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Multiple Forms of Galactosyltransferase from Bovine Milk†

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ABSTRACT: A galactosyltransferase which transfers galactose from UDP-galactose to form β-(1–4)-glycosides was isolated from bovine milk. Two molecular forms, 42,000–44,000 and 55,000–59,000 molecular weight, were demonstrated by electrophoresis on polyacrylamide gels and chromatography on Bio-Gel P-200. Both forms contained carbohydrate. Both forms had similar catalytic properties based on similar apparent K_m's for UDP-galactose, *N*-acetylglucosamine, and α-lactalbumin. Both forms were retained to the same extent on α-lactalbumin–Sepharose columns and were inhibited to an equal extent by heating at 54° and by sulfhydryl inhibitors.

Galactosyltransferase (UDP-galactose:D-glucose 1-galactosyltransferase, EC 2.4.1.22) catalyzes the transfer of galactose from UDP-galactose forming β-(1–4) linkages with free *N*-acetylglucosamine (Brew *et al.*, 1968) or protein-bound β-glycosides which terminate with *N*-acetylglucosamine (Schanbacher and Ebner, 1970). Galactosyltransferase alone has a low affinity for glucose (K_m > 1 M) as the galactosyl acceptor but α-lactalbumin (*ca.* 0.1 mg/ml) lowers the K_m to the millimolar region (Fitzgerald *et al.*, 1970a) though V_{max} is affected only moderately (Morrison and Ebner, 1971). The general reaction is described by Scheme I, where R_1 is –OH or an oligosaccharide and R_2 is –OH or –$NHCOCH_3$.

Galactosyltransferase occurs in most tissues bound to the Golgi apparatus (Coffey and Reithel, 1968a,b) but is found in soluble form in milk and blood (Wagner and Cynkin, 1971; Ebner and McKenzie, 1972; Bella and Kim, 1972). Molecular weight estimates range from 29,000 to 130,000 (Palmiter, 1969) for the soluble enzymes though the bovine milk enzyme molecular weight is 42,000 and contains approximately 12% carbohydrate, including 2% sialic acid (Trayer and Hill, 1971). Evidence obtained in several laboratories suggests that there may be more than one form of the enzyme in milk (Magee *et al.*, 1972; Trayer and Olsen, 1972; Klee and Klee,

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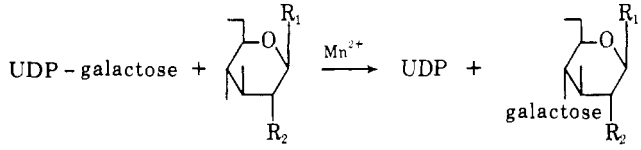
SCHEME I



1972). The present report describes the existence of at least two major forms of galactosyltransferase in bovine milk, each having the same catalytic activities but differing in apparent molecular weights.

Materials

Pyruvate kinase (type I containing 30 units of lactic acid dehydrogenase/mg of protein), NADH, phosphoenolpyruvate, *N*-acetylglucosamine, glyceraldehyde-3-phosphate dehydrogenase, α-chymotrypsinogen, bovine serum albumin, and *p*-chloromercuribenzoate were obtained from Sigma. *N*-Ethylmorpholine and UDP-galactose were purchased from Calbiochem. *N*-Ethylmaleimide was a product of Nutritional Biochemicals and ovalbumin and ultrapure ammonium sulfate were from Mann Laboratories. Bio-Gels were obtained from Bio-Rad Laboratories. Blue Dextran and Sepharose 4-B were from Pharmacia. Electrophoretically pure α-lactalbumin was prepared by chromatography on Bio-Gel P-30 and DEAE-cellulose (Brodbeck *et al.*, 1967) subsequent to an acid precipitation of skim milk (Aschaffenburg and Drewry, 1957).

α-Lactalbumin was coupled to Sepharose 4-B utilizing CNBr (Cuatrecasas *et al.*, 1968; Cuatrecasas, 1970) and the final product contained 1–2 μmol of α-lactalbumin/ml of packed Sepharose (Mawal *et al.*, 1971).

Galactosyltransferase was purified from bovine milk to a specific activity of 1.5–3.5 as described earlier (Fitzgerald *et al.*, 1970a), except the CM-cellulose column was omitted.

Methods

Galactosyltransferase was assayed spectrophotometrically (Fitzgerald *et al.*, 1970b) with a Gilford multiple sample absorbance recorder in the presence of α-lactalbumin using D-glucose as the substrate or in the absence of α-lactalbumin using *N*-acetylglucosamine as the substrate. Blanks were run by omitting the galactosyl acceptor.

Affinity column chromatography was carried out on α-lactalbumin–Sepharose columns with galactosyltransferase solutions diluted with 5–10 volumes of either 50 mM *N*-acetylglucosamine–60 mM *N*-ethylmorpholine (pH 8.0) or 25 μM UDP-galactose–60 mM *N*-ethylmorpholine (pH 8.0). In some experiments, these solutions were made 1 mM in $MnCl_2$ to improve binding of the enzymes to the column. This enzymic solution was applied to an α-lactalbumin–Sepharose column (1 × 10 cm) previously equilibrated with dilution buffer. The column was washed for several fractions (2.2 ml/fraction) with dilution buffer and then the galactosyltransferase activity was eluted with 60 mM *N*-ethylmorpholine (pH 8.0). Active fractions were pooled, precipitated with ammonium sulfate (500 mg/ml), and resuspended in 1–2 ml of 10 mM Tris-Cl (pH 8.2).

Molecular sieve chromatography was carried out on a Bio-Gel P-200 column (1.3 × 108 cm) previously equilibrated with 200 mM ammonium sulfate–2 mM $MgCl_2$–1 mM EDTA–0.5 mM mercapoethanol–20 mM Tris-Cl (pH 7.4). Samples of 0.3–0.6 ml were made 10% in sucrose and 0.1% in Blue Dextran and applied to the column, and 30-drop (1.5 ml) fractions were collected at a flow rate of 1.3–1.5 fractions/hr. Similar patterns were obtained in the absence of Blue Dextran.

Gel electrophoresis was performed with 7.5% polyacrylamide gels using a 5–35-μl sample containing 25–200 μg of protein in 50 mM potassium phosphate (pH 8) which was 10% in sucrose and 0.01% in Bromophenol Blue. A continuous buffer of 50 mM postassium phosphate (pH 8.2) was used. Gels were either stained in 0.5% Amido-Schwarz–7% acetic acid or cut into 1-mm thick sections, mascerated with 0.2 ml of 20 mM glycine–100 mM KCl (pH 7.5), and extracted overnight at 4°. Gel electrophoresis was also carried out in the presence of sodium dodecyl sulfate (Weber and Osborn, 1969) by applying to 11% polyacrylamide gels made up in 0.2% sodium dodecyl sulfate–50 mM sodium phosphate (pH 7.2), 50–250 μg of protein in 35 μl of 2% sodium dodecyl sulfate–2% mercaptoethanol–0.01% Bromophenol Blue–50 mM sodium phosphate (pH 7.2). Bovine serum albumin, ovalbumin, glyceraldehyde-3-phosphate dehydrogenase, chymotrypsinogen, and α-lactalbumin were run in separate gels as molecular weight standards. Electrophoresis was carried out at 12 mA/gel for 2.3 hr which was sufficient for the tracking dye to migrate 8–9 cm. Surgical steel pins were inserted into the gel on the leading edge of the tracking dye. The gels were fixed in 40% methanol–7% acetic acid and washed with two changes of fixative over a 48-hr period. Gels were stained with a periodic acid–Schiff technique for glycoproteins (Zacharius *et al.*, 1969) or with 0.5% Amido-Schwarz in 7% acetic acid. Gels stained with the periodic acid–Schiff technique or with Amido-Schwarz were scanned at 560 or 600 nm, respectively, by a Gilford recording spectrophotometer, Model 2000, equipped with a Gilford gel scanning attachment.

Sialic acid was removed from galactosyltransferase by incubating 0.8 mg of enzyme with neuraminidase, 10^{-3} unit (*Clostridium perfringens*, Sigma) in 0.4 ml of 20 mM sodium acetate–5 mM $CaCl_2$–2 mM mercaptoethanol (pH 5.5) for 3.5 hr at 37°.

Results

Evidence for Two Active Forms. Samples of galactosyltransferase purified by conventional means (Fitzgerald *et al.*, 1970a) frequently exhibited two major bands and one or more minor bands upon polyacrylamide gel electrophoresis. Consistently, the two major bands were capable of forming lactose or *N*-acetyllactosamine (Figure 1). However, elution from hydroxylapatite, CM-cellulose, or DEAE-Sephadex with a variety of gradients failed to resolve the two active forms.

Charge difference effects between proteins can be essentially eliminated upon electrophoresis in sodium dodecyl sulfate gels and galactosyltransferase, purified by affinity chromatography, electrophoresed in sodium dodecyl sulfate gels gave two distinct bands, each staining for carbohydrate and protein (Figure 2). Estimates of the respective molecular weights were 55,000 and 42,000. Increasing the sodium dodecyl sulfate and mercaptoethanol concentrations up to 8% and then heating at 80° for 1 hr did not alter this pattern. Purified samples from different preparations of galactosyltransferase had 55,000:42,000 ratios varying from as high as 7:1 to as low as 1:2 though most had a ratio near 3:1. On the basis of the preceding evidence it could not be ruled out that one of the bands was a contaminant. However, galactosyltransferase applied first to an affinity column equilibrated with UDP-galactose then reapplied to an affinity column equilibrated with *N*-acetylglucosamine demonstrated little change in rela-

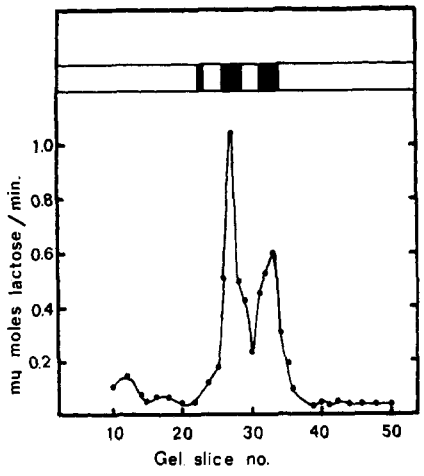


FIGURE 1: Electrophoretic pattern of galactosyltransferase purified by conventional means (Methods). The upper part of the figure is a schematic representation of the staining pattern obtained with Amido-Schwarz. The lower portion of the figure indicates the activity of galactosyltransferase (●) in each 1-mm thick slice of a gel run in a manner identical with the one stained.

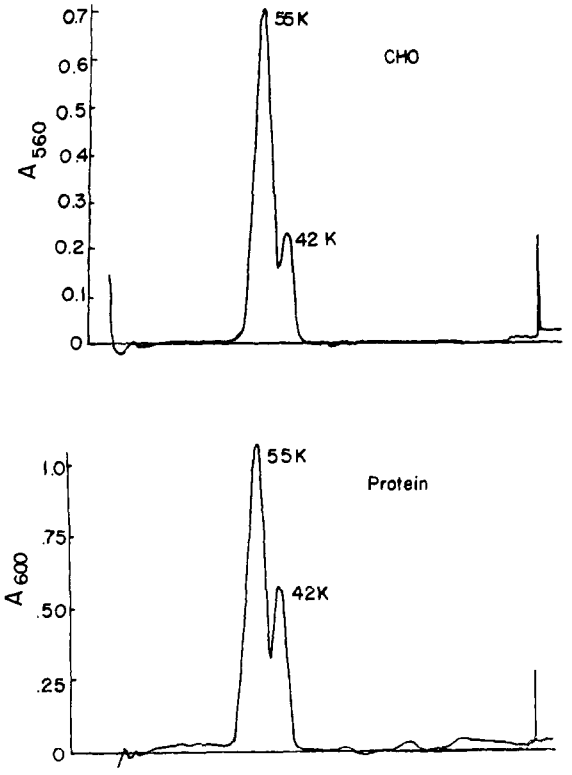


FIGURE 2: Sodium dodecyl sulfate gel electrophoretic patterns of galactosyltransferase purified by conventional means followed by chromatography on an affinity column. Each gel contained 180 μg of protein, one stained with periodate-Schiff reagent for detecting glycoproteins (upper tracing) and the other stained with Amido-Schwarz (lower tracing). The entire gel was scanned and the spike at the right on each scan is the pin marking the leading edge of the tracking dye. 55K and 42K are the 55,000- and 42,000-dalton forms.

tive amounts of the 55,000- and 42,000-dalton proteins. Therefore, each of the two molecular forms observed on sodium dodecyl sulfate gel electrophoresis is capable of forming both a galactosyltransferase–UDP-galactose–α-lactalbumin–Sepharose complex and galactosyltransferase–*N*-acetylglucosamine–α-lactalbumin–Sepharose complex (Mawal *et al.*, 1971).

Galactosyltransferase activity could be eluted from the affinity column in two (or more) major peaks by stepwise elution with buffer containing decreasing concentration of UDP-galactose (Figure 3) but scans of sodium dodecyl sulfate gels of various fractions stained with Amido-Schwarz indicated that the ratios of the amount of higher molecular weight form to the amount of low molecular weight form were the same as in the starting material. Similar results were obtained upon eluting the column with as many as four decreasing concentrations of UDP-galactose or *N*-acetylglucosamine. Since both forms were eluted from the column simultaneously, and in constant ratio, it would appear that both forms have similar affinities for UDP-galactose and for *N*-acetylglucosamine when in the presence of α-lactalbumin.

Galactosyltransferase contains about 2% sialic acid and it is known that covalently bound sialic acid significantly alters the physical properties of some proteins and it was suspected that the two forms of galactosyltransferase observed on gel electrophoresis possibly resulted from identical proteins containing different amounts of sialic acid. However, complete removal of covalently bound sialic acid by neuraminidase resulted in no detectable loss in enzymatic activity and still gave two bands upon gel electrophoresis, though those bands migrated slower than controls. Furthermore both molecular forms appeared on sodium dodecyl sulfate gels and it was observed that the migration rates and relative staining intensities (Amido-Schwarz) were the same as for controls.

Gel filtration on Bio-Gel P-200 resulted in partial resolution of the two catalytically active forms of galactosyltransferase indicating that the two forms do indeed differ in molecular size (Figure 4). Comparison with elution volumes obtained with standard proteins indicated molecular weights of 59,000 and 49,000 for the two forms. The relative activity of the two forms for the respective substrates, glucose and *N*-acetylglucosamine, was similar. Galactosyltransferase isolated from the leading edge of the high molecular weight species (almost exclusively 58,000 form by sodium dodecyl sulfate gels) and from the trailing edge of the lower molecular weight species (almost exclusively 42,000 form by sodium dodecyl sulfate gels) had similar apparent Michaelis constants for *N*-acetylglucosamine (9.0 and 20.2 mM, respectively), UDP-galactose

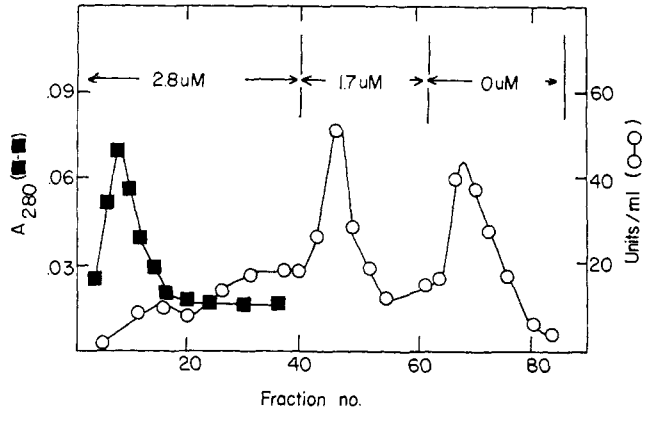


FIGURE 3: Stepwise elution of galactosyltransferase from an α-lactalbumin–Sepharose column. Two milligrams of galactosyltransferase was equilibrated with 2.8 μM UDP-galactose–60 mM *N*-ethylmorpholine (pH 8.0) and applied to the affinity column previously equilibrated with the same buffer. The column was then washed successively with 2.8 μM UDP-galactose–60 mM *N*-ethylmorpholine (pH 8.0), 1.7 μM UDP-galactose–60 mM *N*-ethylmorpholine (pH 8.0), and 60 mM *N*-ethylmorpholine (pH 8.0). Absorbance at 280 mμ (■) and galactosyltransferase activity (O).

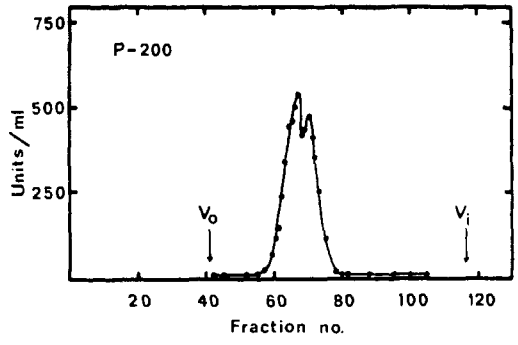


FIGURE 4: Activity profile of galactosyltransferase chromatographed on Bio-Gel P-200. A partially purified sample of enzyme (10.0 units) was applied to a 108 × 1.3 cm column. A flow rate of 1 fraction (1.5 ml)/hr was maintained. Data points are averages of duplicate determinations with *N*-acetylglucosamine as the galactosyl acceptor substrate. The void volume (V_0) and salt peak (V_i) are indicated.

(45.1 and 50.0 μM, respectively), and α-lactalbumin (6 and 4 μM, respectively). Other experiments showed that the apparent K_m for GlcNAc for both molecular forms was nearly identical at 3 different fixed levels of UDP-galactose. In addition to this, enzyme isolated from the leading edge of the P-200 column (high molecular weight form) and from the trailing edge (lower molecular weight form) displayed identical heat inactivation curves at 54° (half-life of 12 min) and both maintained similar rates of inactivation in the presence of 8 mM *N*-ethylmaleimide. With respect to sulfhydryl inactivation, both forms apparently require a free sulfhydryl since 95% of the original activity was recovered upon diluting the enzyme 1:1 with buffer containing 50 mM mercaptoethanol after inhibiting 85% of the galactosyltransferase activity with 0.1 mM *p*-chloromercuribenzoate. Thus, it would appear that the catalytic sites of the two forms of galactosyltransferase are similar, if not identical.

Discussion

Galactosyltransferase previously purified using affinity chromatography displayed, in addition to the previously purified form of 42,000 (Trayer and Hill, 1971), species with molecular weights close to 50,000, though it was not clear whether this represented catalytically active protein (Barker *et al.*, 1972). The present study shows that there are at least two molecular weight forms of galactosyltransferase in bovine milk, one with a molecular weight of 55,000–59,000 and the other exhibiting a molecular weight of 42,000–44,000.

No separation of molecular forms was achieved by affinity chromatography using a stepwise decrease in substrate concentration; however, since approximately equal amounts of the two forms were released from the affinity column with each decreased substrate concentration it is concluded that the two forms have closely similar, if not identical, affinities for the substrates and form the galactosyltransferase–*N*-acetylglucosamine–α-lactalbumin–Sepharose dead-end complex to an equal extent. Both forms contain substantial amounts of carbohydrate, have similar K_m's for the substrates, have similar heat inactivation profiles, and are inhibited to the same extent by sulfhydryl inhibitors. Recent studies have shown that the 42,000 molecular weight form may be derived from the 58,000 molecular weight form by limited trypsin digestion without significant loss of enzymatic activity (Magee *et al.*, 1973).

All the evidence presented indicates that the active centers of the two forms are very similar, if not identical. These results permit further studies on the mechanism of action of the galactosyltransferase even though it is isolated as a mixture of two forms even after purification by affinity chromatography on Sepharose–α-lactalbumin columns.

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