Interactions of Substrates and \( \alpha \)-Lactalbumin with Galactosyltransferase as Measured by Difference Spectroscopy*

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The interactions of Mn\(^{2+}\), nucleotides, monosaccharide substrates, and the modifier protein \( \alpha \)-lactalbumin with galactosyltransferase have been studied by difference spectroscopy. MnCl\(_2\) required for significant binding of UDP-galactose or UDP to the enzyme, does not exhibit specific interactions with tyrosine or tryptophan residues. The interaction of UDP-galactose or UDP with galactosyltransferase in 2 mM MnCl\(_2\) produces difference spectra with a major positive peak at 284 nm, a second positive peak at 298 nm, and a large negative trough at 254 nm, suggesting the involvement of tyrosine and tryptophan residues in the interaction. The interaction of GlcNAc with the enzyme in 2 mM MnCl\(_2\) produces only small nonspecific difference spectra. However, the addition of 100 mM GlcNAc markedly increases the difference extinction coefficient at 284 nm of the UDP-bound enzyme-ligand complex, while the coefficient at 254 nm which arises from UDP remains constant. The results suggest that a conformational change involving tyrosine residues, which does not affect UDP, occurs in the process, enzyme•Mn + UDP + GlcNAc → enzyme•Mn•UDP•GlcNAc. Glucose does not show a similar effect. The interaction of galactosyltransferase and \( \alpha \)-lactalbumin produces difference spectra characteristic of tryptophan and does not affect the difference spectra of galactosyltransferase produced by the interaction with UDP and GlcNAc. This implies that the interaction of the two proteins does not involve the bound UDP on galactosyltransferase and does not affect the conformational change induced by UDP and GlcNAc.

Galactosyltransferase (UDP-galactose:d-glucose, 1-galactosyltransferase, EC 2.4.1.22) from bovine milk catalyzes the transfer of galactose from UDP-galactose to carbohydrate acceptors forming \( \beta \)-(1–4) links with N-acetylglucosamine and its \( \beta \)-glycosides (1, 2). Glucose is a poor galactosyl acceptor with a \( K_m \) above 1 M but, in the presence of \( \alpha \)-lactalbumin, the \( K_m \) of glucose is lowered to the millimolar range (3) which allows for the synthesis of lactose under physiological conditions. The interaction of \( \alpha \)-lactalbumin with galactosyltransferase is a unique control mechanism in which \( \alpha \)-lactalbumin acts as a \( K_m \) modifier of the galactosyl acceptor.

A number of studies have shown that various substrates protect against reagents which inactivate galactosyltransferase. Modification of galactosyltransferase by sulfhydryl reagents, such as \( p \)-chloromercuribenzoate and \( N \)-ethylmaleimide (4), trypsin (4), lactoperoxidase (5), iodine monochloride (6), and ultraviolet light (7) resulted in loss of enzymatic activity. The presence of certain substrates generally protect against inactivation, suggesting that a conformational change occurred upon binding of substrates to galactosyltransferase. Additional evidence suggesting substrate-induced conformational changes possibly involving tryptophan and tyrosine residues were obtained from circular dichroism studies (8) but such studies were essentially limited to Mn\(^{2+}\), UDP-galactose, and UDP as substrates. The implication that aromatic amino acids are possibly involved in substrate-induced conformational changes in galactosyltransferase led to an examination of the interactions of various substrates and \( \alpha \)-lactalbumin with galactosyltransferase by utilizing difference spectroscopy.

**EXPERIMENTAL PROCEDURES**

**Materials**—Galactosyltransferase was isolated from bovine milk as previously described (9) and all preparations were about 70% as the higher molecular weight form (10, 11). Both forms have the same specific activity (10, 11) at room temperature which was 13–19 units/mg of protein. Enzymatic activity was stable for at least 5 h at room temperature and 24 h at 4 °C in 10 mM Tris, 100 mM KCl, 0.5 mM dithioerythritol, pH 7.5. Highly purified bovine \( \alpha \)-lactalbumin was obtained from the Institute of Dairy Science, Faculty of Agriculture, Hokkaido University, Sapporo, Japan. MnCl\(_2\) was treated with dithiothreitol according to the procedure of Morrison and Uhr (12) and the recrystallized salt was dried over P\(_2\)O\(_5\) under vacuum. The MnCl\(_2\) contained 2 mol of water of hydration as determined by heating to constant weight and by the procedure of Morrison et al. (13). UDP-galactose was purchased from Boehringer Mannheim, UDP, GlcNAc, and \( \alpha \)-amin acids were purchased from Sigma. All other reagents used were reagent grade.

**Methods**—Galactosyltransferase was purified on an \( \alpha \)-lactalbumin-Sepharose column just prior to use. The enzyme was dialyzed against 250 ml of 10 mM Tris, 100 mM KCl, 0.5 mM dithioerythritol, pH 7.5, with 4 changes of buffer of 20 h and then centrifuged at 180,000 \( \times \) g for 45 min. Buffers and stock solutions of MnCl\(_2\), GlcNAc, and glucose were passed through 0.45-\( \mu \)m Millipore filters. The MnCl\(_2\) solution was made for each measurement because of its tendency to turn brown. The stock solution of \( \alpha \)-lactalbumin was centrifuged at 180,000 \( \times \) g for 45 min.

Difference spectra were obtained as previously described (14) on a Cary 118 spectrophotometer. The auto-slit mode was used with a constant gain set higher than in regular absorption measurements in order to give reasonable slit widths. All the measurements were made at 23–27 °C in 10 mM Tris, 100 mM KCl, 0.5 mM dithioerythritol, pH 7.5, unless otherwise specified and at least 15 min after mixing of the enzyme and ligands. In most measurements, the concentrations of galactosyltransferase and ligands were chosen so that the absorbance did not exceed 1.0 at any wavelength. Four cylindrical matched quartz cells of 1-cm path length (R\(_1\) and R\(_2\) in the reference beam; S\(_1\) and S\(_2\) in the sample beam) were used to compensate for any absorption of ligands. The titration method was not used to obtain the dependence of UDP-galactose or UDP on the difference spectral intensity but, rather, each experimental point is the result of an independent measurement.

The concentrations of galactosyltransferase, bovine \( \alpha \)-lactalbumin, UDP-galactose, and UDP were determined spectrophotometrically using extinction coefficients of 1.61 (mg/ml)\(^{-1}\) cm\(^{-1}\) at 280 nm (15),

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RESULTS

Difference Spectra of Galactosyltransferase with Small Ligands—Previous studies have shown that Mn$^{2+}$ is required for catalytic activity and must be bound to galactosyltransferase prior to the addition of substrates (20-22) and that maximum activity occurs at about 4 mM MnCl$_2$ (20). The difference spectra of galactosyltransferase were examined between 0.4-4 mM MnCl$_2$. The spectra obtained were small ($\Delta e < 500 \text{ M}^{-1} \text{cm}^{-1}$) and featureless (Fig. 1a). There were no correlations between peak intensities, MnCl$_2$, and enzyme concentration.

The difference spectra of galactosyltransferase with UDP-galactose, UDP, UDP plus GlcNAc, and UDP plus glucose, in the presence of 2 mM MnCl$_2$, are presented in Fig. 1. The difference spectra in the presence of UDP-galactose and 2 mM MnCl$_2$ has a major positive peak at 284 nm, a small positive peak at 298 nm, and a large negative peak at 254 nm. Similar spectra were observed with UDP and 2 mM MnCl$_2$. The difference spectra in the presence of 100 mM GlcNAc and 2 mM MnCl$_2$ had a small positive peak at 288 nm and a second positive peak at 280 nm (spectrum not shown). When GlcNAc, UDP, and 2 mM MnCl$_2$ were present, the peak at 284 nm increased significantly and the peak at 298 nm became a shoulder. Glucose did not alter the shape of the difference spectra in the presence of UDP and 2 mM MnCl$_2$. No difference spectra were observed in the presence of 107 mM UDP-galactose (no MnCl$_2$) (Fig. 1g). The difference spectrum in the presence of 107 mM UDP and 100 mM GlcNAc (no MnCl$_2$) was similar to that observed in the presence of 20 mM MnCl$_2$ and 97 mM UDP-galactose (Fig. 1f). The difference spectrum in the presence of 20 mM MnCl$_2$, 107 mM UDP, and 100 mM GlcNAc was similar to that in the presence of 2 mM MnCl$_2$, 107 mM UDP, and 100 mM GlcNAc. The intensity of the former was about 70% of the latter. The intensity became 92% in the presence of 0.1 mM MnCl$_2$ and 96% in the presence of 0.5 mM MnCl$_2$ as compared to 2 mM MnCl$_2$ when the MnCl$_2$ concentration dependence was examined at the fixed concentrations of 107 mM UDP and 100 mM GlcNAc. Thus, the MnCl$_2$ concentration of 2 mM used in most of the present experiments is high enough to maximize the enzyme-substrate interactions.

Figs. 2 and 3 show the dependence of the difference spectral intensity at 254 and 284 nm on the UDP-galactose or UDP concentration in 2 mM MnCl$_2$ and in the presence or absence of 100 mM GlcNAc or glucose. Fig. 2 also shows the relationship of the absorbance at 288 nm and the concentration of GlcNAc in 2 mM MnCl$_2$. Assuming that galactosyltransferase has only one UDP-galactose or UDP binding site (20, 21), the following equation for a 1:1 enzyme-ligand binding was used to evaluate the apparent dissociation constant ($K_{\text{app}}$) of UDP-galactose or UDP from the complex and the difference extinction coefficient ($\Delta e$):

$$K_{\text{app}} = (\Delta e \cdot E_0)/(\Delta A - 1)(L_0 - \Delta A/\Delta e)$$

where $E_0$ and $L_0$ are the total concentrations of enzyme and ligand, respectively (23). The data in Figs. 2 and 3, except for the GlcNAc concentration dependence, were fitted to the
above equation by the nonlinear least squares method. The parameters obtained are shown in Table I and the theoretical curves are drawn in Figs. 2 and 3. The 286-nm peak was also saturable by UDP-galactose and UDP and gave $K_{Dapp}$ and $\Delta \varepsilon$ values similar to those obtained from the other wavelengths.

Different preparations of galactosyltransferase had the same proportion of the two molecular forms. Other independent measurements have suggested that, unless the specific activity of the enzyme was as low as 7, the differences in $\Delta \varepsilon$ and $K_{Dapp}$ due to differences in specific activity were small except for $\Delta \varepsilon_{284}$ for Mn$^{2+}$ + UDP + GlcNAc, which appeared to be dependent on the specific activity but was still larger than $\Delta \varepsilon_{284}$ for Mn$^{2+}$ + UDP when a comparison was made using the same preparation of galactosyltransferase with a lower specific activity.

The Effect of pH and Ethylene Glycol on the Spectra of UDP-galactose, Tryptophan, Tyrosine, and Phenylalanine—Possible chromophores which could contribute to the difference spectra of galactosyltransferase above 240 nm upon the interaction with ligands are the uracil portion of UDP-galactose and UDP, tryptophan, tyrosine, and phenylalanine residues. Therefore, the absorption changes of UDP-galactose and the aromatic amino acids as a function of pH or of the polarity altered by ethylene glycol were examined. The difference spectra of UDP-galactose as a function of pH are shown in Fig. 4. The shift of the pH from 7.6 to an alkaline pH (pH 8.9 or 9.9) produced a difference spectrum which had a large negative peak at 263 nm. A shift to an acidic pH (pH 2) produced a similar difference spectrum which had a negative peak at 260 nm, although the magnitude was much smaller than that observed at the alkaline pH. Ethylene glycol produced a difference spectrum which had a positive peak at 278 nm and a negative peak at 249 nm. The magnitudes of the two peaks are similar and are comparable to that of the acid difference spectrum. All of these difference spectra showed no significant intensity above 290 nm.

The difference spectrum of L-tryptophan in 20% ethylene glycol at pH 7.3 had a major positive peak at 290.5 nm, a minor positive peak at 283 nm, and a negative peak at 247 nm (spectrum not shown). The difference spectrum of L-tyrosine in 20% ethylene glycol at pH 7.3 had a major positive peak at 284.5 nm and a negative peak at 237 nm. The difference spectrum of L-phenylalanine in 20% ethylene glycol at pH 7.3 was rather complex and occurred from 230-280 nm. The alkaline difference spectra of these amino acids are similar to those produced by ethylene glycol (24). The reversal of the spectra across the base-line is expected for the acid difference spectra (24). In all cases, the negative peaks below 280 nm are similar in magnitude or smaller than the positive peaks.

**Difference Spectra of Galactosyltransferase**—The difference spectra of α-lactalbumin with the ligands of Galactosyltransferase—The difference spectra of α-lactalbumin with the ligands for galactosyltransferase were examined under the following conditions with α-lactalbumin in 2 mM MnCl$_2$ as the reference: 1) buffer (10 mM Tris, 100 mM KCl, 0.5 mM dithioerythritol, pH 7.5) (i.e. no addition); 2) 2 mM MnCl$_2$ plus 97 μM UDP-galactose; 3) 2 mM MnCl$_2$ plus 100 mM GlcNAc; 4) 2 mM MnCl$_2$ plus 100 mM glucose; 5) 2 mM MnCl$_2$ plus 97 μM UDP-galactose plus 100 mM glucose; 6) 2 mM MnCl$_2$ plus 97 μM UDP-galactose plus 100 mM GlcNAc. The protein concentration was 18.7 μM (0.265 mg/ml). Most of the spectra obtained were featureless and contained a broad peak between 260-300 nm (spectra not shown). The difference extinction coefficients were all about 100 μM$^{-1}$ cm$^{-1}$. Thus, the results do not suggest any strong specific interactions of the above ligands with tryptophan or tyrosine residues in α-lactalbumin.

**Interaction of Galactosyltransferase with α-Lactalbumin**—The difference spectra of galactosyltransferase with α-lactalbumin in the presence of 2 mM MnCl$_2$ and 100 mM...
GlcNAc or glucose are shown in Fig. 5, a and b. Because any spectral effects of MnCl₂, GlcNAc, or glucose on galactosyltransferase or on α-lactalbumin are compensated, the difference spectra observed arise from the interaction of galactosyltransferase with α-lactalbumin. In the presence of GlcNAc there is a major positive peak at 290 nm and a second positive peak at 282 nm. The spectrum in the presence of glucose is similar to that observed with GlcNAc, except that the magnitude of the peaks at 290 and 281 nm is about the same. A similar effect of α-lactalbumin was observed in the presence of UDP (Figs. 5, c, d, and e). Spectra c, d, and e in Fig. 5 represent the summation of the effects of the interaction of galactosyltransferase and α-lactalbumin and the effects of various ligands, UDP, GlcNAc, and glucose on galactosyltransferase. The addition of α-lactalbumin greatly enhances the positive intensity when compared to the intensity in the absence of α-lactalbumin and in addition, a shoulder or peak appears at 290 nm (compare spectra c, d, and e in Fig. 5 with spectra c, d, and e in Fig. 1, respectively).

**Discussion**

*Origin of the Difference Spectra—* The difference spectra observed with galactosyltransferase and UDP-galactose or UDP resemble the difference spectra of nucleotide binding to a number of enzymes (23, 25, 26). Such interactions are characterized by a large negative trough below 270 nm. In particular, the interaction of UDP with ribonuclease resulted in a large negative trough at 257 nm (26). In the present study, the interaction of UDP or UDP-galactose with galactosyltransferase produced difference spectra with a large negative trough at 254 nm (Fig. 1). This negative trough at 254 or 257 nm represents the absorption change of UDP or UDP-galactose upon interaction with the enzyme since UDP-galactose can generate similar spectra (Fig. 4). The contributions of tryptophan, tyrosine, and phenylalanine are small at these wavelengths (smaller than the major positive peaks above 260 nm). The pH difference spectra of UDP-galactose have a simple negative peak around 290 nm (Fig. 4) and this may be due to protonation or deprotonation of uracil (26) or to differences in the state of ionization of phosphate groups which might be sterically adjacent to the uracil chromophore. This type of spectrum would be expected if UDP-galactose bound to galactosyltransferase by hydrogen bonding to uracil or through electrostatic interactions between the phosphate groups of UDP-galactose and charged groups in the protein. If UDP-galactose bound to a hydrophobic portion of the enzyme, it would be expected to produce a difference spectrum similar to that observed in ethylene glycol which is characterized by a negative peak at 249 nm and a positive peak at 278 nm, both of which are of similar intensity (Fig. 4). The observation that the interaction of UDP or UDP-galactose produced principally a large negative peak at 254 nm suggests that the interaction is mainly hydrogen bonding or electrostatic in nature. It is possible that the electrostatic interaction involves the bound Mn²⁺ on galactosyltransferase. The fact that the UDP and UMP are effective inhibitors of the enzyme suggests that the difference spectra observed with UDP-galactose and UDP-galactose or UDP-galactose produced principally a large negative peak at 254 nm suggests that the interaction is mainly hydrogen bonding or electrostatic in nature. It is possible that the electrostatic interaction involves the bound Mn²⁺ on galactosyltransferase. The fact that the UDP and UMP are effective inhibitors of the enzyme, while uridine is a poor inhibitor (27, 28), suggests the importance of the phosphate groups in the interaction.

It is unlikely that the peak observed at 278 nm in the difference spectra of UDP-galactose with ethylene glycol shifts to 284 nm in the difference spectra of galactosyltransferase with UDP-galactose. The fact that the value of Δε at 284 nm varies while the value at 254 nm remains constant (Table I) also suggests that the peak at 284 nm does not arise from UDP-galactose or UDP. Thus, the peak at 284 nm may be ascribed to tyrosine residues, which are involved in the binding of UDP-galactose and is consistent with the role of tyrosine(s) in the activity of galactosyltransferase (6). The small positive peak at 298 nm in the difference spectrum of galactosyltransferase may be due to the change in the electrostatic environment of tryptophan residues as previously suggested (29, 30). This is consistent with the possible role of

![Fig. 5. Difference spectra produced by the interactions of galactosyltransferase with α-lactalbumin and with small ligands. All the solutions contained 2 mM MnCl₂. a: R, enzyme + GlcNAc; b: R, α-lactalbumin + GlcNAc; c: R, enzyme + α-lactalbumin + GlcNAc; d: R, α-lactalbumin + glucose; e: R, enzyme + α-lactalbumin + glucose; f: R, enzyme + α-lactalbumin + GlcNAc; g: R, α-lactalbumin + GlcNAc + UDP; h: R, enzyme + α-lactalbumin + UDP; i: R, enzyme + GlcNAc; j: R, α-lactalbumin + UDP. In the presence of GlcNAc, the concentration of UDP was 4.19 μM (specific activity, 13). The concentration of α-lactalbumin was 79 μM. The crosses are the sum of the effects of UDP or GlcNAc or glucose on galactosyltransferase (spectra are shown in Fig. 1) and the interaction of galactosyltransferase and α-lactalbumin (spectra a and b) as follows: x and + associated with spectrum c are the sum of spectrum c in Fig. 1 and spectrum a here. x associated with spectrum d is the sum of spectrum d in Fig. 1 and spectrum a here. x associated with spectrum e is the sum of spectrum e in Fig. 1 and spectrum b here.](image-url)
tryptophan in the binding of UDP-galactose (7). If a trypto-
phan residue is involved in the binding site for UDP-galactose, 
the proximity of a negative charge from the phosphate of 
UDP-galactose would result in such a spectrum. The positive 
peak at 290 nm observed in the difference spectra of galactos-
yltransferase upon interaction with α-lactalbumin may be 
assigned to tryptophan residues, although the peak position is 
a little lower than usually observed for the tryptophan perturbation spectra of proteins. Whether a phenylalanine resi-
due is involved in any of the interactions could not be ascert-
tained from the difference spectra or the derivative spectra.

Interactions of Galactosyltransferase with Small Lig-
ands—Recent kinetic studies have shown that bovine colos-
trum galactosyltransferase has two nonequivalent manganese 
binding sites with dissociation constants for the enzyme-mangan-
ese complex in the micromolar (site I) and the millimolar 
ranges (site II) (31, 32). The presence of two manganese 
binding sites has also been shown in bovine milk galactosyl-
transferase (33, 34). The binding of Mn2+ to site I is independ-
ent of substrates and must occur prior to substrates binding 
and prior to a second Mn2+ binding to site II, which, in 
contrast, is affected by UDP-galactose (32). The difference 
spectra of galactosyltransferase in the presence of 2 mM MnCl2 
did not indicate a specific interaction with tryptophan or tryp-
tophan residues. In this context, Geren et al. (8) did not 
observe any changes in the far and near ultraviolet circular 
dichroism spectra of galactosyltransferase in the presence of 
Mn2+, although Klee and Klee (35) had previously reported a 
small change in the far ultraviolet region.

The presence of UDP-galactose or UDP in addition to Mn2+ 
produced significant difference spectra characteristic of tyro-
sine and tryptophan along with UDP-galactose or UDP. Al-
though previous kinetic studies suggested that Mn2+ alone 
can bind to galactosyltransferase and free UDP-galactose can 
bind to the enzyme-Mn complex (20), it is not certain whether 
the observed difference spectra, and therefore, the Kp and 
Δε values obtained in the present study, correspond to the 
above process. Under the conditions used in the present study 
(2 mM MnCl2, 10–100 μM UDP or UDP-galactose), about 10% 
of UDP-galactose and about 95% of UDP are complexed with 
Mn2+, as estimated from the Kp for Mn-UDP-galactose of 
17.5 μM (34) and the Kp for Mn-UDP of 115 μM (36). The 
difference spectra may be caused by the interactions of Mn-
UDP(galactose) with the enzyme-Mn(I) complex or the free 
enzyme, or by the interaction of free UDP(galactose) with the 
enzyme-Mn(I) complex or the enzyme-Mn(II)-Mn(II) com-
plex. In this paper, only the reaction, enzyme-Mn + 
UDP(galactose) → enzyme-Mn-UDP(galactose), is used in 
referring to the values of Kp and Δε. The MnCl2 concentra-
tion dependence of ΔA shows that in the presence of 100 mM 
GlcNAc and a total concentration of UDP of 107 μM, the 
compositional change from 69 μM UDP and 38 μM MnUDP 
(at 0.1 mM MnCl2) to 6 μM UDP and 101 μM MnUDP (at 2 
mM MnCl2) has little effect on ΔA. This suggests that the 
complex formation of UDP with Mn2+ may not change the 
Kp and Δε values significantly.

In the absence of MnCl2, no spectra or only very small 
difference spectra lacking some of the characteristics of the 
spectra in 2 mM MnCl2 were observed with UDP-galactose or 
UDP plus GlcNAc (Fig. 1). From the intensities 230–290 
nm, it can be concluded that the extent of binding of UDP-
galactose is essentially zero in the absence of MnCl2 and 
GlcNAc. If UDP can bind to galactosyltransferase in the 
absence of MnCl2 and in the presence of 100 mM GlcNAc, the 
extent of binding is only 20% of that in the presence of 2 mM 
MnCl2 and 100 mM GlcNAc. The resultant perturbation of 
tryptophan and tyrosine is negligibly small. It is possible that 
the galactosyltransferase sample used in the present study 
might be contaminated by slight amounts of Mn2+, which 
causcd about 20% binding of UDP to the enzyme in the 
presence of 100 mM GlcNAc. In any case, the present results 
show that Mn2+ is required for significant binding of UDP-
galactose or UDP to galactosyltransferase.

The difference spectra of galactosyltransferase with UDP-
galactose or UDP plus 2 mM MnCl2 suggest that tyrosine and 
tryptophan residues are involved in the binding of UDP-
galactose or UDP to the enzyme-Mn complex. This is con-
sistent with previous results where Clymer et al. (7) observed 
protection by Mn2+ and UDP-galactose against ultraviolet 
photoinactivation which resulted in the destruction of 1 tryp-
tophan/molecule and where Silvia and Ebner (6) found sig-
nificant protection by Mn2+ and UDP-galactose and by other 
combinations of substrates against the modification of tyro-
sine residues by ICl. There may be at least two possible 
explanations for these observations. One is that the tyrosine 
and tryptophan residues are involved in the binding sites for 
UDP-galactose and UDP. The 298-nm peak, suggesting inter-
action of tryptophan with a negative charge, is in accord with 
this view. The other possibility is that the binding of UDP-
galactose or UDP causes a conformational change in galacto-
yltransferase which involves tyrosine and tryptophan resi-
dues that may or may not be immediately located in the direct 
binding site of UDP-galactose or UDP. At present, we cannot 
conclude which is the case. Geren et al. (8) observed significant 
increase in ellipticity in the near ultraviolet CD spectra, but 
only a slight change in the far ultraviolet CD spectra by the 
addition of Mn2+ and UDP-galactose.

Although the values of Δε for UDP-galactose and UDP 
shown in Table I differ somewhat, the significance of the 
difference is not clear. Some possibilities include a difference 
in charge between UDP-galactose and UDP, differences be-
tween the interaction of UDP-galactose and UDP with the 
enzyme, or a difference between binding of UDP-galactose 
and MnUDP with the enzyme. It is interesting to note that 
Geren et al. (8) did observe a change in the near ultraviolet 
CD spectra for galactosyltransferase in the presence of Mn2+ 
and UDP-galactose, but not with Mn2+ and UDP. However, 
Mn2+ and UDP induced more change than Mn2+ and UDP-
galactose in the difference spectra.

The difference spectra observed in GlcNAc plus 2 mM 
MnCl2 can be attributed to nonspecific solvent effects on the 
tryosine residues which are exposed on the surface of galac-
tosyltransferase molecule because they are small [Δεmax = 430 
(m−1 cm)2 at 0.1 M GlcNAc] and increase linearly with the 
GlcNAc concentration (Fig. 2). According to the Kd values 
reported by Powell and Brew (37) and Bell et al. (38), 82–94% 
of galactosyltransferase is in the enzyme-Mn-GlcNAc com-
plex at 10 mM MnCl2 and 100 mM GlcNAc. Therefore, it is 
concluded that the binding of GlcNAc does not involve a 
tyrosine or tryptophan residue.

The change in shape of the difference spectra of galactosyl-
transferase with UDP (Fig. 1) as well as the increase in Δε at 
284 nm (Table I) by the addition of 100 mM GlcNAc suggests 
alternative interactions of UDP and/or GlcNAc with galac-
tosyltransferase. The Δε value (= Δε, Table I) obtained from 
titration by UDP in the presence of 100 mM GlcNAc is 
considered to be associated with the following processes:

\[
\begin{align*}
\text{UDP} & \quad \text{GlcNAc} \\
\text{E·Mn} & \quad \text{E·Mn-UDP} \\
& \quad \text{E·Mn-UDP·GlcNAc} \\
& \quad \text{E·Mn-GlcNAc}
\end{align*}
\]

The galactosyltransferase sample used in the present study 
might be contaminated by slight amounts of Mn2+, which 
causcd about 20% binding of UDP to the enzyme in the 
presence of 100 mM GlcNAc. In any case, the present results 
show that Mn2+ is required for significant binding of UDP-
galactose or UDP to galactosyltransferase.
The letters in parentheses are the extinction coefficient of the respective complex. The same extinction coefficient is assigned for enzyme-Mn-GlcNAc and enzyme-Mn (i.e. $e_1$) because the binding of GlcNAc does not cause any absorption change, as shown above. In the above scheme, two possible ways to form the quaternary complex, enzyme-Mn-UDP-GlcNAc, are considered. It is also possible that in addition to this complex, the enzyme-Mn-UDP complex may be present even at 100 mM GlcNAc and at the infinite concentration of UDP (note that the $\Delta_e$ refers to this condition) because of the relatively large dissociation constant of GlcNAc (37, 38).

If $x$ is the fraction of the enzyme-Mn-UDP-GlcNAc complex under this condition, $\Delta_e$ can be described as follows:

$$\Delta_e = (1 - x)e_{11} + xe_{12} - e_1 + x(e_{11} - e_1),$$

where $\Delta e_2 = e_{12} - e_1$ (i.e. $\Delta e_2$ is the value obtained from titration by UDP in the absence of GlcNAc). The results in Table I show that at 254 nm, $\Delta e_1 = \Delta e_2$, and thus, $e_{11} = e_{12}, e_{11} - e_1 = e_{12} - e_1$ and at 284 nm, $\Delta e_1 > \Delta e_2$, and thus, $e_{11} > e_{12}, e_{11} - e_1 > e_{12} - e_1$. The above relationship between $e_{11}$ and $e_{12}$ shows that the absorbance at 284 nm increases when GlcNAc binds to the enzyme-Mn-UDP complex to form the enzyme-Mn-UDP-GlcNAc complex, while the absorbance at 254 nm does not change significantly. As pointed out earlier, the absorption change at 254 nm arises mainly from UDP itself and reflects the environmental change of UDP. No significant change at 254 nm suggests that the above process does not involve or affect UDP which is already bound to galactosyltransferase prior to the GlcNAc binding. On the other hand, the increase in absorbance at 284 nm shows an apparent difference with the process, enzyme-Mn+GlcNAc $\rightarrow$ enzyme-Mn-GlcNAc, where no absorption change was observed. Possible explanations for the cause of this difference would be that the binding of UDP to the enzyme-Mn complex causes a conformational change in the GlcNAc binding site, which allows some tyrosine residues to be involved in the binding of GlcNAc or that most likely by a conformational change, the binding of UDP results in transition to a state which undergoes an additional conformational change involving tyrosine residues upon binding of GlcNAc to this enzyme-Mn-UDP complex. Another possibility is that the binding of GlcNAc causes displacement of a tyrosine residue near the UDP binding site in such a way that without bound UDP, the polarity of the environment surrounding this residue does not change but that, with bound UDP, the tyrosine residue comes close to the phosphate groups of UDP, which results in positive tyrosine perturbation spectra.

Similarly, the relationship $e_{11} - e_1 > e_{12} - e_1$ at 284 nm reveals a difference between the two processes:

$$\text{UDP} \downarrow$$

$$E \cdot \text{Mn} \rightarrow E \cdot \text{Mn-UDP} (e_{11} - e_1)$$

$$\text{UDP} \downarrow$$

$$E \cdot \text{Mn-GlcNAc} \rightarrow E \cdot \text{Mn-UDP-GlcNAc} (e_{12} - e_1)$$

while the relationship $e_{11} - e_1 = e_{12} - e_1$ at 254 nm suggests no difference in the mode of the UDP binding. Similar explanations may apply to this case. Thus, the present results cannot be explained only by direct interaction of UDP or GlcNAc with the binding sites on galactosyltransferase without causing any conformational changes and suggest that a conformational change must be occurring in the process enzyme-Mn+UDP + GlcNAc $\rightarrow$ enzyme-Mn-UDP-GlcNAc.

The above explanations may account for some of the causes for the change in $K_d$ of one of the substrates by the precedent binding of the other substrate. As shown in Table I, the apparent $K_d$ of UDP decreases in the presence of 100 mM GlcNAc. The measurements at 20 $\mu$m MnCl$_2$ also suggest that GlcNAc facilitates the binding of UDP to galactosyltransferase: if the fraction of the enzyme which is in the enzyme-UDP-galactose complex is estimated from the intensity at 250-260 nm, it is only 8% for 96 $\mu$m UDP-galactose and 5.43 $\mu$m enzyme and it is 64% for 107 $\mu$m UDP and 3.12 $\mu$m enzyme in the presence of 100 mM GlcNAc. (It may be reasonably assumed from the similar $K_d^* = [\text{UDP-galactose}]$ and UDP at 2 mM MnCl$_2$ in the absence of GlcNAc (Table I) that they also have similar $K_d^*$ values at 20 $\mu$m MnCl$_2$ in the absence of GlcNAc.) Powell and Brew (37) reported that the presence of UDP increased the equilibrium association constant of ovalbumin with galactosyltransferase by a factor of 46. (Ovalbumin is considered to interact with galactosyltransferase through the terminal GlcNAc of its carbohydrate moiety.)Bell et al. (38) reported $K_d$ values for various possible reactions, although their results are not apparently in accord with those expected from former results in that GlcNAc has lower affinity to the enzyme-Mn-UDP-galactose complex than to the enzyme-Mn complex and that UDP-galactose has larger affinity to the enzyme-Mn-GlcNAc complex than to the enzyme-Mn complex.

In contrast to GlcNAc, glucose appears to have little effect (Fig. 1; Table I). The apparent ineffectiveness of glucose could be due to the high $K_d$ value of glucose (3) for galactosyltransferase even in the presence of Mn$^2+$ and UDP: the $\Delta e$ value obtained from titration by UDP in the presence of 100 mM glucose (Table I) may be the average of the values for a large amount of the enzyme-Mn-UDP complex and a small amount of the enzyme-Mn-UDP-GlcNAc complex. Therefore, the possibility that the binding of glucose produces a spectral effect similar to GlcNAc cannot be ruled out.

**Interation of Galactosyltransferase and $\alpha$-Lactalbumin**—

The difference spectra of the interaction between galactosyltransferase and $\alpha$-lactalbumin have been observed in the presence of 2 mM MnCl$_2$ and 100 mM GlcNAc or glucose (Fig. 5, a and b) and suggest the involvement of tryptophan residues in the interaction. The shape and magnitude of the difference spectrum in the presence of GlcNAc are a little different from those in the presence of glucose. While the difference in magnitude could be due to the difference in the extent of interaction, the difference in shape might suggest a difference in the mode of interaction. The shape of the spectrum in the presence of GlcNAc is that of a typical tryptophan perturbation spectrum and the involvement of tyrosine residues is not apparent. The origin of the tryptophan perturbation in the interaction is not clear. It is possible that the tryptophan residue(s) are from $\alpha$-lactalbumin and not from galactosyltransferase. There might be a tryptophan residue at the interaction site on either galactosyltransferase or $\alpha$-lactalbumin, which is perturbed by the interaction of the two proteins. Alternatively, the interaction might induce a conformational change involving tryptophan residues in either galactosyltransferase or $\alpha$-lactalbumin. The identification of the tryptophan residues is now in progress.

A similar interaction of galactosyltransferase and $\alpha$-lactalbumin is indicated by the difference spectra in the presence of MnCl$_2$ and UDP (Fig. 5c), MnCl$_2$, UDP, and GlcNAc (Fig. 5d), and MnCl$_2$, UDP, and glucose (Fig. 5e). Although in those spectra the interaction of the two proteins is overlapped by the interactions of galactosyltransferase with UDP and GlcNAc or glucose, they all show a peak or shoulder at 290 nm which is not present in the difference spectra for the interactions with the above small ligands. In order to compare the interactions of galactosyltransferase and $\alpha$-lactalbumin under these conditions (i.e. in the presence of UDP) with
those in the absence of UDP, the sum of the difference spectral intensities for the galactosyltransferase-small ligands interactions (Fig. 1) and for the galactosyltransferase-a-lactalbumin interaction in 2 mM MnCl₂ and 100 mM GlcNAc or glucose (Fig. 5, a and b) is indicated by crosses at several wavelengths in Fig. 5, c, d, and e. (The concentration differences are corrected.) Good agreements are seen in Fig. 5d, whereas disagreements are significant for the positive peaks in Fig. 5, c and e. Because the presence of UDP can facilitate the interaction of galactosyltransferase and a-lactalbumin (37, 38) and because UDP alone may not be as effective as GlcNAc or glucose in forming the galactosyltransferase-a-lactalbumin complex (37, 38), the disagreements may be accounted for by the difference in the extent of the interaction of the two proteins under different conditions and, in the case of Fig. 5e, by the possible effect of glucose binding which is made significant by a-lactalbumin. The agreements in Fig. 5d suggest that the interaction of galactosyltransferase and a-lactalbumin is nearly maximum in 2 mM MnCl₂ and 100 mM GlcNAc. The $K_d$ value of 1.6 $\mu$M reported by Bell et al. (38) for the interaction of enzyme-Mn-GlcNAc and a-lactalbumin predicts 82% interaction under the present conditions. Moreover, the agreements seen in Fig. 5 have important implications on the relation between the interactions of galactosyltransferase with substrates and those with a-lactalbumin, as discussed below.

If the tryptophan residues involved in the interaction of galactosyltransferase and a-lactalbumin interact with the negatively charged phosphate groups of UDP, there should be a significant increase in the intensity above 290 nm in the presence of UDP compared to that in the absence of UDP. If the uracil ring of UDP which is bound to galactosyltransferase interacts with any groups in a-lactalbumin or is involved in a conformational change which might be induced by the interaction, a significant change in the intensity at 252-254 nm should be observed. The absence of those observations in Fig. 5d (and Fig. 5, c and e, for 252- to 254-nm peak) suggests that the tryptophan residues involved in the interaction of galactosyltransferase and a-lactalbumin do not interact with UDP bound on galactosyltransferase and that the interaction of the two proteins does not involve at least the uracil ring of UDP. Moreover, the additivity seen at 284 and 290 nm in Fig. 5d also suggests that the tryptophan residues involved in the interaction of galactosyltransferase and a-lactalbumin are not located in the region which undergoes a conformational change induced by UDP and GlcNAc and that the tyrosine residues involved in the interactions with UDP and GlcNAc are not affected by the interaction with a-lactalbumin. Thus, there is some distinction in the galactosyltransferase molecule between the interaction sites of substrates and those of a-lactalbumin, and the interactions, as far as observed by difference spectra, are not affected by each other. However, this does not mean that those interaction sites are completely separated. Some regions might be overlapped, which could not be detected by difference spectra. The agreements at 252-254 nm also suggest that the interaction of galactosyltransferase with a-lactalbumin does not facilitate the binding of UDP to galactosyltransferase appreciably.

The difference spectra measurements of a-lactalbumin with the ligands for galactosyltransferase did not indicate any strong specific interactions of those ligands with a-lactalbumin. The absence of intensity at 250 to 265 nm in the difference spectra of a-lactalbumin in the presence of 2 mM MnCl₂ plus 97 $\mu$M UDP-galactose ± 100 mM GlcNAc or glucose shows that UDP-galactose does not bind to a-lactalbumin. However, these results cannot rule out the possibility of the interactions between a-lactalbumin and the ligands on the galactosyltrans-ferase molecule (i.e. when there are all bound to galactosyltransferase). The present studies on the interaction between galactosyltransferase and a-lactalbumin have shown that there is no contact between a-lactalbumin and the uracil ring of UDP when they are bound to galactosyltransferase. The primary structure of a-lactalbumin is similar to that of lysozyme (19, 39) and the similarity of their three-dimensional structures has been proposed (40, 41). The proposed three-dimensional structure of a-lactalbumin has a cleft which corresponds to the sugar binding site of lysozyme, although it is more blocked in a-lactalbumin than in lysozyme. Whether such a cleft in the a-lactalbumin molecule plays a role in the binding of glucose and GlcNAc or the galactose moiety of UDP-galactose in the various galactosyltransferase-a-lactalbumin complexes is still a question to be solved.

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REFERENCES

Difference Spectra of Galactosyltransferase