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PURIFICATION BY AFFINITY CHROMATOGRAPHY AND SOME PROPERTIES OF MICROSOMAL GALACTOSYLTRANSFERASE FROM PIG THYROID

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Summary

Membrane-bound 4-β-galactosyltransferase (lactose synthase; UDP galactose: D-glucose 4-β-galactosyltransferase, EC 2.4.1.22) was purified 1500-fold to near homogeneity from pig thyroid microsomes with about 30% yield.

The purified enzyme behaved as a lipophilic protein, rapidly losing activity and aggregating if not supplemented with either Triton X-100 or serum albumin (both of these were equally effective for long-term stabilization).

The enzyme preparation showed an absolute requirement for Mn^{2+}, which could not be replaced by other cations. Catalytic properties were very similar to those reported for soluble forms of the enzyme in biological fluids.

The purified galactosyltransferase showed a major protein band of approx. 74 000 daltons on sodium dodecyl sulfate gel electrophoresis. On gel filtration, enzyme activity was eluted at approx. 70 000 daltons. It is concluded that the membrane-bound thyroid galactosyltransferase is a monomeric protein significantly larger than the soluble forms of this enzyme described earlier; but it resembles recently reported galactosyltransferases from sheep mammary Golgi membranes and liver microsomes.

Introduction

Galactosyltransferase (lactose synthase; UDP galactose:D-glucose 4-β-galactosyltransferase, EC 2.4.1.22) catalyzes the β 1 → 4 transfer of galactose from

Abbreviations: buffer A, 20 mM Tris-HCl (pH 7.4), 25 mM KCl, 5 mM $MgCl_2$, 0.25 M sucrose; buffer B, the same except for 1 mM $MgCl_2$, 1 mM dithiothreitol; buffer C, 40 mM sodium cacodylate-HCl (pH 6.5), 50 mM KCl, 5 mM $MnCl_2$, 0.5 mM dithiothreitol; buffer D, the same except for 100 mM KCl; buffer E, 15 mM Tris-HCl (pH 7.2), 2.5 mM dithiothreitol.

UDP-galactose to *N*-acetylglucosamine (either free or in glycoproteins) or to glucose. The latter reaction, the lactose synthetase activity, is favoured when galactosyltransferase interacts with α-lactalbumin, a property common to soluble and membranous enzymes assayed in a variety of sources [1—3]. Galactosyltransferase is predominantly Golgi-associated in liver [3], thyroid [4] and other tissues producing glycoproteins for export. The proposed presence of the enzyme at cell surfaces in minor amounts (but possibly with specific intercellular functions) remains a matter of controversy [5—9]. Soluble forms of the enzyme, of which the significance and relatedness to membranous galactosyltransferase(s) are unclear, have been extensively studied. By taking advantage of various affinity chromatography procedures, purified preparations were obtained from milk and colostrum [10—17] and from serum [18—20]. More recently, membrane-derived enzymes were also investigated [21—25]. Because of a relative diversity in the observed molecular sizes, it has been proposed that partial proteolysis might create a series of more or less 'intact' but still active species [14,23].

In the present study, thyroid galactosyltransferase was extracted from unfractionated microsomes, which included Golgi membranes, and was purified on α-lactalbumin-Sepharose to near homogeneity. It was found to be larger than most previously reported preparations, thus resembling two recently studied membranous galactosyltransferases [24,25]. It behaved as an hydrophobic enzyme in that it needed to be supplemented with Triton X-100 for maximal activity, but otherwise exhibited catalytic properties common to soluble galactosyltransferases.

Materials and Methods

UDP-[U-^{14}C]Galactose (238—301 Ci/mol) was from The Radiochemical Centre (Amersham, U.K.) or New England Nuclear (Boston, U.S.A.). Unlabeled UDP-galactose, bovine serum albumin (essentially fatty acid-free), α-lactalbumin (more than 90% pure) and ε-aminocaproic acid were from Sigma, *N*-acetylglucosamine from Sigma or Merck. Sodium dodecyl sulfate was from Serlabo; Sephadex G-100 (Superfine), G-150 and G-200 were from Pharmacia.

α-Lactalbumin-Sepharose (approx. 4 mg coupled/ml gel) was prepared essentially as described by others [11] using CNBr-activated Sepharose 4B from Pharmacia. Affinity chromatography takes advantage of the complex formed between galactosyltransferase and α-lactalbumin in the presence of *N*-acetylglucosamine as a substrate of the enzyme [10,26].

Measurement of galactosyltransferase activity. A standardized lactosamine synthetase assay was performed throughout this work, except when specified otherwise. All determinations were in duplicate. Incubation mixtures were in a final volume of 65 μl, including 40 mM sodium cacodylate-HCl (pH 6.5), 50 mM KCl, 5 mM $MnCl_2$, 0.5 mM dithiothreitol, 0.1% (v/v) Triton X-100, 0.1% (w/v) lipid-free serum albumin, 10 mM *N*-acetylglucosamine (omitted for determination of the endogenous activity in crude samples), 0.125 mM UDP-[^{14}C]-Gal (0.04 μCi) and enzyme. After 1 h at 37° C (30 min for crude samples, assayed at 2 enzyme concentrations), reactions were stopped by adding 30 μl 1% (w/v) sodium tetraborate, 50 mM EDTA, and were stored at −20° C. The

reaction mixture (plus 2 washings) was spotted on Whatman 3 MM paper and electrophoresed at 2500 V for 1 h. The segment of paper containing *N*-acetyl-[^{14}C]lactosamine, near the origin, was counted in 10 ml scintillation fluid (5 g PPO and 0.1 g dimethyl POPOP per l toluene; counting efficiency: 68%).

Enzyme purification from thyroid microsomes. Pig thyroid glands (usually 240 g) were sliced and homogenized in 3 vols. cold buffer A with an Ultra-Turrax (3 times at half-maximum speed for 20 s). The temperature was kept at 0—4° C during the preparation procedure. After 10 min centrifugation at 10 000 × *g*, supernatants were submitted to 90 min centrifugation at 105 000 × *g*. Microsomal pellets were suspended in buffer A for washing, sedimented and stored at —20° C for 18 h before extraction.

In order to release galactosyltransferase, microsomes from 240 g thyroid (400—500 mg protein) were suspended in 100—200 ml buffer B and either ultrasonicated [27] using a Branson apparatus B-12 (6 experiments) or treated for 45 min with Triton X-100 (0.2% (v/v) in 2 experiments, 0.5% (v/v) in 2 others). After 1 h centrifugation at 140 000 × *g*, the supernatants contained from about 45% (sonicated extracts) to 98% (0.5% Triton X-100 extracts) of the initial activity.

These extracts were immediately dialyzed for 20 h (3 changes) against the buffer used for affinity chromatography, buffer C made 10 mM *N*-acetylglucosamine, and, for detergent extracts, also containing Triton X-100 at the concentration used for extraction (pilot assays showed that this detergent did not interfere with affinity chromatography on α-lactalbumin-Sepharose). With sonicated extracts, some precipitated material was discarded at the end of the dialysis, by centrifugation at 14 000 × *g* for 20 min. The column of α-lactalbumin-Sepharose (3 × 45 cm) (re-utilized several times, a 2 M KCl washing being performed after each use; stored in the presence of 0.04% sodium azide) was pre-equilibrated with buffer C containing 10 mM *N*-acetylglucosamine (and eventually Triton X-100 as indicated above). After charging the extract, 5-ml fractions were collected, measured for galactosyltransferase and for absorbance at 280 nm, (sonicated extracts). In the case of Triton X-100 extracts, the washing preceding the elution of the exchanged enzyme was either without detergent (2 experiments) or at 0.02% detergent (i.e. above the critical micellar concentration, but less than in the applied extract, 2 experiments). Elution was achieved by omitting *N*-acetylglucosamine; in all cases, a symmetrical activity peak emerged in the same position at about 4 void volumes.

In two preparations, 10 mM ε-aminocaproic acid was introduced from the extraction step to the end of the purification as a protective agent against proteolysis [15,24].

Eluted active fractions were pooled. Small aliquots were removed, stabilized for enzyme activity (by adding either 0.02—0.5% (v/v) Triton X-100, if not present, or 1 mg/ml lipid-free serum albumin) and stored in liquid N_2. The remaining material was usually dialyzed against buffer E and concentrated using a Sartorius membrane (pore diameter 5 nm) under N_2, and/or alternatively by surrounding a dialysis bag with Sephadex G-200.

When a second affinity chromatography (1.5 × 7.5 cm column) on α-lactalbumin Sepharose was carried out (3 samples), it was performed as soon as possible with the unconcentrated material from the first column made 10 mM *N*-acetylglucosamine.

Protein estimation. Protein content was determined by a modification of the method of Lowry et al. [28] which comprised alkylation of dithiothreitol [29] and addition of sodium dodecyl sulfate when Triton X-100 was present [30]. Bovine serum albumin was used as standard.

For one purified preparation, protein content was calculated from an amino acid analysis kindly performed by Dr. H. Rochat and G. Martinez (with a Beckman analyzer); the sample was dialyzed against 0.5 M ammonium acetate, lyophilized and subjected to a 6 M HCl hydrolysis at 110°C for 24 h, before analysis.

Polyacrylamide gel electrophoresis. Electrophoresis was carried out essentially by the method of Weber and Osborn [31].

Analytical gel filtration. Gel filtration experiments were carried out on calibrated columns (1 × 88 cm) of Sephadex G-100 and Sephadex G-150 using a detergent-free solvent (buffer D). 0.5 ml samples of purified galactosyltransferase in the same buffer (stabilized for enzyme activity by one of the procedures mentioned above and kept frozen until use) were applied to these columns. 0.45 ml fractions were collected in 25 μl serum albumin, Triton X-100 and *N*-acetylglucosamine (at concentration of 19-fold that of each compound in the standard assay). After mixing, 0.1-ml aliquots were removed, received UDP-[^{14}C]galactose and were incubated for 3—6 h at 37°C. The markers for calibration were pig thyroglobulin, serum albumin and its dimer, ovalbumin and cytochrome *c*.

Results

Purification procedures

After pilot assays suggesting a negligible release of thyroid microsomal galactosyltransferase in the presence of high KCl concentrations, we chose to extract the enzyme either by ultrasonication or by Triton X-100 *.

The results of two representative experiments are summarized in Table I (sonicated extract) and Table II (0.5% Triton X-100 extract). An almost total release from microsomes was possible with the detergent instead of about 50% solubilization by sonication. The proportion of enzyme not exchanged on the column (probably vesicle-bound enzyme) was usually less for Triton X-100 extracts than for sonicated ones. Final recoveries in enzyme units were over 30% for Triton X-100 extracts and approx. 25% for sonicated extracts. The purification in comparison to starting microsomes was approx. 1500. The purified enzyme was very unstable (except when Triton X-100 was present in the eluting buffer) and this may have caused some underestimation of actual enzyme recovery.

Molecular properties of the purified galactosyltransferase

Polyacrylamide gel electrophoresis. After the affinity chromatography step, and whatever the initial extraction procedure, electrophoresis in 5% polyacrylamide gels in the presence of 0.1 M sodium dodecyl sulfate (Fig. 1) revealed a

* Nonidet P-40, and S 10-7 [32], a gift from Dr. Egan, also gave a good solubilization of the enzyme activity.

TABLE I

PURIFICATION OF GALACTOSYLTRANSFERASE AFTER SONIC EXTRACTION

Number of comparable experiments: 6.

Steps in purification	mUnits (nmol Gal/min)	Protein (mg)	Spec. act. (mU/mg protein)	Enzyme recovery (%)	Purification (-fold)
Suspension of microsomes from 240 thyroid	244	416	0.6	100	
After sonication	252	416	0.6	100	
Supernatant after ultracentrifugation	116	196	0.6	46	
Dialyzed and clarified (14 000 × *g*)	91	130	0.7	37	1.2
Unretained on α-lactalbumin-Sepharose	25	n.d.	n.d.	10	
Recovered purified from α-lactalbumin-Sepharose	61	0.060 *	1020	25 *	1700 *

* Protein was determined by a modification of the method of Lowry et al. [28], after pressure-concentration of the diluted purified enzyme.

major, somewhat diffuse, Coomassie Blue-stained band, of average apparent molecular weight of 74 000. Two minor bands of molecular weights 64 000 and 52 000 were always observed. Addition of ε-aminocaproic acid during the preparation did not suppress the 64 000 dalton material. Heavier minor species of apparent molecular weight of 92 000 and 160 000 were occasionally present, especially after purifying sonicated extracts, in the absence of Triton X-100. Omitting previous heating of the samples with β-mercaptoethanol did not greatly modify the electrophoresis patterns. Neither did a second affinity chromatography of the purified enzyme. That most of this material was galactosyltransferase was inferred from a gel electrophoresis without sodium dodecyl sulfate.

Electrophoresis on a similar gel but without sodium dodecyl sulfate, in Tris-glycine buffer (pH 8.3) was performed with an enzyme initially stabilized by 0.02% Triton X-100. As shown in Fig. 2, Coomassie Blue revealed a single spot with a mobility identical to the enzyme activity peak determined in a parallel gel.

Analytical gel filtration. When we first examined on a Sephadex G-100

TABLE II

PURIFICATION OF GALACTOSYLTRANSFERASE AFTER EXTRACTION BY 0.5% TRITON X-100

Number of experiments: 2. Triton X-100 was not maintained during elution.

Steps in purification	mUnits (nmol Gal/min)	Protein (mg)	Spec. act. (mU/mg protein)	Enzyme recovery (%)	Purification (-fold)
Suspension of microsomes from 240 g thyroid	345	425	0.81	100	
After adding Triton X-100	350	425	0.82	102	
Supernatant after ultracentrifugation	339	294	1.15	98	1.4
Dialyzed	320	260	1.23	86	1.5
Recovered purified from α-lactalbumin-Sepharose	110	0.090 *	1220	32	1500

* Protein determination from amino acid analysis.

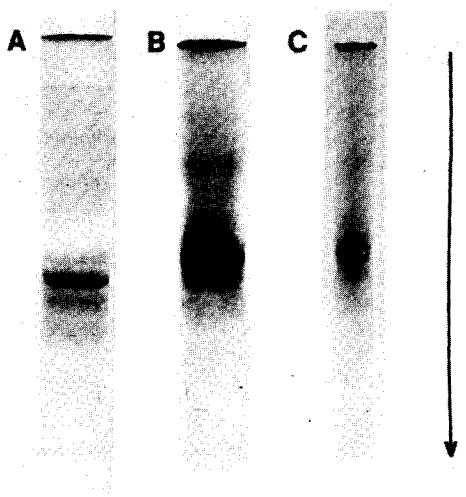

Fig. 1. Sodium dodecyl sulfate polyacrylamide gel electrophoresis of purified galactosyltransferase. Stained with Coomassie Blue. 5% polyacrylamide gels in 0.05 M Tris-0.38 M glycine (pH 8.3) containing 0.1% sodium dodecyl sulfate. Tracking dye: Bromophenol Blue. Markers for calibration were pig thyroglobulin, γ-globulin, serum albumin and its dimer, and ovalbumin (respective M_r: 330 000, 155 000, 67 000 and 134 000, 45 000). Dialyzed galactosyltransferase samples were concentrated under pressure and/or with Sephadex G-200. A, galactosyltransferase purified from a sonicated extract, first affinity chromatography; B, the same after a second affinity chromatography. A and B were treated for 2 h at laboratory temperature with 1% (v/v) β-mercaptoethanol and 1% sodium dodecyl sulfate. C, galactosyltransferase purified from a Triton X-100 extract, ε-aminocaproic acid being present during the purification; treated for 25 min at 100°C with 2% β-mercaptoethanol and 2% sodium dodecyl sulfate.

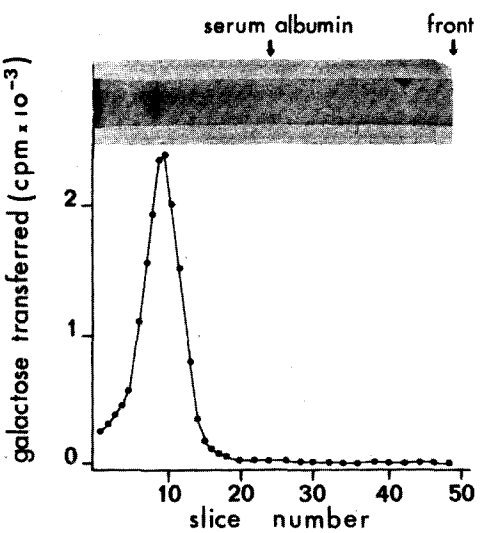

Fig. 2. Polyacrylamide gel electrophoresis of purified galactosyltransferase. Upper part is a Coomassie Blue stained gel; lower part represents the activity in each 1 mm thick slice of a duplicate gel (estimated by incubating in 0.1 ml of the usual incubation mixture). 5% polyacrylamide gels in Tris-glycine as in Fig. 1, except for no sodium dodecyl sulfate. The sample analyzed was purified from a sonicated extract and concentrated under pressure.

column run in buffer D the unsupplemented galactosyltransferase eluted from α-lactalbumin-Sepharose in buffer C, it was found to have high tendency to self-aggregate in the form of a material of poor activity which was excluded from the column.

In contrast, it was possible to study the purified galactosyltransferase stabilized by Triton X-100 or serum albumin on calibrated columns developed without detergent. Using samples purified from sonicated extracts or from Triton X-100 extracts, these experiments revealed more or less symmetrical activity peaks centred on approx. 70 000 daltons.

Enzymic properties of the purified galactosyltransferase

Enzyme properties were investigated with purified samples (in buffer C) supplemented with 1 mg/ml serum albumin or 0.02—0.05% (v/v) Triton X-100: a series of controls have shown us the efficiency of either addition for preventing any loss of enzyme activity, for as long as several months in liquid N_2. In their absence, enzyme activity was lost in a few days of storage at 0° C and by freezing.

The standard assay that was adopted, after testing a range of conditions, also included serum albumin and Triton X-100. Whereas for the purified enzyme the detergent certainly helps to maintain a conformation necessary for activity by interacting with an hydrophobic region of its molecule, when using microsomes, it first acts by dissociating the enzyme from its membranous environment.

In contrast to unpurified galactosyltransferase, purified preparations were totally free of contaminating UDP-galactose-pyrophosphatase. The activity in a standard assay was found to increase linearly with time for up to 15 h or more (Fig. 3). The reaction was faster at 37° C than at 31 or 27° C. A relatively broad pH optimum, from pH 6.4—7.6 was evaluated, with sodium cacodylate-HCl and

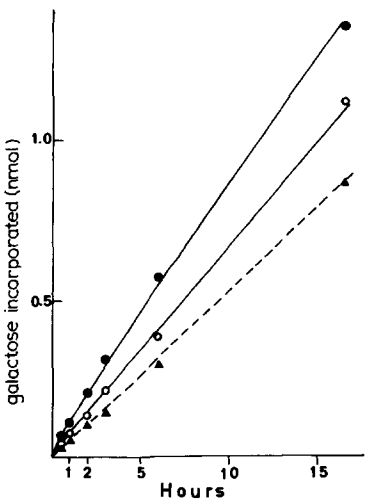

Fig. 3. Galactose transfer to *N*-acetylglucosamine as a function of incubation time. Purified enzyme stored frozen in buffer B, supplemented with serum albumin. Incubation at 37° C (●———●), 31° C (○———○) and 27° C (▲———▲).

Tris-HCl buffers. In the presence of 10 mM *N*-acetylglucosamine as galactose acceptor, the K_m for UDP-Gal was found to be $8 \cdot 10^{-5}$ M, a value close to that already reported for a much less purified thyroid galactosyltransferase with the same acceptor [27]. In our conditions of assay, the K_m for *N*-acetylglucosamine was close to $4 \cdot 10^{-3}$ M; excess of substrate was inhibitory.

Although during this work, thyroid galactosyltransferase was routinely assayed for its lactosamine synthetase activity, evidence was obtained that the same purified enzyme was able to transfer galactose from UDP-galactose to desialyzed-degalactosylated fetuin. It was totally inactive for transferring *N*-acetylneuraminic acid from CMP-AcNeu to desialyzed fetuin or desialyzed thyroglobulin. No galactose transfer from UDP-galactose to an exogenous dolichol (from Sigma) could be observed.

Dialyzing for 15 h against a 10 mM EDTA-buffer C without $MnCl_2$, then twice for 3 h against the same but without EDTA, rendered the purified enzyme (a sample supplemented with serum albumin) totally inactive in the absence of added cations. Full activity was restored by introducing 5—10 mM $MnCl_2$ in the assay. In our standard working conditions, a 5 mM Mn^{2+} concentration was sufficient for maximal activity.

Discussion

These results show that a relatively simple and rapid method enabled us to isolate a highly purified thyroid 4-β-galactosyltransferase, an enzyme intrinsically associated with thyroid endomembranes. This purification relies upon an adaptation to either ultrasonic, or preferably Triton X-100, microsomal extracts of an affinity chromatography proposed by others for soluble forms of the enzyme in various biological fluids. Considering the instability and hydrophobic behaviour of the membranous galactosyltransferase, we conclude that the following optimized technique should be adopted: (1) a first affinity chromatography of a Triton X-100 extract on α-lactalbumin-Sepharose performed entirely in the presence of this detergent, (2) a second exchange of the recovered activity but on a small column, Triton X-100 still present, (3) discontinuing the detergent before the elution, or adjusting its concentration as a function of the experiments to come, thus obtaining finally a concentration of the enzyme with minimal risks of aggregation and losses.

Although rigorous characterization of the purified thyroid galactosyltransferase was not possible at the present time because of the small quantities prepared (approx. 100 μg protein per 240 g thyroid) our data suggest that it was nearly pure. On sodium dodecyl sulfate gel electrophoresis it revealed a major, somewhat diffuse, constituent of average apparent molecular weight 74 000, accompanied by a minor one of 64 000. Apparently in agreement with these results, when serum albumin or Triton X-100-stabilized samples were examined on molecular sieve columns developed in the absence of detergent they revealed activity peaks at values close to 70 000 daltons.

It is known that gel filtration is sometimes capable of removing all detergent associated with hydrophobic proteins [33]. This may indeed be the case for Triton X-100-supplemented samples as nearly similar apparent molecular weights were found for the samples from sonicated extracts, stabilized with

essentially lipid-free serum albumin. Alternatively some lipids might remain tightly enzyme-bound in the latter samples and some Triton X-100 in the former; further work is necessary to ascertain these points.

Molecular weights calculated from the sodium dodecyl sulfate polyacrylamide gel electrophoresis might be over estimated due to the possible presence of carbohydrates in galactosyltransferase [1,34]. In other experiments, we have repeatedly observed that approx. 85% of our purified activity could be retained on Concanavalin A-Sepharose; it has not yet been determined whether the approx. 15% unretained enzyme contains carbohydrate units unrecognized by the lectin, or no carbohydrate.

It is possible that the approx. 64 000 and 52 000 dalton species result from a limited proteolysis of the larger major component: a prolonged storage of the microsomes (2 weeks instead of one night at −20° C) was found to increase the proportion of the minor species.

It is now recognized for the multiple forms of 4-β-galactosyltransferase in milk and colostrum that they derive from a glycoprotein of average molecular weight of 55 000, more or less convertible by proteolysis into smaller, still active, glycosylated species of molecular weight as low as approx. 43 000 [1]. The addition of ϵ-aminocaproic acid, an inhibitor of plasmin-like proteases, during the purification of the milk enzyme, results in an increased proportion of the large component. In this work 10 mM ϵ-aminocaproic did not have a great effect on molecular sizes. The less studied soluble forms of galactosyltransferase found in serum, show molecular weights values of 43 000—85 000 [18—20,24].

It is interesting that in two recent reports on membrane galactosyltransferases, it was concluded that large sized molecules exhibit (as reported here for the thyroid enzyme) instability in aqueous solvents [24,25]. It is tempting to speculate, as proposed by Smith and Brew [25], when comparing the sheep mammary gland Golgi enzyme with that significantly smaller from colostrum, that an hydrophobic peptidic extra-segment, cleavable by proteolysis, might be responsible for anchorage of fully-sized galactosyltransferase to membranes. In certain circumstances intact species could then be delivered into biological fluids such as serum [20], and, in other cases, products of proteolytic cleavage. Structural studies of reasonably pure membrane galactosyltransferase are needed to solve these problems.

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