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CHARACTERIZATION OF HUMAN PLATELET UDPGLUCOSE-COLLAGEN GLUCOSYLTRANSFERASE USING A NEW RAPID ASSAY *

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Summary

A rapid and specific assay has been developed for UDPglucose-collagen glucosyltransferase (UDPglucose: 5-hydroxylysine-collagen glucosyltransferase, EC 2.4.1.66) using galactosylhydroxylysine (Gal-Hyl) as acceptor. Studies with intact human platelets and isolated plasma membranes indicated that about 5—10% of the total activity was surface bound and the rest was of cytoplasmic origin. The two forms of the enzyme had similar broad pH optima (6.5—8.0), K_m values for UDPglucose (5 μM) and Gal-Hyl (approx. 4 mM) and for optimal manganese concentrations (25 mM).

The soluble form of the enzyme was purified 80-fold. The reaction mechanism was determined as being rapid equilibrium random BiBi + dead end complex or ordered BiBi with UDPglucose being the first substrate to bind. Using Gal-Hyl bound in purified α1 chain of chick skin collagen, a K_m value three orders of magnitude less (2 μM) was found than for free Gal-Hyl and the manganese requirement decreased to 2 mM. These results suggest that the binding to the enzyme of Gal-Hyl in the collagen molecule is enhanced by the presence of the protein portion so that the enzyme may be capable of recognizing not only the carbohydrate side chains but also the primary structure of collagen.

Introduction

The adhesion of blood platelets to collagen is the primary step in hemostasis. This process may be mediated by the formation of an enzyme-substrate com-

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Abbreviations: Gal-Hyl, galactosylhydroxylysine; Glc-Gal-Hyl, glucosylgalactosylhydroxylysine.

plex between the cell surfact enzyme, UDPglucose-collagen glucosyltransferase (UDPglucose: 5-hydroxylysine-collagen glucosyltransferase, EC 2.4.1.66) and Gal-Hyl residues present in collagen or basement membrane which has been exposed by damage to vascular endothelium [1]. This concept is an extension of a more general hypothesis proposed by Roseman [2] that intercellular adhesion is mediated by the formation of enzyme-substrate complexes between cell-surface glycosyltransferases and complex carbohydrate acceptors on adjacent cell surfaces. The uniqueness of this hypothesis resides in the fact that such enzyme-substrate interactions should be subject to the control mechanisms associated with enzymatic reactions. However, the proposed role of these enzymes requires that the initial formation of an enzyme-acceptor complex occur in the absence of free sugar nucleotides since these are not present in plasma. Thus, elucidation of the validity of the glucosyltransferase theory in cellular physiology requires a knowledge of the reaction mechanism of the various cell-surface enzymes and their specific acceptors.

One difficulty in studying the interaction of cell-surface glycosyltransferases with their substrates has been the inability to obtain enzyme preparations devoid of activities which compete for the carbohydrate substrates. Furthermore, such investigations have been hindered by the lack of large quantities of purified complex carbohydrates from the cell surface for use as specific acceptors.

These problems have now been successfully solved in relation to the studies of the interaction of collagen: glucosyltransferase of human platelets with its specific acceptor. We have used, as a model system, the soluble form of human platelet UDPglucose-collagen glucosyltransferase which possesses activity that appears to be essentially identical to the membrane-associated enzyme. As a specific acceptor, we have used galactosylhydroxylysine purified from marine sponge collagen by a large scale procedure [3]. In this paper we report the characterization of the platelet collagen glucosyltransferase using a rapid and specific assay. Preliminary accounts of this work have appeared elsewhere [4—6].

Materials and Methods

Substrates. For the standard assay of UDPglucose-collagen glucosyltransferase, the acceptor was Gal-Hyl prepared from marine sponge [3]. A higher molecular weight acceptor was prepared as previously described [7] by partial acid hydrolysis of bovine tendon collagen (Sigma, Type I) in order to obtain collagen fragments enriched in Gal-Hyl residues. UDP-[U-^{14}C]glucose (200 Ci/mol) was purchased from either Amersham/Searle (Chicago, Ill.) or New England Nuclear (Boston, Mass). ADP-[U-^{14}C]glucose (234 Ci/mol), TDP-[U-^{14}C]glucose (50 Ci/mol) and UDP-[U-^{14}C]glucose (83 Ci/mol) were obtained from ICN Pharmaceuticals, Inc. (Cleveland, Ohio). Unlabeled sugar nucleotides were from Sigma Chemical Co. (St. Louis, Mo) and pure α1 chain from chick skin collagen was kindly provided by Dr. Andrew H. Kang, Veterans Administration Hospital, Memphis, Tenn.

Platelet fractions. Platelet concentrates prepared from single units of blood (approx. 450 ml) collected from normal volunteer donors in plastic collection

containers using citrate/phosphate/dextrose as anticoagulant were obtained from the Washington, D.C. Red Cross Regional Blood Center. The human platelet preparations, essentially free of contaminating leukocytes (<2%) and red cells, were prepared and homogenized by the glycerollysis technique as previously described [8]. Four fractions were obtained [8]: (a) a soluble fraction which remains above the sucrose layer; (b) a plasma membrane fraction which remains at the sucrose interface; (c) the sucrose layer itself, and (d) the debris fraction which forms a pellet at the bottom of the tube.

The membrane fraction was washed with 0.05 M Tris · HCl, pH 7.4, and suspended in 20% glycerol containing 0.15 M NaCl for storage at −40° C. For the determination of the subcellular distribution of the transferase activity the remaining fractions were dialyzed against 0.05 M Tris · HCl, 1 mM EDTA, and 2 mM mercaptoethanol, pH 7.4, to ensure the complete removal of sucrose which as found to be an inhibitor of the enzyme. These fractions could be stored at −40° C for 1—2 months without loss of enzyme activity.

Standard assay for UDPglucose-collagen glucosyltransferase. UDPglucose-collagen glucosyltransferase was assayed by incubating, in a total volume of 0.1 ml, a mixture containing 12 mM Gal-Hyl, 0.02 mM UDP-[U-^{14}C]glucose (62.5 μCi/μmol), 0.015 M $MnCl_2$, 0.15 M NaCl, 2 mM mercaptoethanol, 1.0 mM EDTA, and 0.1 M Tris · HCl (pH 7.4). NaCl was omitted and 0.1 mM dithiothreitol was substituted for mercaptoethanol when the purified soluble enzyme was assayed. Following incubation (30 min, 37° C) the reactions were terminated by the addition of 0.5 ml of cold 1% phosphotungstic acid in 0.5 M HCl and the precipitated protein was removed by centrifugation. Aliquots (0.5 ml) of the supernatant solutions, which contained the the acid-soluble product, were diluted to 8.0 ml so that a final pH of between 1.0 and 2.0 was obtained.

Cones of the Amicon Centriflo apparatus (Amicon Corp., Lexington, Mass.) were fitted at the bottom of the cone with small discs cut from sheets of porous plastic (Bel-Art Products, Pequannock, N.J.), and 1-g portions of dry AG-50W × 4 resin, H^+ (Bio Rad Laboratories, Richmond, Calif.) were added to the cones. The diluted solutions were applied to the top of the resin in the Centriflo cones and centrifuged for 5 min at 300 rev./min in a Sorvall GLC-centrifuge. The resin was washed with 50 ml of distilled water, and the wash discarded. The product, together with unreacted Gal-Hyl, was then eluted from the resin by washing twice with 2.5 ml of 1.0 M NH_4OH. The volumes of the NH_4OH eluates were measured and 2-ml aliquots were counted in xylene/dioxane/cellosolve scintillation mixture [9].

When partially acid-hydrolyzed bovine tendon collagen was used as a glucose acceptor, the phosphotungstic acid precipitates were washed three times with 1-ml aliquots of 10% trichloroacetic acid to remove unreacted UDP-[U-^{14}C]glucose, dissolved in 0.5 ml of 2.5 M NaOH and hydrolyzed in sealed tubes at 105° C for 20 h. The hydrolysates were diluted to 8 ml and adjusted to pH 2. When the α1 chain of chick skin collagen was utilized as the glucose acceptor, the acid-precipitable product was hydrolyzed in 4 M KOH for 20 h at 105° C and neutralized with 4 M $HClO_4$. The resulting $KClO_4$ was removed by centrifugation and the supernatant was combined with three 2-ml washes of the precipitate, diluted to 8.0 ml and adjusted to pH 2. In each case, the radioactive products were isolated on 2.5 g of AG-50W as described above. These studies were

performed using the soluble form of the glucosyltransferase.

Assay of intact platelets for glucosyltransferase activity. To obtain intact platelet preparations free of plasma components with minimal change in biochemical and physiological properties, platelet concentrates (5 ml, $4 \cdot 10^{10}$ cells/ml) were subjected to gel filtration on Sepharose 2B according to the procedure of Tangen and Berman [15]. Aliquots (0.05 ml) of gel-filtered platelet suspension ($2 \cdot 10^8$—$5 \cdot 10^8$ cells/ml) were assayed for glucosyltransferase in a final volume of 0.1 ml after 1 h incubation at 37°C as described, using the eluting solution [15] as the buffer. No change in enzyme activity was observed when the purified, soluble enzyme was assayed in this buffer system. Total enzyme activity was determined from homogenates of intact platelets prepared by sonication in the presence of 2 mM EDTA. To determine the amount of activity released by the platelet preparations during incubation, assay mixtures without substrates were prepared and incubated for 1 h. Platelets were then removed by centrifugation and aliquots (0.05 ml) of the supernatants were assayed for total activity. Surface-associated activity was the difference between total activity in intact platelet preparations and total activity in supernatants from intact, incubated platelets.

Identification of product. The radioactive product was characterized in the NH_4OH effluents remaining after aliquots were taken to measure radioactivity. The reaction products were pooled and were concentrated by flash evaporation, neutralized, and desalted on a column (1.4 × 80 cm) of Bio-Gel P-2 which had been equilibrated in distilled water. The radioactive fractions from this column were pooled and 5 μmol of purified Glc-Gal-Hyl (glucosylgalactosylhydroxylysine) [3] were added. This mixture was then concentrated by flash evaporation, adjusted to pH 2.0 with dilute HCl and chromatographed by a modification of the ion-exchange chromatographic method of Cunningham et al. [10]. A jacketed column (0.9 × 60 cm) of AG-50W × 8 was equilibrated in 0.2 M sodium citrate at pH 3.25. The column temperature was maintained at 45°C and the flow rate was maintained at 60 ml/h. Hydroxylysine and its glycosides were eluted in a second buffer of 0.2 M sodium citrate, pH 4.25.

When high molecular weight collagen fractions were used as glucose acceptors the alkali-stable products of the reaction were concentrated in a flash evaporator and 26 μmol of purified Glc-Gal-Hyl and 30 μmol of Gal-Hyl were added. This solution was adjusted to pH 2 and applied to a column (0.9 × 57 cm) of AG-50W × 8 (H^+ form) equilibrated in distilled water. The column was then developed using a modification of the procedure for the separation of Glc-Gal-Hyl and Gal-Hyl [3], as follows. After washing with 190 ml of 0.1 M HCl a linear gradient (1200 ml) of HCl from 0.1 to 1.0 M was used to elute the mixture of alkaline-stable reaction products and hydroxylysine glycosides. The column effluents were assayed for neutral sugar by the phenolsulfuric acid method [11] and aliquots were counted to determine the total radioactivity in each fraction.

The radioactive fractions were pooled and desalted on Bio-Gel P-2. The desalted radioactive product was concentrated and submitted to high voltage paper electrophoresis in pyridine/acetate buffer at pH 3.5 [12] using purified Glc-Gal-Hyl and hydroxylysine as standard markers. Hydroxylysine and its glycosides were located with ninhydrin spray (1% ninhydrin in acetone with

5% glacial acetic acid) and the radioactivity was localized by counting 0.5-cm portions of the paper strip.

To determine the nature of the radioactive component, the reaction product was hydrolyzed in 2 M HCl for 4 h at 100°C, and HCl was removed by repeated lyophilization from distilled water. The hydrolysate was submitted to paper chromatography in butanol/pyridine/water (5 : 4 : 3, v/v) for 47 h with glucose and galactose as standards. The neutral sugars were localized by the method of Trevelyan et al. [13] and the radioactivity was localized as described.

Purification of soluble enzyme. The soluble fractions from 65 units of platelet concentrates (270 ml, 6 mg protein/ml) were pooled and treated at 4°C with a solution of saturated $(NH_4)_2SO_4$ which had been adjusted to pH 7 with NH_4OH. The protein which precipitated between 28 and 42% saturation was dissolved in 0.05 M Tris · HCl, 1 mM EDTA, 0.1 mM dithiothreitol, pH 7.4 (Buffer 1). The $(NH_4)_2SO_4$ fraction was dialyzed against Buffer 1 and applied to a column (2.5 × 30 cm) of either Bio-Gel A-DEAE or Whatman DE-52 equilibrated in Buffer 1 at 4°C. The column was then developed with a linear gradient of 0—0.2 M NaCl in Buffer 1 and the fractions containing glucosyltransferase activity were pooled, concentrated by ultrafiltration over an XM-100A membrane (Amicon, Inc.) and stored at —40°C.

Further purification of the enzyme could be achieved by application of the concentrated enzyme preparation to a column (1.5 × 80 cm) of Bio-Gel A-1.5 M equilibrated in 0.05 M cacodylate, pH 7.2, 1 mM EDTA, 0.2 M NaCl, at 4°C (Buffer 2). The fractions containing glucosyltransferase activity were pooled, concentrated and stored at —40°C. Protein concentrations were measured by the method of Warburg and Christian [14]. The results of this purification are shown in Table I.

The enzyme obtained from the DEAE-cellulose columns was extremely stable to freezing (—40°C) and thawing cycles and lost only 10% of its activity at room temperature for 18 h but the enzyme preparation from the Bio-Gel A-1.5 M column maintained full activity for only 2—4 weeks at —40°C. Due to its greater stability the enzyme purified through the DEAE-cellulose column step was used for the kinetic studies.

TABLE I

PURIFICATION OF SOLUBLE UDPGLUCOSE-COLLAGEN GLUCOSYLTRANSFERASE FROM HUMAN PLATELETS

Fraction	Protein (mg)	Units */mg	Relative Specific Activity	Relative total Activity
Soluble fraction	1615	1.44	1.0	1.00
$(NH_4)_2SO_4$ (28—42%)	882	2.73	2.0	1.03
DEAE-cellulose	64.0	23.3	16	0.64
Bio-Gel A-1.5 M	10.2	113.2	79	0.50

* One unit of activity is defined as the amount of enzyme required for synthesis of 1 nmol Glc-Gal-Hyl/h at 37°C.

Results

Identification of product

Both the membrane-associated and soluble cytoplasmic forms of the enzyme gave identical products. The reaction product formed using Gal-Hyl and UDP-[U-^{14}C]glucose as substrates gave a single peak of radioactivity on Bio-Gel P-2 and co-chromatographed with pure Glc-Gal-Hyl on AG-50W X 8 (Fig. 1). All components were quantitatively recovered from both column steps. Glc-Gal-Hyl and the reaction product gave identical migration rates on high voltage paper electrophoresis. Paper chromatography of an acid hydrolysate of the purified reaction product showed that glucose and galactose were the only sugars present and that the radioactivity migrated with an R_F identical to that of glucose. These data indicate that the only product measured in the NH_4OH effluents obtained during the assay procedure was ^{14}C-labeled Glc-Gal-Hyl. The product of the reaction using partially hydrolyzed bovine tendon collagen or purified α1 chain from chick skin collagen as substrates was similarly identified as Glc-Gal-Hyl.

These results indicated that this assay does not require a purification step after isolation of the alkaline-stable reaction product on AG-50W resin. This is presumably a result of the high affinity of the product for AG-50W at extremely low values of pH [3]. The use of higher molecular weight acceptors necessitated the removal of unreacted UDP-[U-^{14}C]glucose from the phosphotungstic acid-precipitable product prior to alkaline hydrolysis due to the formation of non-specific ^{14}C-labeled compounds during hydrolysis of the complete reaction mixture (results not shown).

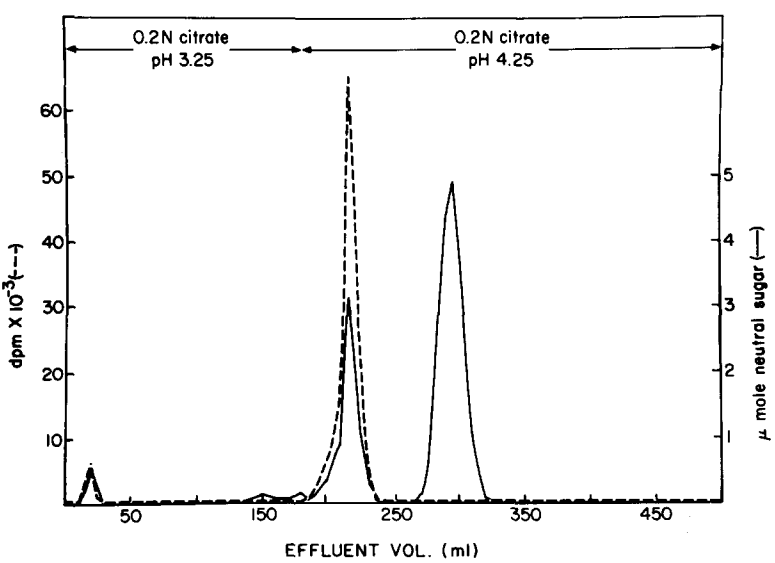

Fig. 1. Co-chromatography of Glc-Gal-Hyl and radioactive reaction product on AG-50W X 8 equilibrated in the first buffer at 45°C and eluted at 60 ml/h. Fractions (5 ml) were assayed for neutral sugar (———) and radioactivity (- - - - -). The neutral sugar peak at 215 ml corresponded to pure Glc-Gal-Hyl. The neutral sugar peak at 295 ml represents unreacted Gal-Hyl which was isolated with the radioactive product during the assay procedure.

Subcellular distribution of platelet glucosyltransferase

The subcellular distribution of the glucosyltransferase is shown in Table II. These data indicate that approx. 80% of the total platelet activity is found in the soluble fraction while only 5% of the total activity is associated with the plasma membrane fraction. The remaining activity was found in the sucrose and debris fractions which represent fragments from intact platelets and the intracellular granules and organelles [8].

In order to establish that the membrane-associated activity did not arise from contamination with soluble enzyme during homogenization, intact platelets were separated from plasma by gel filtration [15]. Under these conditions the surface-associated activity comprised 11% of the total activity determined after disrupting the platelets by sonication (Table III).

Properties of UDPglucose-collagen glucosyltransferase

(a) Membrane-associated activity. When either whole homogenates or various subcellular fractions were assayed, the transferase activity was directly proportional to both time of incubation and protein concentration. Manganese was found to be the most effective metal ion with partial activity being observed with cobalt and magnesium (64 and 14%, respectively). No significant activity was observed with calcium, cadmium or zinc or in the absence of metal ion. The optimal Mn^{2+} concentration was approx. 25 mM with no significant decrease in enzymatic activity with concentrations as high as 80 mM. The membrane-bound glucosyltransferase had a rather broad pH optimum between 7.0 and 8.0. Plots of $1/v$ vs. 1/[substrate] were linear for each substrate, giving a K_m for UDPglucose of 5 μM and a K_m for Gal-Hyl of 3.8 mM.

The activity associated with the plasma membrane was completely inhibited by UTP and UDP at a concentration of 6 mM. Sucrose (0.42 M), *p*-chloromercuribenzoate (10 and 6 mM) inhibited the activity 88, 54 and 54%, respectively. These compounds have been previously described as inhibitors of the rat kidney enzyme [12]. D-Glucosamine (100 mM), Glc-Gal-Hyl (50 mM) or *N*-ethylmaleimide (10 mM) did not inhibit the plasma membrane-associated enzyme.

(b) Soluble activity. The activity in the presence of various divalent cations was essentially the same as that for the plasma membrane-associated activity.

TABLE II

DISTRIBUTION OF UDPGLUCOSE-COLLAGEN GLUCOSYLTRANSFERASE ACTIVITY DURING PLATELET PLASMA MEMBRANE ISOLATION

Fraction	Units */mg protein	Total units **	Relative Total Activity (%)
Lysate	0.99	54	100
Soluble	0.92	43	78
Plasma membrane	0.51	2.6	5
Sucrose layer	0.43	6.4	12
Debris	0.92	13	24

* See Table I.

** Based on the number of platelets in 450 ml of blood.

TABLE III

UDPGLUCOSE-COLLAGEN GLUCOSYLTRANSFERASE ACTIVITY OF GEL-FILTERED PLATELETS

	Units */10^9 cells	Percent Total Activity
Sonicated platelets	2.38	100.0
Intact platelets	0.45	18.9
Supernatant from intact platelets	0.18	7.6
Surface associated activity	0.27	11.3

* See Table I.

Manganese was the most effective metal but could be partially replaced by cobalt, nickel and magnesium. Very little activity was observed in the presence of zinc, calcium, cadmium or copper. The optimal manganese concentration was 25 mM; however, unlike the membrane-associated activity, the soluble enzyme was inhibited at concentrations of Mn^{2+} above 50 mM. The pH optimum was relatively broad being between pH 6.5 and 7.5.

TDPglucose had about 60% of the activity of UDPglucose in the standard assay while GDPglucose and ADPglucose had negligible activity: the K_m for TDPglucose was approx. 29 μM.

Initial velocity studies

A plot of reciprocal velocities of the soluble enzyme versus reciprocal concentrations of Gal-Hyl at various fixed concentrations of UDPglucose and at a fixed concentration of Mn^{2+} (Fig. 2A) gives a family of lines which intersect at a common point. Similarly, a plot of reciprocal velocities versus reciprocal concentrations of UDPglucose at fixed concentrations of Gal-Hyl and at a fixed concentration of Mn^{2+} (Fig. 2B) also gives a family of lines intersecting at a common point. This result rules out any mechanism which requires the dissociation of one product before the addition of the second substrate [16]. Secondary plots (Figs. 2C and 2D) of these results were linear indicating that only a single enzyme mechanism is operating in this reaction.

Double reciprocal plots of $1/v$ vs. $1/[Mn^{2+}]$ at several constant concentrations of UDPglucose gave an intersecting pattern in the range of 1—3 mM Mn^{2+}. These data are consistent with a sequential addition of Mn^{2+} to the enzyme from chick embryo as described by Myllylä [17]. Detailed initial velocity analyses with respect to Mn^{2+} resulted in curved double reciprocal plots which are not readily interpretable due to interaction of Mn^{2+} with both substrates.

Inhibition studies

Both reaction products, UDP and Glc-Gal-Hyl, were tested as inhibitors of the soluble transferase activity. Glc-Gal-Hyl showed no inhibition of transferase activity at levels as high as 75 mM, with UDPglucose present at its K_m concentration. UDP was found to be a competitive inhibitor with respect to UDPglucose (Fig. 3A) and a non-competitive inhibitor with respect to Gal-Hyl (Fig. 3B). The secondary plots (Figs. 3C and 3D) of these results were also linear.

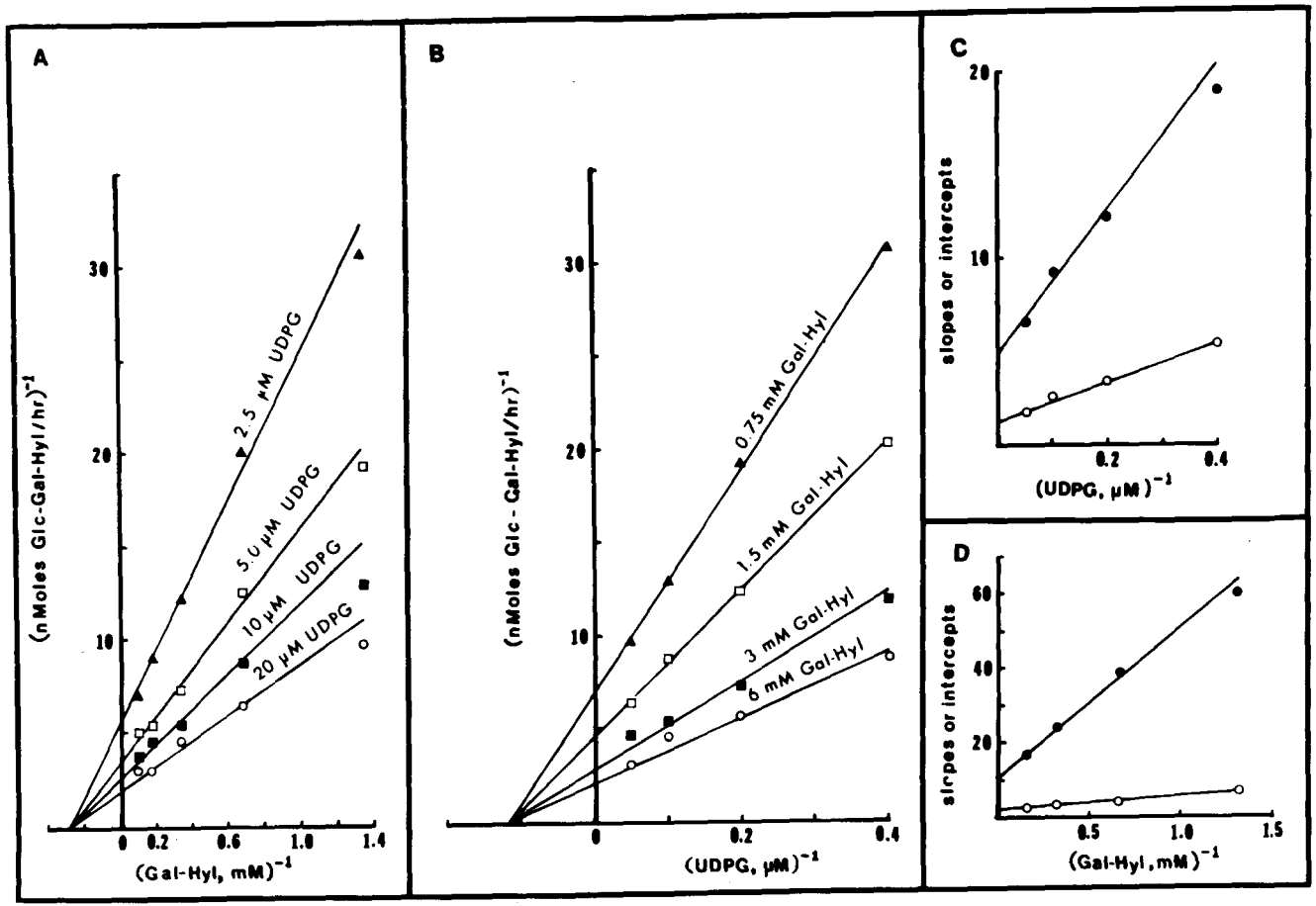

Fig. 2. Initial velocity patterns with Gal-Hyl (A) and UDPglucose (B) as the variable substrate. Replots of slopes (●) and intercepts (○) for A and B are shown in C and D, respectively. The standard assay was used with substrate concentrations as indicated and 17 μg enzyme protein.

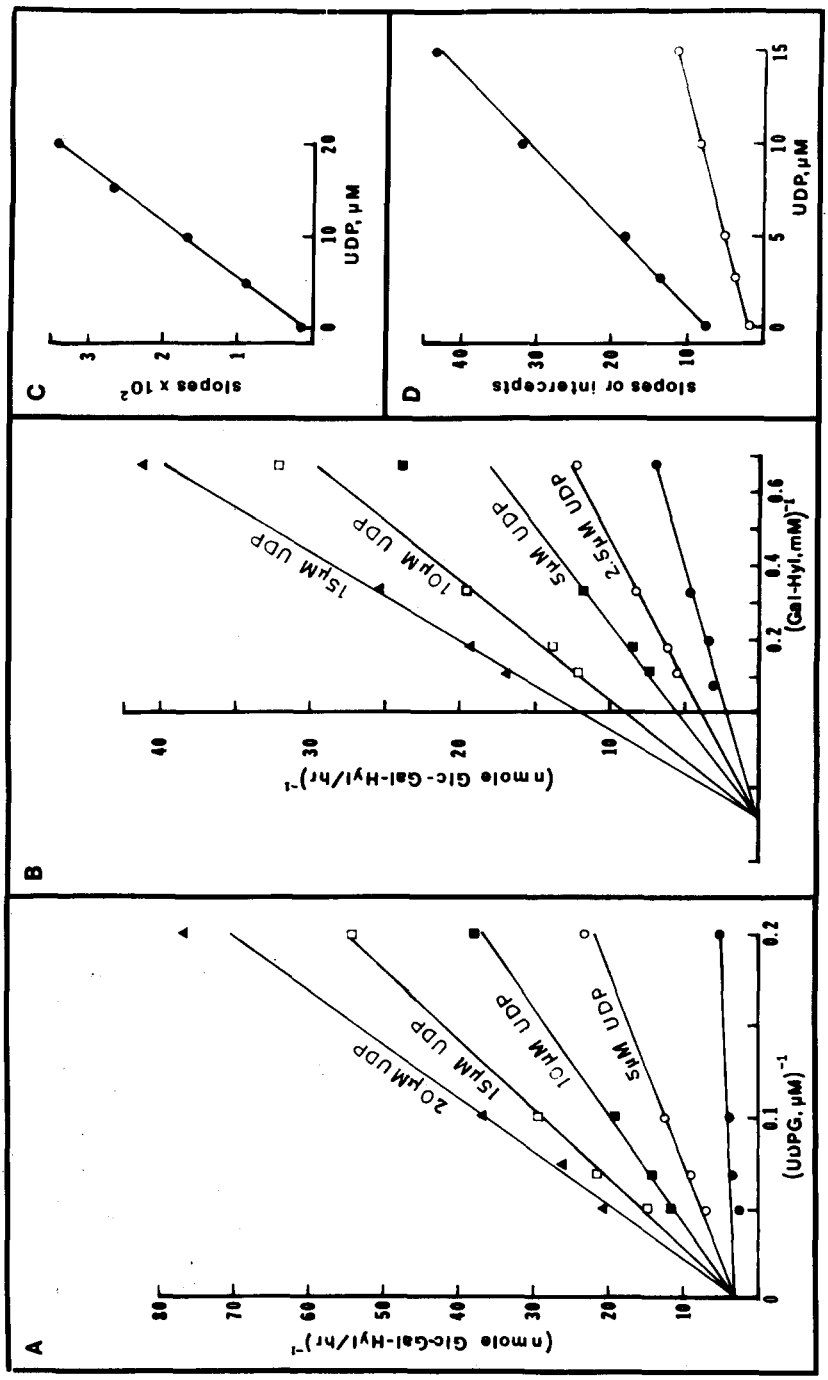

Fig. 3. Inhibition of transferase activity by UDP with UDP glucose (A) or Gal-Hyl (B) as the varied substrate. In the complementary experiment, Gal-Hyl was at a concentration of 12 mM and UDPglucose at a concentration of 20 μM. Replots of slopes (●) and intercepts (○) for A and B are shown in C and D, respectively. The standard assay was used with inhibitor concentrations as indicated and 17 μg enzyme protein.

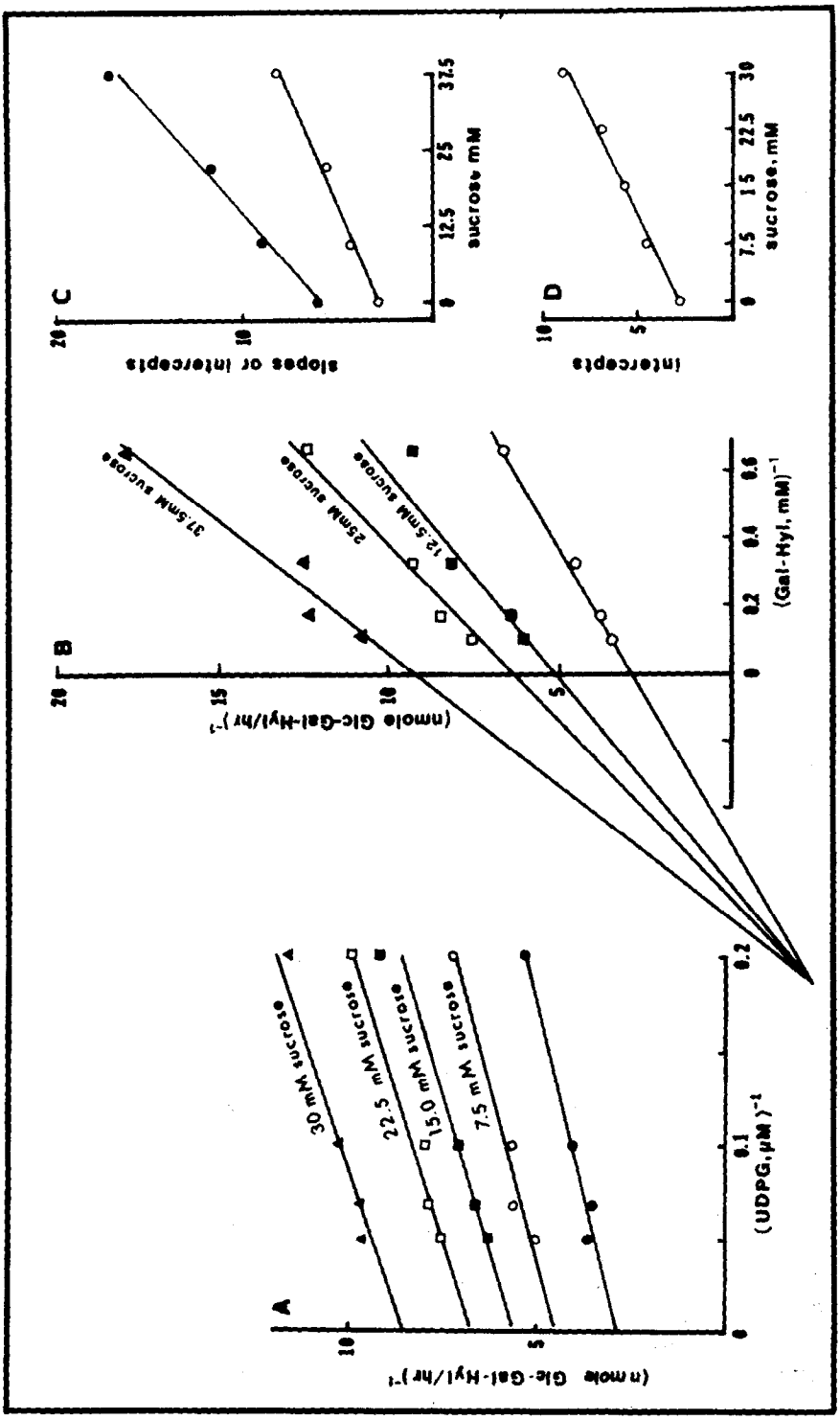

Fig. 4. Inhibition of transferase activity by sucrose using UDPglucose (A) or Gal-Hyl (B) as the varied substrates. Replots of slopes (●) and intercepts (○) of B and A are shown in C and D, respectively. The standard assay was used with inhibitor concentration as indicated and 17 μg enzyme protein.

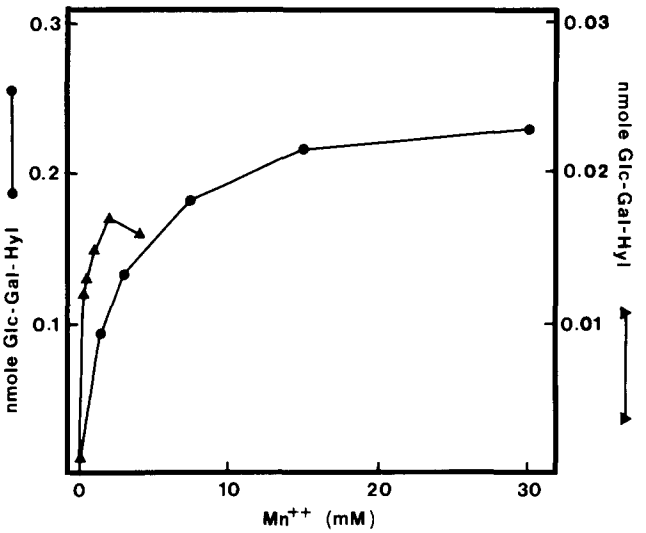

Fig. 5. Comparison of manganese optimum using Gal-Hyl bound in the α1 chain of chick skin collagen (▲) and free Gal-Hyl (●). Incubation with α1 chain as substrate (10 mg/ml) was for 1 h in a final volume of 0.05 ml with 5 μg enzyme protein. All other reaction components were at the same concentration as in the standard assay system. Incubation with Gal-Hyl as substrate was as described for the standard assay using 17 μg enzyme protein.

Sucrose, which has been shown to be an inhibitor of rat kidney cortex glucosyltransferase [12] is an uncompetitive inhibitor of the platelet enzyme with respect to UDPglucose (Fig. 4A) and a non-competitive inhibitor with respect to Gal-Hyl (Fig. 4B). The secondary plots (Figs. 4C and 4D) of these results were linear.

The α1 chain of chick skin collagen as acceptor for glucosyltransferase

A plot of reciprocal velocities versus reciprocal concentrations of chick skin collagen α1 chain at a fixed level of Mn^{2+} and with UDPglucose in excess is linear and gives a K_m for the α1 chain of approx. 1.5 mg/ml. Based on the compositional analysis of this material as reported by Kang et al. [18], which indicated that the α1 chain contained 0.025% Gal-Hyl, the K_m for Gal-Hyl in the α1 chain is 2.2 μM. The optimum Mn^{2+} concentration using the α1 chain was 2 mM rather than 25 mM as observed with free Gal-Hyl (Fig. 5). The possibility that this is due to binding of free Mn^{2+} by Gal-Hyl cannot be ruled out. The binding of Mn^{2+} by collagen has been demonstrated by Myllylä [17].

Discussion

UDPglucose-collagen glucosyltransferase has been isolated from various rat tissues [19], HeLa cells [20], guinea pig skin [21], bovine artery [22], chick embryo [23] and human platelets [7,24].

Two different assay systems have previously been used for this enzyme; one, which measures the incorporation of glucose from UDPglucose into a trichloroacetic acid precipitable fraction has been widely used but is non-specific and, in the case of platelets at least, suffers from a lack of sensitivity and the complica-

tion of incompletely identified endogenous acceptors [7,25]. The second is a highly specific assay using a partially purified glycopeptide fraction from collagenase and pronase digests of basement membrane [12] but it requires alkaline hydrolysis prior to a 6-day chromatographic step for the isolation of the product, Glc-Gal-Hyl. The assay system described in this report used purified Gal-Hyl as the specific glucose acceptor and all radioactivity isolated on AG-50W resin was in the form of Glc-Gal-Hyl. This eliminated the presence of competing carbohydrate acceptors and the necessity for tedious isolation procedures to obtain the specific reaction product which is required when higher molecular weight acceptors are used. During the progress of this work Myllylä et al. [23] reported an assay using calf skin gelatin as the carbohydrate acceptor. This assay system is essentially the same as that described by Spiro and Spiro [12], except that the final 6-day chromatographic step was replaced by high voltage paper electrophoresis for product isolation. Although this procedure decreased the time required to process samples, it requires six paper electrophoresis apparatuses to process 36 samples in 2 days [23]. Using our assay system with two Sorvall GLC table top centrifuges 32 samples can be easily processed in 1 day. The rapid processing makes this assay amenable to detailed kinetic studies while its sensitivity permits enzyme activity to be measured in the platelets from as little as 10 ml of whole blood.

Collagen-glucosyltransferase in the platelet apparently occurs mainly in soluble form but with 5—10% occurring as a membrane-bound form as determined from studies with intact platelets and isolated membranes. In two previous studies the enzyme was reported to be in particulate form with 50% [24] and 90% [7] or the enzyme activity associated with the plasma membrane and with relative purifications of 18- and 100-fold, respectively. These differences from the present study are probably due to the presence of sucrose in the various fractions used for enzyme analysis in the previous investigations [7,24]. Since sucrose is an inhibitor of the transferase activity and was present in very high concentrations in the subcellular fractions and the lysate, erroneously low values were presumably observed in all fractions with the exception of the washed plasma membrane fraction.

The presence of 80% of the total platelet activity in the soluble fraction suggested that the enzyme was present both in membrane-associated and cytoplasmic forms. However, another possibility was that the enzyme was removed from the exterior surface of the plasma membrane during the isolation procedure since this loss of a surface component is known to occur with glycocalicin, a component of the platelet glycocalyx or exterior coat [26]. Moreover, platelets are known to readily undergo the release reaction in response to a variety of stimuli resulting in the expulsion of certain subcellular components to the extracellular space. Since a surface location for the enzyme is crucial to its possible role in platelet:collagen adhesion, the glucosyltransferase activity expressed at the surface of intact platelets was determined in platelets separated from plasma by gel filtration so as to effect minimum damage to biochemical or physiological properties which may occur with repeated centrifugation and resuspension of platelet preparations. The value of 11% for the surface-expressed activity agrees relatively well with the value of 5% for the isolated plasma membrane fraction and the difference probably reflects the yield of plasma mem-

brane with the remaining membrane-associated activity being in the debris fraction.

The level of collagen glucosyltransferase activity in human platelets is approx. 90 nmol/h per g of cells or 1.2 μmol/h per g platelet protein. The purification of UDPglucose-collagen glucosyltransferase from human platelets resulted in an 80-fold increase in specific activity, relative to the initial soluble fraction.

The collagen glucosyltransferase activity from human platelets is similar to that isolated from rat kidney cortex [12] and chick embryo [23,27] which have also been investigated using specific assay systems. These enzymes have essentially identical pH optima (6.5—7.5) and divalent cation requirements with Mn^{2+} being most efficient in all cases. The K_m values for UDPglucose are in a similar range of 5—30 μM and all of these enzyme preparations are capable of using denatured collagen fractions as well as low molecular weight collagen glycopeptides as the glucose acceptor.

Very little is known about the kinetic mechanisms of glycosyltransferases involved in the synthesis of complex carbohydrates of glycoproteins. Our kinetic data obtained using Gal-Hyl as the model glucose acceptor for UDPglucose-collagen glucosyltransferase is compatible with an ordered mechanism based on inspection of initial velocity patterns. Inspection of product inhibition patterns using the method of Cleland [16] suggests that the reaction may be ordered BiBi with UDPglucose as the first substrate or rapid equilibrium random BiBi + dead end EBQ complex; The inability to obtain product inhibition data with Glc-Gal-Hyl prevented the differentiation of these pathways by classical kinetic methods. However, an ordered BiBi mechanism with the sugar nucleotide as the first substrate has been described for the galactosyltransferase involved in the synthesis of lactose and *N*-acetyllactosamine [28]. This mechanism has also been proposed more recently by Myllylä [17] for the collagen glucosyltransferase from chick embryo. This author's conclusions are consistent with our kinetic studies which had been presented in preliminary form earlier [4—6].

The sucrose inhibition pattern, if interpreted within the framework of the ordered mechanism proposed by Myllylä [7] would indicate that this inhibitor is an analog of the disaccharide product, Glc-Gal-Hyl [16]. This is consistent with their structural similarity since both sucrose and Glc-Gal-Hyl possess a non-reducing, terminal glucopyranosyl residue. Thus, available data concerning the mechanism of UDPglucose-collagen glucosyltransferase would favor the ordered BiBi reaction with UDPglucose as the first substrate to bind.

The application of the hypothesis that cell-surface glycosyltransferases are involved in cell-cell interactions [2] places certain constraints on acceptable kinetic mechanisms for these enzymes. In its application to the adhesion of platelets to collagen [1], this theory requires that the first step in the kinetic mechanism be the interaction of collagen glucosyltransferase with galactosylhydroxylysine present in collagen and that the addition of UDPglucose follows as a subsequent step necessary for the formation of product but not for the formation of the adhesion complex. While this point has been emphasized in regard to platelet collagen adhesion [1,4,6], it frequently has not been recognized in application to the general hypothesis [2] that glycosyltransferases may be involved in intercellular adhesion.

Although the available kinetic data for collagen glucosyltransferase do not

support a role for carbohydrate, as such, in platelet collagen adhesion as originally described, studies of the reaction using the α1 chain of chick skin collagen show significantly different properties. In this acceptor, the collagen-bound Gal-Hyl has a K_m of approx. 2.2 μM which is three orders of magnitude less than in the case of free Gal-Hyl. Furthermore, the Mn^{2+} requirement decreased from an optimal value of 25 mM to a value of 2 mM with this acceptor. Since most glucosyltransferases so far studied demonstrate optimum Mn^{2+} concentrations in the non-physiological range (1—25 mM), it is possible that the in vitro assays are not representative of in vivo reactions [29]. This suggests that the use of native or endogenous substrates may be necessary for the investigation of these enzymatic reactions.

The lowered K_m value of Gal-Hyl bound in the α1 chain may indicate either that the binding of Gal-Hyl residues themselves to the enzyme is enhanced when they are in the polypeptide chain or that the α1 chain may be binding at sites other than the substrate site and thus altering the kinetic properties of the enzyme. The latter suggestion is supported by the work of Risteli at al. [30] who showed that collagen glucosyltransferase from chick embryo was bound in the absence of UDPglucose to collagen fragments which had been conjugated with Sepharose. Preliminary data from our laboratory using the platelet enzyme are in agreement with these results. Furthermore, Anttinen and Kivirikko [31] demonstrated that the chick embryo transferase was displaced most efficiently from a UDPglucose derivative of agarose with collagen peptides. The inability of high concentrations of Gal-Hyl to substitute for the collagen peptides [31] indicates that this enzyme has a high affinity for the protein portion of collagen at a site(s) other than the substrate site. Thus, UDPglucose-collagen glucosyltransferase appears to be a "collagen-binding" protein which may not require carbohydrate side chains for initial interaction.

Myllylä [17] and Menashi et al. [32] have shown that collagen in its triple-helical conformation does not act as a substrate for the collagen glucosyltransferase of chick embryo [17] and human platelets [32]. However, these observations do not preclude the ability of this enzyme to bind specifically to collagen since the enzyme is known to bind to insolubilized denatured collagen in the absence of cofactors necessary for transferase activity [30]. This protein could, therefore, function as the collagen receptor of the human platelet as proposed by Puett et al. [33] who suggested that the platelet surface receptor recognized the three-dimensional structure of collagen as well as the Gal-Hyl residues.

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