Manganese(II) and Spin-Labeled Uridine 5′-Diphosphate Binding to Bovine Galactosyltransferase†

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ABSTRACT: The kinetically observed Mn(II) activation as well as inhibition has been clarified for bovine galactosyltransferase. An electron spin resonance (ESR) titration of $MnCl_2$ with galactosyltransferase alone at pH 8.0 clearly shows the existence of at least two metal ion binding sites with microscopic dissociation constants of 0.84 ± 0.1 and 9.0 ± 1.0 m*M*, respectively. The second site corresponds with either published kinetic constant for Mn(II) of 8.5 m*M* (inhibition) or 3.40 m*M* (activation). The contribution of the binary complex Mn(II)–UDPGal is of lesser significance, as concluded by its ESR measured K_{diss} of 14.5 ± 1.1 m*M* at pH 8.0. A spin-labeled inhibitor analog of UDPgalactose, UDP-4-*O*-(2,2,6,6-tetramethyl-4-piperidinyl-1-oxy), or UDP-R, was synthesized as a competitive inhibitor for UDPGal. It was shown from inhibition kinetics to be almost as potent an inhibitor as UDPGlu. The K_i values at pH 8.0 in the *N*-acetyllactosamine and lactose reactions were 0.38 ± 0.04 and 0.63 ± 0.06 m*M*, respectively, as compared with 0.10 ± 0.01 and 0.094 ± 0.009 m*M* for UDPGlu. An ESR titration of UDP-R with galactosyltransferase at pH 8.0 yielded direct physical dissociation constants of 0.40 ± 0.07 and 0.53 ± 0.08 m*M* in the absence and presence of α-lactalbumin, respectively. No other substrates (glucose or *N*-acetylglucosamine) nor Mn(II) were present.

Galactosyltransferase (uridine diphosphate-D-galactose: D-glucose 1-galactosyltransferase, EC 2.4.1.22) of bovine milk catalyzes two basic reactions. Most general is the galactosyltransferase reaction with monosaccharide, polysaccharide, or glycoprotein acceptors with *N*-acetylglucosamine as the acceptor at the nonreducing end:

$$\text{UDPGal} + \text{GlcNAc} \xrightarrow{A,\ \text{Mn(II)}} \text{UDP} + N\text{-acetyllactosamine} \quad \text{(I)}$$

where *A* is galactosyltransferase (*A* protein).[1] In the presence of the modifier protein, α-lactalbumin, the "lactose synthetase" reaction proceeds with glucose as the acceptor:

$$\text{UDPGal} + \text{glucose} \xrightarrow{A,B,\text{Mn(II)}} \text{UDP} + \text{lactose} \quad \text{(II)}$$

where *B* is α-lactalbumin (*B* protein). Recent kinetic studies (Morrison and Ebner, 1971a–c) have indicated an absolute sole specificity for divalent manganese as the metal ion. Only one early report by Babad and Hassid (1966) is contrary where a significant degree of activation (25%) was observed with Mg(II). A complete discussion of metal ion studies is found in a recent review by Ebner (1973).

The kinetic results of Morrison and Ebner (1971a–c) showed that Mn(II) associates first with the *A* protein in a linear ordered mechanism. The kinetically derived *A* protein–Mn(II) dissociation constant K_{im} is approximately 1.2–1.4 m*M* (see also Khatra et al., 1974). At Mn(II) concentrations above ca. 2.5–4 m*M* second-order effects are displayed in both reactions I and II, probably reflecting the binding of one or more additional Mn(II) cations to the *A* protein or its complexes along the reaction pathway.[2] While donor specificity is limited to galactosyluridine nucleotides (UDPGal and dUDPGal), UDPGlu has been shown to be a potent competitive inhibitor for UDPGal (Morrison and Ebner, 1971a–c; Ebner, 1973).

In this initial report of our conformational studies of this lactose synthesizing protein–protein complex we report ESR studies of Mn(II) binding and the synthesis, kinetics, and binding of a spin-labeled substrate analog. For more background on spin labeling or specific applications to enzyme systems the reader is referred to outside reviews or texts (Berliner, 1974, 1975).

Experimental Procedure

Materials

2,2,6,6-Tetramethylpiperidinyl-1-oxy was obtained from Aldrich Chemical Co. as the amine and oxidized to the nitroxide by standard methods (Briere et al., 1965). Phosphoenolpyruvate (PEP), *p*-nitrophenyl phosphate, 2-cyanoethyl phosphate, and UMP-morpholidate were from Sigma. UDPGal was from Calbiochem. *N*-Methylmorpholine, tri-*n*-octylamine, UMP, and dicyclohexylcarbodiimide

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[1] Abbreviations used are: *A* protein, galactosyltransferase; *B* protein, α-lactalbumin; GlcNAc *N*-acetylglucosamine; NMM, *N*-methylmorpholine; PEP, phosphoenolpyruvate; UDP-R, uridine 5′-diphosphate 4-(2,2,6,6-tetramethylpiperidinyl-1-oxy); UDPGal and UDPGlu, UDPgalactose and -glucose, respectively.

[2] There is currently considerable controversy over this phenomenon. Morrison and Ebner (1971a) report Mn(II) inhibition above 4 m*M* at pH 8.0 (0.1 *M* NMM, 30°), while Powell and Brew (1974) report Mn(II) activation above 2.5 m*M* at pH 7.4 (0.05 *M* sodium cacodylate, 37°). While this controversy has not yet been resolved, the results in the present work are consistent with either mechanism.

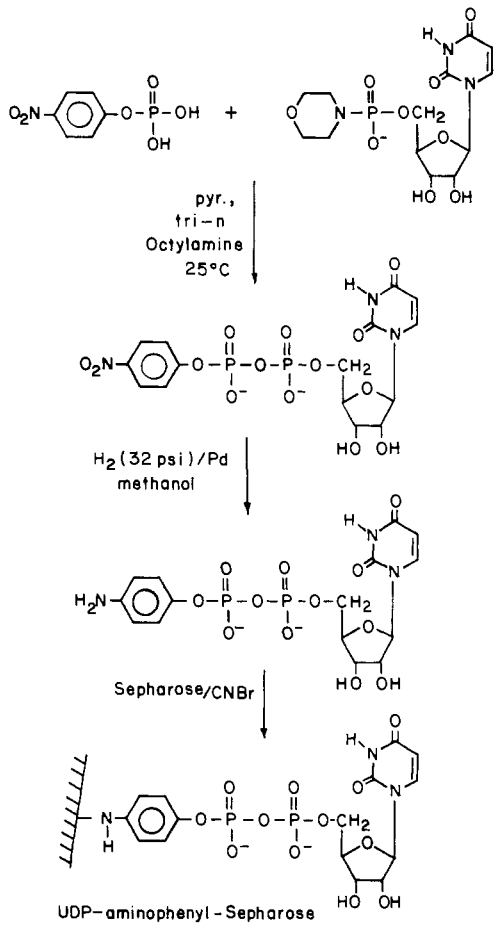

FIGURE 1: Synthesis of UDP-aminophenyl-Sepharose.

were purchased from Aldrich. Cyanogen bromide, acrylamide, and sodium dodecyl sulfate were purchased from Eastman Organic Chemicals. NADH was from P.L. Biochemicals and GlcnNAc was from Pfanstiehl Laboratories. Chelex 100 was from Bio-Rad Laboratories. Sepharose 4B was a Pharmacia product.

Proteins. Pyruvate kinase (type 1) and alkaline phosphatase (type II) were purchased from Sigma Chem. Co.; α-lactalbumin was purchased from Nutritional Biochemicals (ICN). Galactosyltransferase was purified from bovine milk as described below.

Methods

Preparation of α-Lactalbumin-, UDP-hexanolamine-, and UDP-aminophenyl-Sepharose. The Sepharose-α-lactalbumin and Sepharose-UDP-hexanolamine affinity columns were made by the procedures of Trayer and Hill (1971) and Barker et al. (1972), respectively.

UDP-aminophenyl-Sepharose. This was synthesized by the general scheme of Ebner (personal communication) and procedures from Cuatrecasas et al. (1968, 1969a,b) and Winer (1972). The synthesis is depicted in Figure 1. Berglund and Eckstein (1972) have used a similar method for preparing ATP-aminophenyl-Sepharose.

UDP-*p*-nitrophenyl ester was prepared by first converting 3.5 g (9.4 mmol) of disodium *p*-nitrophenyl phosphate to the pyridinium salt by passage through a (380 ml) Dowex 50 W-X12 cation exchange column (200–400 mesh) in the pyridinium form. The evaporated white crystalline solid was dissolved in 10 ml of freshly distilled pyridine and 15 ml of tri-*n*-octylamine and then concentrated to an oil; 10 ml of dry pyridine was added and again evaporated to dryness. After repeating this pyridine evaporation step several times the final dry pyridine solution was mixed with 6 g (8.7 mmol) of UMP-morpholidate. After several steps of a 10-ml absolute pyridine addition followed by evaporation to dryness, the final reaction mixture in 50 ml of pyridine was allowed to stand in a dark desiccator at room temperature for 5 days. Paper chromatography on Whatman No. 1 with 95% ethanol–1 *M* NH_4OAc (pH 7.0) (5:2) indicated sufficiently complete reaction. The product may be further purified on DEAE-cellulose eluting as the last of three peaks on a linear (0.04–0.15 *M*) KCl gradient at pH 3.5 (Winer, 1972). After evaporating the reaction mixture to an oil, followed by addition of 6 g of lithium acetate (as an aqueous solution) the solution was extracted with ether to remove excess tri-*n*-octylamine; 10 ml of H_2O was added to the oil solution followed by further extractions with 150-ml portions of ether.

The final concentrated oil of UDP-*p*-nitrophenyl was dissolved in 200 ml of 50% aqueous methanol. To this solution was added 1 g of 10% palladium on charcoal followed by hydrogenation at 32 psi for 70 min. The reduced solution (UDP-*p*-aminophenyl) was filtered over Celite to remove the catalyst. The evaporated filtrate, an oil, was then dissolved in 200 ml of water.

Sepharose 4B which had been freshly activated with 200 g of cyanogen bromide (Cuatrecasas, 1970; Cuatrecasas et al., 1968) and well washed with ice-water was suspended in the UDP-*p*-aminophenyl solution above to give a total volume of 1.2 l. The suspension was stirred overnight at 4°, packed into a column, and washed with several liters of water. The column was then equilibrated with 0.0125 *M* sodium cacodylate-HCl buffer (pH 7.4) containing 0.025 *M* $MnCl_2$ and 5 m*M* mercaptoethanol. The stoichiometry of ligand bound to the Sepharose was determined by either treating 2 ml of settled gel with alkaline phosphatase, type II (contains alkaline pyrophosphatase), in 2 ml of 0.1 *M* glycine buffer (pH 9.5), or by incubating an aliquot of settled gel in dilute NaOH for several hours. The hydrolyzed nucleotide was estimated at ca. 1 μmol of UDP/ml of gel by absorption at 262 nm.

Synthesis of UDP-R. The preparative scheme, adopted from the synthesis of the adenyl analog by Weiner (1969), is depicted in Figure 2. The phosphopiperidine nitroxide, 2,2,6,6-tetramethyl-4-phosphopiperidinyl-1-oxy, was synthesized after Weiner's (1969) method adopted from Tener (1961). To the phosphonitroxide (ca. 3 mmol) pyridin in a small volume of pyridine, 2 ml of tri-*n*-octylamine was added followed by several evaporation–addition steps with 10-ml portions of absolute pyridine. To a final dry 5-ml pyridine solution was added 410 mg (0.6 mmol) of UMP-morpholidate. This solution was also evaporated and reconstituted with dry pyridine several times to ensure dryness. The reaction proceeded at room temperature in 5 ml of pyridine for several (~5) days and was terminated by the addition of water followed by 300 mg of lithium acetate. The tri-*n*-octylamine was extracted with ether. The aqueous fraction was applied on a Dowex AG 1-X2 (200–400 mesh) column in the chloride form. The product was eluted as the second to last band using a 3-l. gradient of 0.01–0.5 *M* LiCl in 0.1 *M* NH_4Cl. The fractions were pooled and concentrated to a small volume for desalting on a Bio-Gel P-2 (2.5 × 47 cm) column. Further purification was achieved by again eluting the product solution through another Dowex AG 1-X2 (1.6 × 12 cm) column with a 1-l. gradient of 0.05–0.6 *M* LiCl in

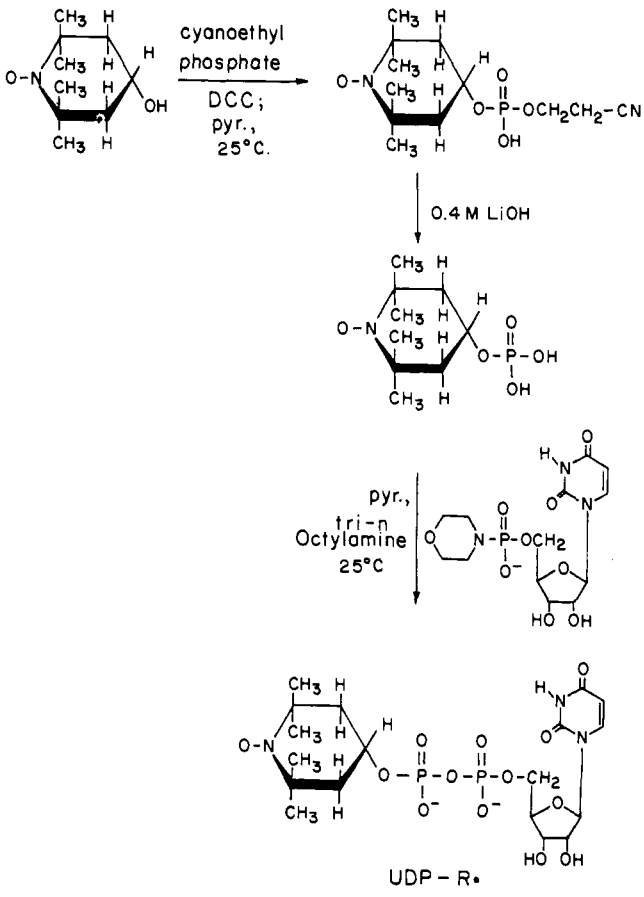

FIGURE 2: Synthesis of UDP-R.

0.003 *N* HCl (25 ml/hr flow rate). The product fractions were pooled, adjusted to pH 10 with LiOH, and desalted on Bio-Gel P-2. The solution was evaporated and reconstituted with methanol, and the product was reprecipitated with ether. The residue obtained after centrifugation was redissolved in methanol and reprecipitated with ether several times. Paper chromatography using 1-butanol–acetone–acetic acid–5% NH_4OH–water (45:15:10:10:20) gave one spot with an R_f value of 0.35; yield, 16%. Characterization was based on a spectrophotometric measurement of uridine concentration (ϵ for uridine = 8.5×10^{-3} M^{-1} cm^{-1} at pH 9.5, Fox and Shugar, 1952); nitroxide concentration by comparing the ESR peak heights with those of a standard ("spin count"), and the Lowry and Lopez (1946) test for inorganic phosphate after treatment of UDP-R with (pyrophosphatase contaminated) alkaline phosphatase (type II), at pH 9.5, 1 m*M* $MgCl_2$. The results: uridine–phosphate–nitroxide = 1.00:2.14:1.06, agreed well with theory. Physical properties of UDP-R: uv_{max} at 262 nm; ϵ 9.2×10^3 M^{-1} cm^{-1}.

Isolation of Galactosyltransferase. The purification scheme adopted utilizes the affinity chromatography columns of Barker et al. (1972) with modifications based on Ebner et al. (1973). Raw bovine milk was obtained from the O.S.U. Dairy Barn within 2 hr after milking followed immediately by cream separation and acidification to pH 4.2–4.5 for casein precipitation. An ammonium sulfate (390 g/l.) precipitate of the resultant whey solution was chromatographed on either a UDP-hexanoyl- or a UDP-aminophenyl-Sepharose affinity column, followed by ammonium sulfate precipitation (560 g/l.), and finally two to three

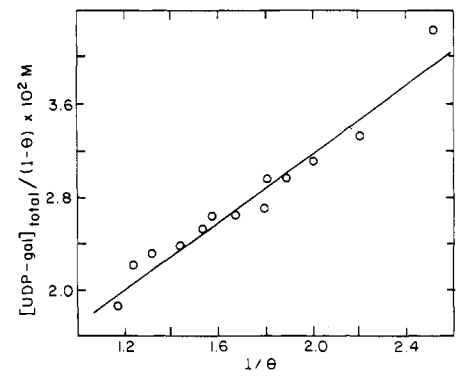

FIGURE 3: Binary complex formation of Mn(II)-UDPGal. An ESR titration of 0.51 m*M* $MnCl_2$ in 0.08 *M* NMM (pH 8.0) (0.08 *M* KCl) with 2.75–25.6 m*M* UDPGal at 26 ± 2°. The calculated K_{diss} is 14.5 ± 1.1 m*M*.

passes through an α-lactalbumin-Sepharose column. A more detailed procedure will be supplied on request.

Final purified enzyme concentration was estimated spectrophotometrically using an extinction coefficient, $E_{280\,nm}(0.1\%)$ 1.61 (Trayer and Hill, 1971). Disc gel electrophoresis on 7.5% polyacrylamide (0.2% sodium dodecyl sulfate) by the method of Weber and Osborn (1969) gave patterns similar to that reported by Barker et al. (1972), Magee et al. (1974), and Magee and Ebner (1974), showing the two bands associated with the 58,000 and 42,000 molecular weight forms.

Galactosyltransferase activity was measured spectrophotometrically by the coupled assay method of Fitzgerald et al. (1970). Specific activity was 1.7 units/mg at 25° in our best purification which compares well with Fitzgerald et al. (1970). A molecular weight of 50,000 was used as an average estimate of the two catalytically identical forms (Magee et al., 1974).

ESR measurements of equilibrium dissociation constants were carried out for Mn(II)–UDPGal, Mn(II)–galactosyltransferase and UDP-R–galactosyltransferase by titrating 0.025–0.05-ml samples in small quartz capillaries (0.9–1.1 mm × 100 mm) in a configuration similar to that used by Taylor (1969). The purified enzyme was always pretreated with Chelex 100 before any ESR measurements to remove extraneous metal ions. In all binding studies the narrow six line spectrum in the case of Mn(II) or three-line spectrum in the case of nitroxide was assumed to reflect free (unbound) paramagnetic species, the bound Mn(II) or nitroxide spectrum contributing little or none to the "free" spectrum peak height. All ESR measurements were carried out on a Varian E-4 spectrometer at X-band frequency.

Results

Mn(II)–UDPGal Complex. In order to quantitate the contribution, if any, of Mn(II)–UDPGal complex formation to the observed substrate inhibition at high Mn(II) concentrations, we measured the Mn(II)–UDPGal dissociation constant by ESR at pH 8.0, 0.08 *M* NMM containing 0.08 *M* KCl at 26 ± 2°. Figure 3 shows the ESR data plotted for a titration of 0.51 m*M* $MnCl_2$ with 2.75 m*M* to 25.6 m*M* UDPGal. The data are plotted as defined below where the subscripts f and b denote "free" and "bound" species, respectively, where

$$\text{fraction of free manganese} = \theta = \frac{[\text{Mn(II)}]_f}{[\text{Mn(II)}]_{total}}$$

and

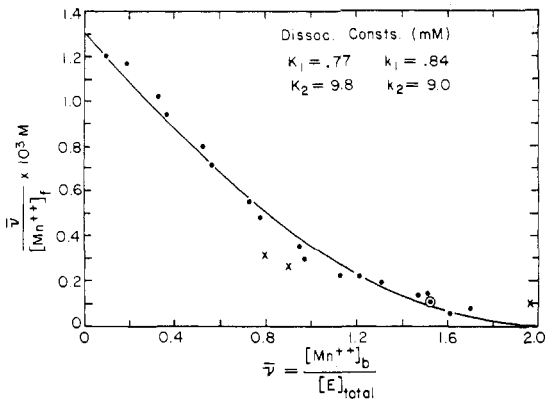

FIGURE 4: Scatchard plot of the ESR data for Mn(II) binding to galactosyltransferase. The concentration of *A* protein was 0.058 m*M* in 0.1 *M* NMM (pH 8.0) containing 10% (w/v) $(NH_4)_2SO_4$ at 26 ± 2°. The solid curve represents a computer fit to a simple two-site model for a 50,000 mol wt protein. The apparent dissociation constants were K_1 = 0.77 m*M*, K_2 = 9.8 m*M*. The microscopic dissociation constants were k_1 = 0.84 m*M*, k_2 = 9.0 m*M*. The points designated (x) were omitted in the final calculation.

$$K_{diss} = \frac{[Mn(II)]_f[UDPGal]_f}{[Mn(II)\cdot UDPGal]_b}$$

then

$$K_{diss} = \frac{[UDPGal]_{total}}{1-\theta} - [Mn(II)]_{total}$$

A plot of $[UDPGal]_{total}/(1-\theta)$ vs. $1/\theta$ gives a slope of K_{diss}. A least-squares fit of the data yields a K_{diss} of 14.5 ± 1.1 m*M*, which is in the range of the approximate value of 7.5 m*M* reported by Khatra et al. (1974) and also in the range for Mn(II)–UDPGlu of ca. 19 m*M* obtained from proton relaxation enhancement experiments by Dwek (1973).

Mn(II)–A Protein Complex. A 0.058 m*M* solution of galactosyltransferase was titrated with 0.08–27 m*M* $MnCl_2$ (pH 8.0) (0.1 *M* NMM, 10% $(NH_4)_2SO_4$) at 26 ± 2° by ESR. The Scatchard plot for this complex (Figure 4) clearly shows the existence of at least two binding sites for Mn(II) on the *A* protein. The solid theoretical curve represents the best fit to a multiple equilibrium program (ABSFIT) written in Fortran for a Nova lab computer by Drs. Dan Leussing and Steve Hershey of this Department. The program uses the Marquardt gradient expansion algorithm (Bevington, 1969) and was fit to a simple two site model. The apparent dissociation constants obtained were K_1 = 0.77 ± 0.04 m*M*, K_2 = 9.8 ± 1.0 m*M*, respectively, while the microscopic binding constants (Klotz and Hunston, 1971) were calculated as k_1 = 0.84 ± 0.1 m*M* and k_2 = 9.0 ± 1.0 m*M*, respectively.

Inhibition Kinetics and Equilibrium Binding. The spin-labeled substrate analog, UDP-R, was designed to mimic UDPGlu as a competitive inhibitor and also to serve as a spin probe in our ESR and nuclear relaxation enhancement studies in progress. The suitability of UDP-R as a competitive inhibitor for UDPGal in the absence (reaction I) or presence (reaction II) of α-lactalbumin is shown in the double reciprocal plots in Figure 5, respectively. The data fit the following expression for the ordered mechanism proposed by Morrison and Ebner (1971a–c) where the constants have the usual meaning for an ordered reaction:

$$\frac{1}{v} = \frac{K_a}{V}\left[\left(\frac{K_{im}}{M} + 1 + \frac{I}{K_i}\right)\left(\frac{K_{ia}K_b}{K_aB} + 1\right)\right]\frac{1}{A} + \frac{1}{B}\left[\frac{K_b}{B} + 1\right]$$

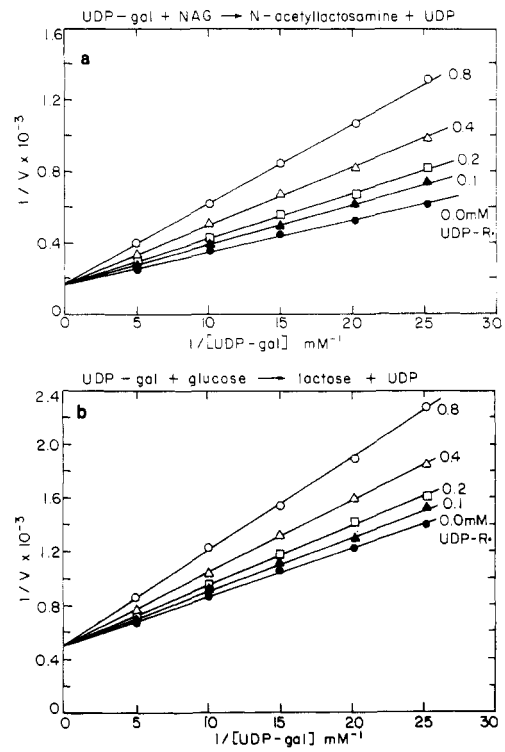

FIGURE 5: Linear competitive inhibition of reaction I (a) and reaction II (b) by UDP-R with UDPGal as the variable substrate in 0.1 *M* NMM (pH 8.0) at 36 ± 0.5°. The concentrations of $MnCl_2$ and GlcnNAc were 4.00 and 20.0 m*M*, respectively, for reaction I while the concentrations of $MnCl_2$, glucose, and α-lactalbumin were 3.73 m*M*, 7.35 m*M*, and 10.3 μ*M*, respectively, for reaction II. The same concentration of enzyme (11.6 μg/ml) was used in all experiments. Inhibitor concentrations (m*M*) are noted on each line. Velocities are expressed as μmol/min.

where *M* = [Mn(II)], *A* = [UDPGal], *B* = [GlcnNAc or glucose], and *I* = [UDP-R]. Secondary plots yield the apparent inhibition dissociation constant, K_{is}, where the "true" UDP-R inhibition constant

$$K_i = K_{is}\left(\frac{M}{K_{im} + M}\right)$$

was calculated using the published K_{im} values for Mn(II) (at 30°) from Morrison and Ebner (1971a,b).[3] The kinetically derived inhibition constants for UDP-R at 36 ± 0.5°, pH 8.0 (0.1 *M* NMM), were 0.38 ± 0.04 m*M* for reaction I and 0.63 ± 0.06 m*M* for reaction II.

Free UDP-R in solution gives a typical nitroxide spectrum with three narrow lines. Upon titrating a fixed concentration of UDP-R with increasing amounts of *A* protein in the absence of Mn(II) or other substrates, the apparent peak height decreases with increasing protein concentration as shown in Figure 6. If one assumes that the contribution of the "bound" *A* protein–UDP-R spectrum contributes negligibly (particularly at the high-field line) to the "free" spectrum, a direct calculation of $[UDP\text{-}R]_{bound}$ can be made and plotted by the method of Scatchard (1949) as

[3] While there is currently some controversy over the point of binding of α-lactalbumin in the reaction sequence and also over the existence of a Mn(II)·UDP complex as the dissociating product (see Khatra et al., 1974; Brew et al., 1975), this should not change the expression for K_i. We did not examine inhibition kinetics while varying other substrates of reactions I and II, but assumed that the structural similarity between UDPGlu and UDP-R leaves the ordered substrate binding mechanism unchanged.

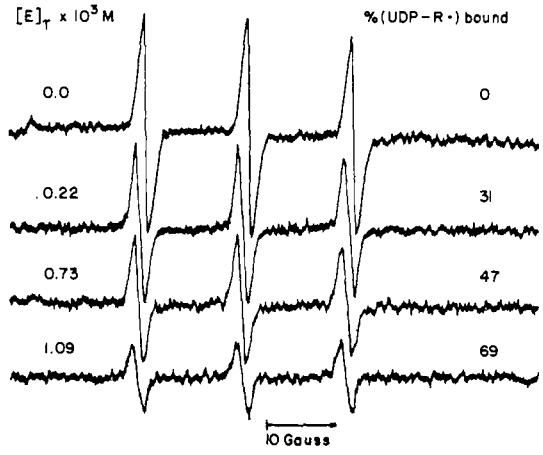

FIGURE 6: Paramagnetic resonance spectra of UDP-R in the absence and presence of galactosyltransferase at X band, 26 ± 1°. The total UDP-R concentration was 1.39×10^{-5} *M* in 0.1 *M* NMM (pH 8.0) containing 10% (w/v) $(NH_4)_2SO_4$ to stabilize the enzyme. No other substrates, including Mn(II), were present. The left column gives galactosyltransferase concentration for each spectrum. Gain and modulation amplitudes were identical for all spectra.

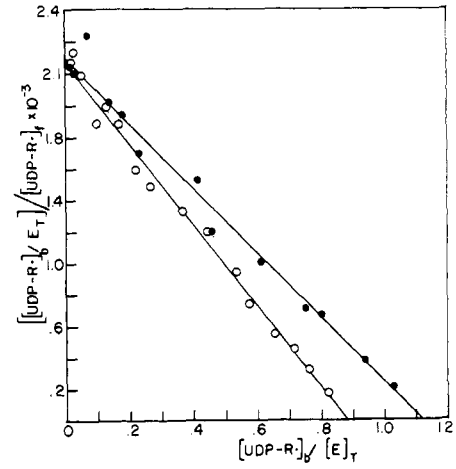

FIGURE 7: Scatchard plot of the ESR data for UDP-R binding to galactosyltransferase in the absence (○) and presence (●) of α-lactalbumin. The concentration of *A* protein was held constant at 8.4×10^{-5} *M* in 0.1 *M* NMM (pH 8.0) and 10% (w/v) $(NH_4)_2SO_4$ at 26 ± 1°. The concentration of UDP-R was varied from 7.8×10^{-5} to 5×10^{-3} *M*. No other substrates nor Mn(II) was present. The data in closed circles (●) are in the presence of 73 μ*M* α-lactalbumin.

shown, for example, in Weiner's (1969) work with the adenosine analog of UDP-R. In Figure 7 are Scatchard plots for the binding of UDP-R to galactosyltransferase in the absence and presence of α-lactalbumin, respectively. The data show that only 1 mol of UDP-R is bound/mol of *A* protein and that the stoichiometry also remains as unity in the presence of α-lactalbumin. The dissociation constants obtained from a linear least-squares fit were 0.40 ± 0.07 and 0.53 ± 0.08 m*M* in the absence and presence of α-lactalbumin, respectively (pH 8.0, 26 ± 2°). These dissociation constants may be compared with the kinetically derived values and compared with the kinetic data for UDPGlu (see Discussion).

Discussion

Manganese Binding to A Protein. At first glance there may appear to be two possible general mechanisms for the kinetically observed activation or inhibition by Mn(II). One involves the binding of a second mole of Mn(II) to a second physically distinct binding site on the *A* protein which is coupled in some way to the catalytic site. Without knowledge of additional physical information a second possibility might be the removal of free UDPGal by Mn(II) from the reaction mixture as the binary complex, Mn(II)-UDPGal. However, the latter complex has already been shown to be catalytically significant with the human enzyme (Khatra et al., 1974). A Dixon plot of the data for the bovine enzyme at high Mn(II) concentrations (K. E. Ebner, personal communication) yields an inhibition constant K_I of 8.5 m*M* while Powell and Brew (1974) recently found an activation constant K_{ia} of 3.4 m*M*. These are both in agreement with our physically determined "second site" binding constant of 9.0 m*M*. The K_{diss} of 14.5 m*M* for the Mn(II)-UDPGal complex (Figure 3) suggests that this latter complex should contribute less until Mn(II) concentrations exceed 15 m*M*.

Therefore, the manganese inhibition (or activation) can at least in part be assigned to a second metal binding site on the *A* protein as found from the ESR studies in Figure 4. Table I summarizes the known kinetic and physical constants from the literature and from this work. With the exception of the (second) "inhibitory" site on the human enzyme (Khatra et al., 1974) the reported kinetic constants

Table I: Binding of Manganese (II) to Galactosyltransferases.

Source and Method	First Mn(II)	Second Mn(II)
Soluble bovine enzyme (Kinetics)		
Reaction I	K_{im} = 1.35 ± 0.14 m*M*[a]	K_I = 8.5 m*M*;[b] K_{ia} = 3.40 m*M*[c]
Reaction II	K_{im} = 1.24 ± 0.20 m*M*[d]	
(ESR)	k_1 = 0.84 ± 0.1 m*M*[e]	k_2 = 9.0 ± 1.0 m*M*[e]
Bovine colostrum enzyme (Kinetics)		
Reaction I	K_{im} = 0.705 m*M*[f]	
Membrane bound sheep mammary gland golgi enzyme (Kinetics)		
Reaction 1	K_{im} = 1.22 m*M*[f]	
Soluble human enzyme (Kinetics)		
Reaction I	K_{im} = 1.42 m*M*[g]	50 m*M* ≤ K_{im} ≤ 100 m*M*[g]

[a] Morrison and Ebner (1971a), 30°. [b] Calculated from the original data in (*a*) supplied by K. E. Ebner (personal communication), 30°. [c] Powell and Brew (1974). [d] Morrison and Ebner (1971b), 30°. [e] This work, 26°. [f] Smith et al. (1975), 37°. [g] Khatra et al. (1974), 37°.

for the various enzyme sources are in good agreement with actual physical constants for Mn(II) binding to the soluble bovine galactosyltransferase. While the two Mn(II) sites may be "coupled", the ambiguity in a choice of models reflects our simpler choice of the independent site microassociation constants calculated from Figure 4. A more complete description of the relationship between these sites awaits further NMR and ESR studies underway.

UDP-R Binds at the UDPGal Site. The kinetic inhibition data of Figure 5 strongly suggest that UDP-R is an excellent "UDPGal site" directed reversible inhibitor in either the absence or presence of α-lactalbumin. This phenomenon is corroborated quantitatively in the direct physical binding experiments for UDP-R and *A* protein alone and in the presence of α-lactalbumin (Figure 7). While both the kinet-

Table II: UDP-R Inhibition and Binding Data.

	UDP-glucose (30°C)[c]	UDP-R (36°C)
	Reaction I (GlcNAc Reaction)	
$K_{is(app)}$[a]	0.23 ± 0.01 m*M*	0.51 ± 0.04 m*M*
$K_{i(true)}$[a]	0.10 ± 0.01 m*M*	0.38 ± 0.04 m*M*
$K_{i(mean)}$[b]	(0.089 ± 0.006 m*M*)	
$K_{diss(ESR)}$		0.40 ± 0.07 m*M*
	Reaction II (Glucose Reaction)	
$K_{is(app)}$[a]	0.21 ± 0.01 m*M*	0.84 ± 0.05 m*M*
$K_{i(true)}$[a]	0.094 ± 0.009 m*M*	0.63 ± 0.06 m*M*
$K_{i(mean)}$[b]	(0.061 ± 0.002 m*M*)	
$K_{diss(ESR)}$		0.53 ± 0.08 m*M*

[a] These are the constants determined from kinetic plots where UDPGal is the variable substrate. [b] Obtained as the weighted mean average of K_i as determined from kinetic plots of (a) UDPGal, (b) Mn(II), and (c) GlcNAc or glucose as variable substrate, respectively. [c] Data of Morrison and Ebner (1971a–c).

ically and physically determined dissociation constants are quite close with or without added α-lactalbumin, the trend toward slightly poorer binding the "α-lactalbumin complex" might be rationalized by some steric restriction to binding in the ternary complex relative to the binary complex (*A* protein–UDP-R). It is also quite noteworthy that the physically determined dissociation constants were determined in the *absence* of Mn(II) while agreeing well with the kinetically (Mn(II) present) determined constants. Affinity chromatography experiments with Sepharose-α-lactalbumin indicate that the presence of UDP-R alone retards *A* protein on the immobilized α-lactalbumin (S. Wong and L. J. Berliner, unpublished experiments).

Table II summarizes the kinetic and binding data for UDP-R as compared with published data for UDPGlu with bovine galactosyltransferase. Since, on the whole UDP-R binds only slightly worse than UDPGlu, while considering the vague similarity between the piperidinoxy group and a hexose, one must conclude that the uridylyl group accounts for the bulk of the binding affinity of galactosyltransferase for this spin label. This is of course no surprise when one compares the inhibition constant of UDP of 0.067 m*M* (Hill et al., 1972). This may be further corroborated by our ESR measurements in progress of *A* protein–UDP-R complexes in the presence and absence of α-lactalbumin and other substrates. Spectral observation of the *A* protein–spin label complex alone has been operationally very difficult since saturation may be limited by the solubility of the enzyme. Further spectral analyses encompassing computer simulations and spectral subtractions may be necessary to "isolate" the bound UDP-R spectrum.

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