rate of transfer of sialic acid to this galactosyl residue. Thus, the rate of transfer of sialic acid to growing oligosaccharide chains could control the degree of completion of oligosaccharide chains during synthesis.

The sialic acid in the terminal position of plasma glycoproteins plays a central role in determining when these molecules will be removed from the circulatory system. The removal of sialic acid residues results in the rapid clearance of these glycoproteins from blood by the liver (Morrell et al., 1971). The mechanisms responsible for the termination of oligosaccharide chain synthesis are largely unknown. Since sialic acid and fucose are usually found at the nonreducing terminal end in many glycoproteins, it is possible that their attachment may serve as a signal for the termination of synthesis. The results obtained in the present study suggest that the transfer of sialic acid to growing oligosaccharide chains during the synthesis of glycoproteins may function to terminate the synthesis of oligosaccharide chains. The addition of sialic acid to some of the terminal galactosyl residues in a glycoprotein essentially stops the transfer of galactose to other incomplete oligosaccharide chains in the same molecule. The activity of galactosyltransferase is thereby regulated, in part, by the action of sialyltransferase. This effect could explain the marked heterogeneity so often observed in the outer portion of the oligosaccharide chains of many secreted glycoproteins.

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Cytochrome P-450LM$_2$ Mediated Hydroxylation of Monoterpene Alcohols†

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ABSTRACT: The enzymatic hydroxylation of geraniol and nerol by rabbit liver microsomal preparations has been demonstrated to occur exclusively at the C-10 (*E*)-methyl group of both acyclic monoterpene alcohols. This activity was greater in microsomes obtained from barbiturate pretreated rabbits than from β-naphthoflavone treated or control microsomal preparations. Kinetic parameters, V_{max} and K_m, for geraniol hydroxylation were consistent with these findings. Additional evidence for cytochrome P-450 mediation included O_2 and NADPH dependence and sensitivity toward typical P-450 heme protein inhibitors such as SKF-525A, metyrapone, CO, deoxycholate, and cytochrome *c*. Upon incubation of a reconstituted system containing highly purified cytochrome P450LM$_2$, Pb-derived P-450 reductase, and dilauroylphosphatidylcholine with geraniol and nerol, V_{max} values of 0.30 nmol of 10-hydroxygeraniol per min per nmol of P-450LM$_2$ and 0.25 nmol of 10-hydroxynerol per min per nmol of P-450LM$_2$, respectively, were obtained. In general, turnover numbers were comparable to those of the microsomes from rabbits administered phenobarbital. Upon reconstitution K_m values for geraniol and nerol (24 and 19 μM, respectively) were 5–10 times lower than the apparent K_m values observed with microsomes, but similar to microsomal dissociation constants derived from binding studies. Purified P-450LM$_4$ was incapable of supporting either monoterpene hydroxylation corroborating data from differential drug treatment. The interactions of representative hemi-, mono-, sesqui-, diterpene, and polyprene alcohols with the cytochrome P-450 fraction of microsomes from phenobarbital-induced animals were tested by optical difference spectroscopy. Many compounds, including the substrate nerol, elicited a type I binding spectrum. The modified type II spectrum was generated by geraniol, 10-hydroxygeraniol, 10-hydroxynerol, and 3-isopentenyl alcohol. In contrast, solanesol, a nonaprenyl alcohol, failed to produce a change in the Soret band of the heme protein.

In mammals cytochrome P-450 dependent monoxygenases are not only capable of hydroxylating an array of structurally different xenobiotics but also a variety of endogenous lipids (Dus, 1976). Included among the latter are many isoprenoid compounds. While cytochrome P-450 systems have not been implicated in the pathway for cholesterol biosynthesis in mammals, they do participate extensively in steroid metabolism. In the adrenal cortex, conversion of cholesterol to corticosteroid hormones entails P-450 heme protein dependent hydroxylations at carbons 11, 17, 18, 20, 21, and 22. Their involvement has also been observed in the synthesis of steroid sex hormones, vitamin D, and bile acids in mammals (Hayaishi, 1974; Gunsalus et al., 1975), of ecdysones in insects (Bollenbacher et al., 1977) as well as in the conversion of diterpenes to giberellins (Murphy & West, 1969) and of monoterpenes to indole alkaloids (Meehan & Coscia, 1973) in higher plants. Finally the studies of Gunsalus & co-workers (1975) on cytochrome P-450 dependent hydroxylation of the monoterpene camphor in bacteria have provided the most detailed knowledge of the mechanism of action of this system.

Investigations on mammalian biotransformation of terpenes extend as far back as a century ago (Williams, 1959). Analysis of urinary metabolites of administered terpenoids have indicated that allylic hydroxylation, oxidation, and glucuronidation are common modes of converting a hydrophobic molecule to a more water-soluble and hence excretable molecule. Examples of such detoxification processes have been observed in the conversion of *d*-limonene to 13 identified metabolites and, in contrast, the transformation of geraniol to predominantly 1,10-dicarboxylic acids (Hildebrandt's acid; Kodama et al., 1976; Kühn et al., 1936). Despite the numerous studies on mammalian terpene metabolism, involvement of cytochrome P-450 has only been based on circumstantial evidence such as the induction of this heme protein after chronic administration of terpenes (Ariyoshi et al., 1975; Bang & Ourisson, 1975; Cinti et al., 1976) and terpene-induced optical difference spectra with microsomal preparations (Leibman & Ortiz, 1973). These difference spectra have been characteristic of substrate–cytochrome P-450 interactions which can be correlated with spin state changes of the latter (Sligar, 1976).

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