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INACTIVATION OF GALACTOSYLTRANSFERASE BY LACTOPEROXIDASE AND *N*-ACETYLIMIDAZOLE

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Summary

Galactosyltransferase (UDPgalactose:D-glucose 4-β-D-galactosyltransferase, EC 2.4.1.22) was totally inactivated by iodination with lactoperoxidase (donor:hydrogen-peroxide oxidoreductase, EC 1.11.1.7). Substrates protected against inactivation. The presence of 10 mM Mn^{2+} and 1 mM UDPgalactose gave partial protection which was enhanced by the addition of 10 mM *N*-acetylglucosamine, but not by glucose. These results are consistent with a conformational change upon binding of UDPgalactose. Only monoiodotyrosine and diiodotyrosine were identified in the pronase digest of iodinated galactosyltransferase. Galactosyltransferase was also inactivated with *N*-acetylimidazole and partial activity was restored by treating acetylated galactosyltransferase with hydroxylamine. These results suggest that tyrosine(s) is essential for galactosyltransferase activity.

Introduction

Galactosyltransferase (UDPgalactose:D-glucose 4-β-D-galactosyltransferase, EC 2.4.1.22) catalyzes the transfer of galactose from UDPgalactose to carbohydrate acceptors forming β-(1,4) linkages with free *N*-acetylglucosamine [1] (Reaction I) or protein-bound β-glycosides which terminate with *N*-acetylglucosamine [2,3].

$$\text{UDPgalactose} + \textit{N}\text{-acetylglucosamine} \xrightarrow{Mn^{2+}} \textit{N}\text{-acetyllactosamine} + \text{UDP} \quad \text{(I)}$$

At low concentrations, glucose is a poor substrate for the galactosyltransferase ($K_m > 1$ M), but in the presence of α-lactalbumin (approx. 0.1 mg/ml), the K_m

for glucose is lowered to the millimolar region [4] while the V is essentially unchanged [5] (Reaction II).

$$\text{UDPgalactose} + \text{glucose} \xrightarrow[\alpha\text{-lactalbumin}]{Mn^{2+}} \text{lactose} + \text{UDP} \quad \text{(II)}$$

At least two catalytically active molecular weight forms (58 000 and 42 000) of the galactosyltransferase can be isolated from bovine milk [6—8] and the lower molecular weight form appears to arise from a trypsin-like proteolytic cleavage of the higher molecular weight form [9]. Both forms have very similar catalytic properties [8] and they contain 13—15% carbohydrate [10].

Previous studies have shown that galactosyltransferase is inactivated, but not completely, by *p*-chloromercuribenzoate, *N*-ethylmaleimide and trypsin [11]. In addition, the enzyme is completely inactivated by ultraviolet light with the concomitant loss of one tryptophan residue [12]. In the previous cases, substrates, and in particular UDPgalactose, protected against the inactivating reagent providing evidence for a conformational change. Direct evidence for such a conformational change upon addition of substrates was observed in circular dichroism studies [13].

In particular, changes in ellipticity of galactosyltransferase at 265 nm and 287.5 nm were saturable with Mn^{2+}-UDPgalactose. Such changes are consistent with the involvement of tryptophan and tyrosine residues. Accordingly, the present study examines the role of tyrosine modifying agents upon the activity of galactosyltransferases in the presence of various substrates.

Materials

Pyruvate kinase (ATP:pyruvate 2-*O*-phosphotransferase, EC 2.7.1.40) (type I containing 30 units of lactate dehydrogenase per mg protein), NADH, phospho*enol*pyruvate, *N*-acetylglucosamine, bovine serum albumin, monoiodotyrosine, and diiodotyrosine were purchased from Sigma Chemical Company. Iodohistidine was prepared by iodination of histidine with lactoperoxidase and purified on a Dowex 50-X8 (NH_4^+) column by eluting with 6.5 M NH_4OH. Catalase (CTS, 40 000 units per mg) was obtained from Worthington, and UDPgalactose and Pronase (Grade B) were from Calbiochem. *N*-Acetylimidazole was from Pierce Chemical Company; hydroxylamine hydrochloride was from Fisher and barbituric acid was from Eastman. Bio-Rex 70, 200—400 mesh, was purchased from Bio-Rad Laboratories and $Na^{125}I$ was obtained from ICN Pharmaceuticals, Inc.

Lactoperoxidase was purified from bovine skim milk by the procedure of Rombauts et al. [14] through a chromatography step with Bio-Rex 70. α-Lactalbumin was purified from bovine skim milk according to Brodbeck et al. [15]. Galactosyltransferase was prepared according to Fitzgerald et al. [4] and the lyophilized preparation was stored, dessicated at —10°C. Stock solutions (1—2 mg/ml) were prepared by dissolving the galactosyltransferase in 0.25 glycylglycine (pH 8.5); 20 mM Tris-HCl (pH 7.5); or 30 mM potassium barbiturate (pH 7.5), containing 200 mM KCl, and the solution was centrifuged at 40 000 × g for 15 min. The absorbance at 280 nm of a 1 mg/ml solution was 1.53 [16], and the ratio of 58 000 molecular weight form to 44 000 molecular

weight form was approx. 3 : 1 [8]. Both forms have similar catalytic activity [8,9].

Methods

Iodination of the galactosyltransferase with lactoperoxidase and assay of residual activity were carried out in the same 1 ml cuvette. Iodination was in a 0.5 ml vol. containing 50 mM Tris-HCl (pH 7.2)/10 mM $MnCl_2$/200 mM KCl/0.2 mM KI/1—6 μg/ml lactoperoxidase/8—20 μg/ml galactosyltransferase/0.2 mM H_2O_2. Iodination was initiated by rapidly adding the H_2O_2 with a disposable capillary pipette and was allowed to proceed for 0.5—0.6 min at room temperature. The reaction was stopped by rapidly adding 0.2 ml catalase (hydrogen-peroxide:hydrogen-peroxide oxidoreductase, EC 1.11.1.6) (Worthington, CTS, 40 000 units/mg). The reagents for the galactosyltransferase assay [17] were then added to the iodination mixture, and the volume was increased to 1 ml and contained 0.33 mM NADH/1.59 mM phospho*enol*pyruvate/0.10 mM UDP-galactose/20 mM *N*-acetylglucosamine. For Reaction II; glucose and α-lactalbumin was substituted for *N*-acetylglucosamine as indicated in Table I. For control assays (no iodination), the H_2O_2 or lactoperoxidase was omitted or the catalase was added prior to adding H_2O_2. Similar values were obtained by all the methods. Endogenous NADH oxidation was determined by omitting *N*-acetylglucosamine or glucose and was subtracted from the assays. Results are expressed as nmol UDP formed/min at 22°C.

Substrates added during the iodination reaction (0.5 ml total volume) were generally at twice the concentration used in the assay, so that the final assay concentration (in 1.0 ml) remained constant. Mn^{2+} (10 mM) was included in all iodination reactions because it helped maintain low endogenous NADH oxidation in the subsequent assays.

Inactivation of galactosyltransferase by *N*-acetylimidazole was carried out in conical centrifuge tubes in a volume of 100—200 μl of 30 mM potassium barbiturate, (pH 7.5). Solid *N*-acetylimidazole was added to a final concentration of 50 mM and aliquots of the incubation mixture were removed and assayed for galactosyltransferase activity as previously described [12,17]. Reactivation with hydroxylamine is described in Fig. 4.

Iodination of galactosyltransferase, bovine serum albumin, and α-lactalbumin was carried out by incubating 20 μg/ml of the appropriate protein in 1 ml containing 50 mM Tris-HCl (pH 7.2)/0.01 mM $K^{125}I$ (100 μCi/0.01 mol)/5 μg/ml lactoperoxidase/0.2 mM hydrogen peroxide and was stopped by adding 0.01 ml catalase. 100 μl water and 5 μl Pronase (EC 3.4.24.4) (0.5 mg/ml in 0.25 M Tris-HCl (pH 7.4) were added to each lyophilized sample and digestion was carried out at 37°C for 48 h with an extra 5 μl of the Pronase solution added at 24 h. The digestions were stopped by boiling for 1 min, and 5 μl of each sample and an aliquot of the $K^{125}I$ solution were counted in 10 ml Bray's solution [18] with a Packard liquid scintillation spectrometer or in a Beckman Biogamma counter. 5 μl of each digest were spotted on an Eastman chromatogram sheet (cellulose without fluorescent indicator) along with mono- and diiodotyrosine and KI standards and the sheet was chromatographed (ascending) in *n*-butanol/glacial acetic acid/water (78 : 5 : 17, v/v) [19] for 3.5 h.

Radioactivity on the thin-layer sheets was determined with a Nuclear Chicago Actigraph strip scanner. The standards were detected with the ferric chloride/ferricyanide/arsenious acid spray [20,21].

Iodoamino acids were chromatographed by modification of the method of Block and Mandle [22]. The pronase digest was acidified with 0.5 ml formic acid and applied to a 0.9 × 20.0 cm Aminex 50w-X4 (NH_4^+ form) column equilibrated with 0.2 M ammonium formate in 30% ethanol (pH 3.9). The iodoamino acids were eluted by an increasing pH gradient formed by 150 ml of 0.2 M ammonium formate in 30% ethanol (pH 3.9) and 150 ml of 0.2 N ammonium hydroxide in 30% ethanol. Flow rate was 0.25 ml/min and 2 ml fractions were collected and counted in a Beckman counter. Amino acid analyses were done as previously described [6].

Results

Milk lactoperoxidase catalyzes the iodination of free tyrosine [19] and tyrosines bound in proteins [19,23,24] and peptides [25]. The time course of the inactivation of galactosyltransferase by lactoperoxidase is presented in Fig. 1. The iodination reaction was first carried out in a 0.5 ml volume in a 1 ml cuvette, and immediately afterwards residual galactosyltransferase activity was measured in a final 1 ml volume by adding the reagent for the galactosyltransferase assay to the cuvette as described in Methods. Fig. 1 shows that galactosyltransferase is rapidly inactivated by iodination with lactoperoxidase. A plot of log relative activity vs. time was linear, indicating that inactivation of galactosyltransferase was a pseudo-first-order process. As shown in Fig. 1, when

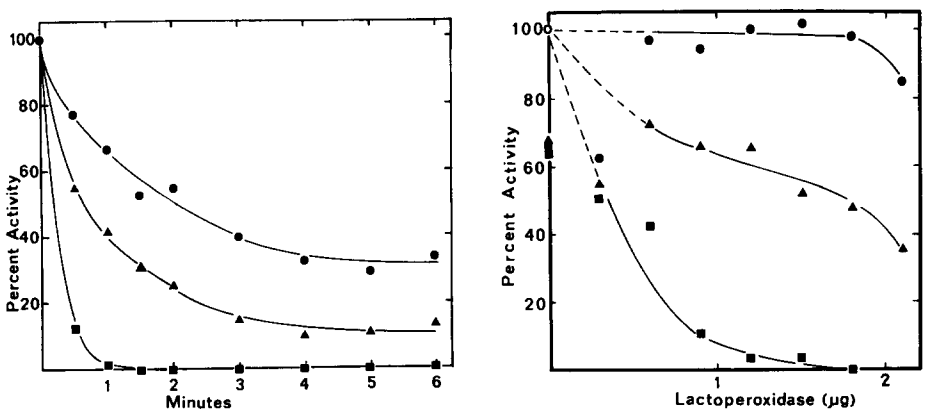

Fig. 1. Time course of inactivation of galactosyltransferase by iodination with lactoperoxidase. Galactosyltransferase (5 μg) was iodinated with 2 μg lactoperoxidase and assayed for residual activity as described. Galactosyltransferase assay reagents present during iodination were: ■———■, 10 mM Mn^{2+}; ▲———▲, 10 mM Mn^{2+} and 1 mM UDPgalactose; ●———●, 10 mM Mn^{2+}, 1 mM UDPgalactose and 40 mM *N*-acetylglucosamine. Activity control (100%) was 6.62 nmol UDP/min.

Fig. 2. Galactosyltransferase activity vs. amount of lactoperoxidase. The iodination of 7 μg galactosyltransferase for 2 min and assays were as described. Additions during iodination were: ■———■, 10 mM Mn^{2+}; ▲———▲, 10 mM Mn^{2+} and 1 mM UDPgalactose; ●———●, 10 mM Mn^{2+}, 1 mM UDPgalactose and 40 mM *N*-acetylglucosamine. Assay control (100%, 7.62 nmol UDP/min) was obtained by omitting H_2O_2 from the complete iodination reaction mixture.

manganese, UDPgalactose and *N*-acetylglucosamine were included during iodination with lactoperoxidase, protection of the galactosyltransferase against inactivation was observed. If only UDPgalactose and manganese were present, an intermediate level of protection occurred. The pseudo-first-order constants of inactivation were: Mn^{2+}, 1.8 min^{-1}; Mn^{2+} and UDPgalactose, 0.7 min^{-1}; and for Mn^{2+}, UDPgalactose and *N*-acetylglucosamine, 0.3 min^{-1}, respectively.

Fig. 2 shows the galactosyltransferase activity as a function of the lactoperoxidase concentration during a 2 min iodination period. Again, protection against inactivation occurred when Mn^{2+}, UDPgalactose and *N*-acetylglucosamine were included during iodination with lactoperoxidase. Higher levels of lactoperoxidase, greater than 2 μg, resulted in rapid loss of activity. However, some inactivation of the galactosyltransferase occurred during the 20 min incubation period even though iodination did not occur (lactoperoxidase was omitted). This loss of activity was not observed at higher levels of lactoperoxidase, greater than 0.6 μg/ml, and in the presence of substrates. The nonspecific inactivation was not due to H_2O_2 since loss of activity occurred when it was omitted from the incubation mixture. It appeared that the nonspecific inactivation was due to some other property of the galactosyltransferase, such as sensitivity to temperature changes or to dilution.

To test the effect of various substrate combinations on the inactivation of galactosyltransferase by the lactoperoxidase reaction, the substrate was added to the 0.5 ml iodination mixture. After iodination, the remaining assay reagents were added and the volume was increased to 1.0 ml. Hence, the concentration of substrates during iodination was twice the normal assay concentration. The results of these studies are presented in Table I. In the reaction with *N*-acetylglucosamine as the carbohydrate acceptor (Expt. 1), Mn^{2+}, UDPgalactose and *N*-acetylglucosamine gave good protection and UDPgalactose plus Mn^{2+} provided an intermediate level of protection when compared with Mn^{2+} alone. Inactivation of galactosyltransferase in the absence of Mn^{2+} was not measured, because under conditions used in the protection experiments inactivation in the presence of Mn^{2+} was very rapid and because having the $MnCl_2$/KCl solution

TABLE I

EFFECT OF SUBSTRATES ON INACTIVATION OF GALACTOSYLTRANSFERASE BY LACTOPEROXIDASE

Reaction mixtures contained 6 μg galactosyltransferase and 2 μg lactoperoxidase. Expt. 1, 100% activity was 6.64 nmol UDP/min; Expt. 2, 6.80 nmol UDP/min.

Experiment	Substrates present during iodination	Activity percent of control Iodination time	
		1 min	2 min
1	a. 10 mM Mn^{2+}	15	2
	b. 10 mM Mn^{2+}, 40 GlcNAc	20	3
	c. 10 mM Mn^{2+}, 1 mM UDPgalactose	62	41
	d. 10 mM Mn^{2+}, 1 mM UDPgalactose, 40 mM GlcNAc	98	79
2	a. 10 mM Mn^{2+}	8	
	b. 10 mM Mn, 40 mM Glc	4	
	c. 10 mM Mn^{2+}, 1 mM UDPgalactose	41	
	d. 10 mM Mn^{2+}, 40 mM Glc, 1 mM UDPgalactose	41	

present during iodination helped maintain blanks (endogenous NADH oxidation) consistently low. Although UDPgalactose plus Mn^{2+} gave an intermediate level of protection, *N*-acetylglucosamine plus Mn^{2+} did not. When glucose was substituted for *N*-acetylglucosamine (Ext. 2), no added protection by glucose (40—480 mM during iodination) over UDPgalactose and Mn^{2+} was observed. This might be expected since glucose at this concentration is a poor substrate unless α-lactalbumin is present [4].

When 400 μg/ml α-lactalbumin, or serum albumin and various substrates were present during iodination, a high level of protection was observed. The protection observed was roughly proportional to the amount of bovine serum albumin and α-lactalbumin and was probably due to the increased availability of tyrosines for iodination, since there was a 20-fold increase in the number of tyrosines when α-lactalbumin and/or bovine serum albumin were present in the iodination mixture.

When galactosyltransferase was incubated with lactoperoxidase and $K^{125}I$, incorporation of label into protein was observed. Under similar conditions (Methods), the following mole atoms I incorporated/mol protein were 4.4 for bovine serum albumin, 0.7 for α-lactalbumin and 9.6 for galactosyltransferase. Different proteins vary in their susceptibility to iodination by lactoperoxidase [23]. Upon pronase digestion and TLC of the iodinated α-lactalbumin, bovine serum albumin and galactosyltransferase, both mono- and diiodotyrosine were detected. Anion exchange chromatography of pronase digests of iodinated galactosyltransferase revealed both monoiodotyrosine and diiodotyrosine in about equal molar ratios. No other iodinated amino acids, including iodohistidine, were detected. Amino acid analysis of galactosyltransferase showed no loss of any amino acids, other than tyrosine modification. No evidence was found for tryptophan destruction and thus it appeared that only tyrosine was modified by iodination with lactoperoxidase.

The lactoperoxidase inactivation of galactosyltransferase suggests that tyrosine residues are critical to galactosyltransferase activity. To further test this hypothesis, the effect of *N*-acetylimidazole, which acetylates the hydroxyl groups of tyrosine [26], as well as ε-amino groups, on galactosyltransferase activity was determined. Fig. 3 shows the time course of inactivation of 1 mg/ml galactosyltransferase with 50 mM *N*-acetylimidazole, a molar ratio of about 1 : 5000. Additional experiments showed a pattern of inactivation and protection by substrates similar to that which occurred with lactoperoxidase-catalyzed inactivation, except that the time required for inactivation was longer. Mn^{2+}, UDP, and *N*-acetylglucosamine afforded good protection and Mn^{2+} and UDPgalactose provided intermediate protection. For this experiment, UDP was substituted for UDPgalactose when *N*-acetylglucosamine was present, because the time required for inactivation was long enough for UDPgalactose and *N*-acetylglucosamine to have completely reacted. Both UDPgalactose and UDP react with the E-Mn^{2+} form of the enzyme [27]. Attempts to inactivate 1 mg/ml galactosyltransferase at lower *N*-acetylimidazole concentrations were unsuccessful.

Tyrosine residues which have been *O*-acetylated by reaction with *N*-acetylimidazole are readily deacetylated by reaction with hydroxylamine [26], and if tyrosine residues are involved, concomitant regain of activity can be ob-

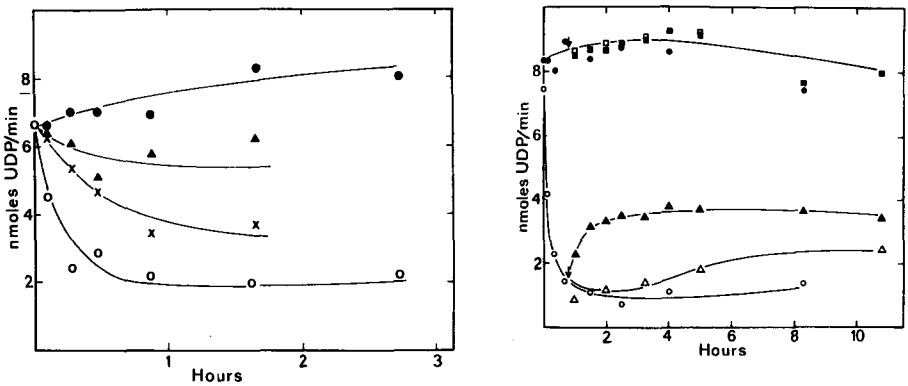

Fig. 3. Inactivation of galactosyltransferase by *N*-acetylimidazole. Galactosyltransferase (1 mg/ml) was incubated in 100 μl of 20 mM Tris-HCl, (pH 7.4), containing the following additions: ○———○, 50 mM *N*-acetylimidazole; X———X, 50 mM *N*-acetylimidazole, 5 mM $MnCl_2$ and 0.5 mM UDPgalactose; ▲———▲, 50 mM *N*-acetylimidazole, 5 mM $MnCl_2$, 0.35 mM UDP and 20 mM *N*-acetylglucosamine; ●———●, no additions. At the times indicated, 7 μl aliquots were assayed for residual galactosyltransferase activity.

Fig. 4. Regain of galactosyltransferase activity after inactivation with *N*-acetylimidazole. Incubations were in 200—400 μl of 30 mM potassium barbiturate, pH 7.5: ○———○, incubation A, 1 mg/ml galactosyltransferase and 50 mM *N*-acetylimidazole; ●———●, incubation B, 1 mg/ml galactosyltransferase. At the time indicated by the arrow (48 min), portions of incubations A and B were transferred to conical tubes and further incubated in 30 mM potassium barbiturate, pH 7.5 with the following additions: ▲———▲, 50 mM hydroxylamine, 5 mM $MnCl_2$, 0.25 mM UDP, 20 mM *N*-acetylglucosamine and 100 μl incubation A in 400 μl total volume; △———△, 5 mM $MnCl_2$, 0.25 mM UDP, 20 mM *N*-acetylglucosamine and 50 μl incubation A in 200 μl total volume; ■———■, 50 mM hydroxylamine, 5 mM $MnCl_2$, 0.25 mM UDP, 20 mM *N*-acetylglucosamine and 100 μl incubation B in 400 μl total volume; □———□, 50 mM hydroxylamine and 50 μl incubation B in 200 μl total volume. 5.5 μl of incubations A and B and 22 μl of the other incubations were assayed at the times indicated. Hydroxylamine hydrochloride stock solution was adjusted to pH 7.4 with KOH before addition to the incubations.

served. Fig. 4 shows that galactosyltransferase inactivated with 50 mM *N*-acetylimidazole can be reactivated partially by incubation with 50 mM hydroxylamine. However, recovery of activity was not complete since only about 40% of the initial activity was regained. Mn^{2+}, UDP and *N*-acetylglucosamine were included during incubation with hydroxylamine to protect the galactosyltransferase against possible inactivation by hydroxylamine, even though there was no detectable effect of hydroxylamine on the galactosyltransferase activity at a concentration of 50 mM.

N-acetylimidazole inactivation of galactosyltransferase supports the proposal that critical tyrosine residues are inactivated by lactoperoxidase-catalyzed iodination. Since only 40% of the galactosyltransferase activity could be regained after *N*-acetylimidazole inactivation, it is possible that *N*-acetylimidazole may also be acetylating another residue such as lysine [26], which is essential for activity and is not removed by hydroxylamine.

Discussion

Galactosyltransferase was inactivated rapidly and completely in a first-order manner by lactoperoxidase-catalyzed iodination. Amino acid analysis showed that the only amino acids altered were tyrosine. ^{125}I was incorporated readily

into galactosyltransferase. Only mono- and diiodotyrosine were detected upon pronase digestion of the iodinated galactosyltransferase and subsequent separation of the amino acids on ion-exchange columns or by TLC. Approx. equal amounts of mono- and diiodotyrosine were obtained. No iodohistidine was detected. Lactoperoxidase can iodinate L-tyrosine and L-3-iodotyrosine in the presence of I^- and H_2O_2 to form L-3-tyrosine and L-3,5-diiodotyrosine, respectively [19]. Tyrosine-containing peptides [23,25] and numerous proteins [23,24] are readily iodinated at tyrosine residues. The available evidence shows that galactosyltransferase is iodinated at tyrosine residues and that iodination leads to a rapid and complete loss of activity, suggesting a role of tyrosine(s) in galactosyltransferase activity.

As shown in Fig. 1, galactosyltransferase was partially protected against inactivation, by iodination, by the presence of 10 mM Mn^{2+} and 1 mM UDPgalactose during iodination. Better protection was obtained with 10 mM Mn^{2+}, 1 mM UDPgalactose, and 40 mM *N*-acetylglucosamine. The extent of protection by substrates was dependent upon the ratio of lactoperoxidase to galactosyltransferase and time of iodination. The presence of glucose, 40—480 mM, in the presence of 10 mM Mn^{2+} did not provide any protection as compared to Mn^{2+} alone. Glucose, up to 480 mM, did not enhance the protection observed with Mn^{2+}-UDPgalactose. The combination of Mn^{2+} and *N*-acetylglucosamine also afforded no protection against inactivation.

Mn^{2+}-UDPgalatose appears to be the critical ligand required for protection since no protection is observed in its absence and by other substrates such as *N*-acetylglucosamine and glucose. Previous studies have shown that Mn^{2+}-UDPgalactose caused a change in the ellipticity of galactosyltransferase at 265 and 287.5 nm, consistent with a conformational change involving tyrosines and tryptophans [13]. In addition, Mn^{2+}-UDPgalactose protects against the destruction of a single tryptophan by photoinactivation [12] and protection against inactivation by trypsin and sulfhydryl reagents [11].

No evidence was obtained to suggest that a combination of glucose and α-lactalbumin would protect galactosyltransferase to iodination in the absence or presence of Mn^{2+}-UDPgalactose. Such experiments were complicated by the observation that α-lactalbumin was an excellent substrate for lactoperoxidase and that it was also inactivated by iodination with lactoperoxidase.

Galactosyltransferase was also inactivated by *N*-acetylimidazole, which acetylates the hydroxyl groups of tyrosine and the ε-amino groups of lysine. A similar pattern of protection to acetylation by Mn^{2+}, UDP and *N*-acetylglucosamine was observed as was found with lactoperoxidase inactivation. UDP is effective since it binds with the same enzyme form as UDPgalactose and is a product rather than a reactant. *O*-Acetyltyrosine residues can be de-*O*-acetylated with hydroxylamine and treatment of the galactosyltransferase, inactivated with *N*-acetylimidazole, resulted in a regain of about 40% of the original galactosyltransferase activity. These results further support the proposal that a tyrosine residue(s) is involved in the activity of galactosyltransferase. Other amino acids essential for full activity may be modified by *N*-acetylimidazole since the de-*O*-acetylation with hydroxylamine did not result in complete regain of galactosyltransferase activity and hydroxylamine under the same conditions did not inhibit the enzyme.

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