Metal Ion and Substrate Binding to Bovine Galactosyltransferase†

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ABSTRACT: Bovine milk galactosyltransferase was examined by ESR and NMR proton relaxation measurements to determine the stoichiometry and nature of manganese and UDP-Gal substrate binding. The ESR and NMR data clearly showed the binding of two Mn(II) per mol of enzyme in the ternary complex (enzyme·manganese·UDP-Gal). The affinity of the enzyme for manganese is much higher in the presence of UDP-Gal than in its absence. A deenhancement was observed in both water and UDP-Gal proton relaxation rates upon ternary complex formation [enzyme·Mn(II)·UDP-Gal] relative to the metal–substrate [Mn(II)·UDP-Gal] binary complex, yet the temperature dependence of the water proton relaxation rate was consistent with fast exchange. A simple model was proposed which accounted for the pronounced deenhancement, involving a slow conformational interconversion of an initially formed, rapidly exchanging conformer of the enzyme·Mn(II)·UDP-Gal complex to a second form which contributes negligibly to the relaxation.

Bovine galactosyltransferase (GT)[1] functions in complex carbohydrate biosynthesis

$$\text{UDP-Gal} + \text{GlcNAc} \xrightarrow{\text{Mn(II)}} \text{UDP} + \textit{N}\text{-acetyllactosamine} \quad (1)$$

as UDP-galactose:*N*-acetylglucosamine β-4-galactosyltransferase (EC 2.4.1.38) while in complex with α-lactalbumin as lactose synthase

$$\text{UDP-Gal} + \text{glucose} \xrightarrow{\text{Mn(II)}} \text{UDP} + \text{lactose} \quad (2)$$

or UDP-galactose:D-glucose D-galactosyltransferase (EC 2.4.1.22).

Recently, Powell & Brew (1976) have shown *kinetically* that several other cations [but not Mg(II)] function as activators for this enzyme. Of particular interest was the evidence from these studies of two Mn(II) sites with kinetically derived dissociation constants in the micromolar (site I) and the millimolar ranges (site II), respectively (Powell & Brew, 1976; E. T. O'Keefe, R. L. Hill, and J. E. Bell, unpublished experiments). Of further curiosity was the rather high dissociation constant(s) for binary Mn(II)·GT ($K_d \geq 1$ mM) from kinetic [see also Morrison & Ebner (1971) and Hill et al. (1972)] and direct physical measurements (Berliner & Wong, 1975) when physiological Mn(II) levels in milk are submicromolar in contrast to millimolar levels of Mg(II) or Ca(II) (Davies, 1936; Altman & Dittmer, 1971). It is perhaps also important to add that the enzyme form used in the kinetic studies above was that from bovine colostrum, which contains primarily a single high M_r form of ~52 000–55 000, while bovine milk contains a mixture of this high M_r form plus two additional active forms of M_r 48 000 and 42 000 (Powell & Brew, 1974).

We have been particularly interested in Mn(II) binary binding to GT in order to utilize this paramagnetic cation in nuclear relaxation measurements as a powerful mapping probe of GT–substrate complexes. We present here Mn(II) binding and stoichiometry results from water proton and UDP-Gal substrate proton relaxation experiments.

Materials and Methods

Materials. UDP-Gal, ATP, phosphoenolpyruvate, and NADH were from Sigma Chemical Co. *N*-Acetylglucosamine (GlcNAc) was from Pfanstiehl Chemical Co. Fresh raw milk was obtained from the Ohio State University dairy barn.

Proteins. Pyruvate kinase (type 1) and α-lactalbumin were from Sigma Chemical Co. Galactosyltransferase was purified as described by Geren et al. (1976) using hydrophobic chromatography with norleucine–Sepharose, followed by one or two purification steps on an α-lactalbumin–Sepharose affinity column. The purified enzyme had a specific activity of 15–20 units/mg[2] at 30 °C. NaDodSO$_4$ gel electrophoresis indicated the presence of only the two standard higher M_r forms (55 000 and 48 000)[3] with no impurities present. Stock solutions of 1–5 mg/mL were made by precipitating the eluted enzyme in 85% saturated ammonium sulfate (Ultrapure, Schwarz/Mann), followed by centrifugation and dialysis of the resuspended precipitate vs. buffer.

Methods. NAL or lactose synthase activity was measured by the spectrophotometric coupled assay of Fitzgerald et al. (1970). GT concentration was estimated spectrophotometrically by using an extinction coefficient $E_{280}^{0.1\%} = 1.61$ (Trayer & Hill, 1971).

NMR. All experiments were run on a Bruker HFX-90 with a Nicolet BNC-12 computer interface at 30 ± 1 °C unless noted otherwise. D_2O was used as an internal heteronuclear lock. Water proton T_1 experiments were performed in 40-μL spherical cells, while for UDP-Gal T_1 measurements, 5-mm tubes (Wilmad 507) were used. Buffers were pH 7.4 0.05 M Tris-HCl, 0.1 M $(NH_4)_2SO_4$, and 2 mM ϵ-aminocaproic acid in 20 or 100% D_2O. T_1 values were determined graphically from a plot of 15–20 τ values (180°–τ–90° pulse sequence) over the range $0.1T_1 < \tau < 2T_1$, where the last two delays were at $\tau = 5T_1$ (Farrar & Becker, 1971). T_2 was estimated from the line width at half-maximal height $\nu_{1/2}$ by the relation T_2

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[1] Abbreviations used: GT, galactosyltransferase; GlcNAc, *N*-acetylglucosamine; UDP-Gal and UDP-Glc, UDP-galactose and -glucose, respectively; NAL, *N*-acetyllactosamine; ESR, electron spin resonance.

[2] The enzyme unit is conventionally defined as the production of 1 μmol of UDP per min.

[3] These two forms have been demonstrated in past work to exhibit identical kinetic and other physical properties (Magee et al., 1974).

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$= 1/(\pi\nu_{1/2})$ for a Lorentzian line shape.

Samples of UDP-Gal (1 mM) were treated with Chelex 100 (Bio-Rad Laboratories) before use to remove all trace paramagnetic impurities.

ESR. Experiments were carried out as "titrations" of free uncomplexed Mn(II) in capillary cells (Taylor, 1969; Berliner, 1977) typically containing 40-μL aliquots of a 500-μL sample. Each spectrum was followed by one of a standard Mn(II) solution (pH 7.4 0.05 M Tris-HCl, 0.1 M $(NH_4)_2SO_4$, and 2 mM ϵ-aminocaproic acid) taken under the same conditions to correct for slight changes in instrumental response.

While control ESR and NMR experiments did not reveal any significant Mn(II)·sulfate complexation, the stoichiometry of enzyme–metal binding was directly calculable since all experiments were compared to standards under these same buffer conditions.

Magnetic Resonance Theory

Water proton relaxation caused by paramagnetic metals can be analyzed as follows. Measurement of the relaxation rate in the absence and presence of the paramagnetic ion gives us the paramagnetic contribution

$$\frac{1}{T_{ip}} = \frac{1}{T_i(\text{obsd})} - \frac{1}{T_i(\text{dia})} \quad \text{where } i = 1 \text{ or } 2 \qquad (3)$$

$1/T_i(\text{obsd})$ and $1/T_i(\text{dia})$ are the measured relaxation rates in the presence and absence of a paramagnet. The molar relaxation rate of the ion is given by

$$R_M = (1/T_{ip})/[M] \qquad (4)$$

where [M] = concentration of manganese. When manganese binds to ligands, the molar relaxation rate will be a weighted sum over all species in solution

$$R_M = \sum_i R_M^i f^i \qquad (5)$$

where R_M^i is the specific molar relaxation rate for a particular manganese-containing species and f^i is the fractional concentration of that particular species.

If manganese is complexed with large molecules (e.g., proteins), the relaxation often increases. Enhancement factors $\epsilon = R_M/R_M(H_2O)$, where $R_M(H_2O)$ is for aqueous (free) manganese, have been found to be useful in analyzing the experimental data in terms of dynamic and structural properties of the complexes formed.

Where ligands other than water are studied, the paramagnetic contribution to the relaxation rate is determined as above and expressed as

$$R_M(L) = \frac{\sum_i [L^i] R_M^i(L)}{[L_0]} \qquad (6)$$

$[L_0]$ is the total concentration of ligand, $[L^i]$ is the concentration of the ligand–manganese complex, and $R_M^i(L)$ is the specific intrinsic relaxation rate of that complex (Andree, 1978). In the case of a single-step binary complex, $1/R_M^i(L)$ equals the conventionally defined T_{2M} or τ_M of the complex for fast and slow exchange cases, respectively (Swift & Connick, 1962; Dwek, 1973). The more general introduction of a specific intrinsic relaxation rate provides a parameter characteristic for a particular complex without introducing any assumption about the relaxation and binding mechanisms.

Results

Binding Studies. Figure 1 shows a typical titration of a solution containing fixed concentrations of GT (126 μM) and

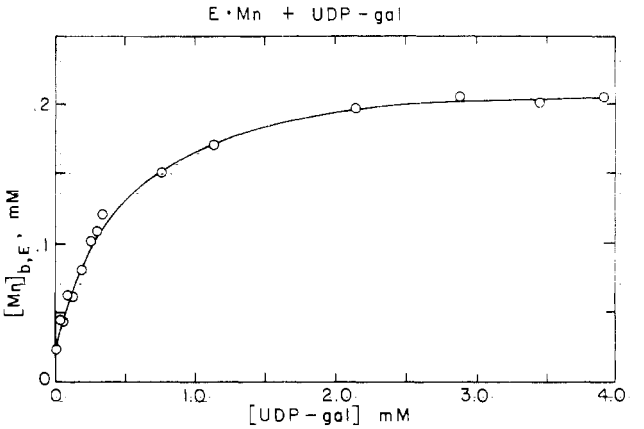
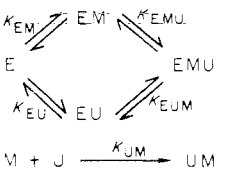


FIGURE 1: ESR titration of Mn(II) binding to GT in the presence of UDP-Gal. Conditions were $MnCl_2$ (347 μM) and GT (126 μM; sp act. ~20 units/mg). Buffer was 0.05 M Tris-HCl, pH 7.4, 0.1 M $(NH_4)_2SO_4$, and 2 mM ϵ-aminocaproic acid, 20 ± 2 °C. The concentration of enzyme-bound Mn ($[Mn]_{b,E}$) was corrected for the Mn(II)·UDP-Gal complex by using a dissociation constant of 17.5 mM for this complex.

Scheme I[a]



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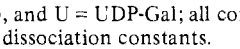


(8)

[a] E = GT, M = Mn(II), and U = UDP-Gal; all constants are defined as microscopic dissociation constants.

$MnCl_2$ (347 μM) with increasing UDP-Gal. The concentration of enzyme-bound Mn(II) is shown on the ordinate as calculated by subtracting the measured concentration of free manganese and the amount of the binary Mn·UDP-Gal complex from the total concentration. Under the conditions of the experiment, a very good estimate for the binary Mn·UDP-Gal complex was obtained from [Mn·UDP-Gal] = [UDP-Gal]-$[Mn^{2+}]_{free}/K_{diss}$, where K_{diss} for this complex was found to be 17.5 mM at pH 7.4 which compares well with the value of 14.5 mM reported earlier at pH 8.0 (Berliner & Wong, 1975).

The stoichiometry of Mn(II) bound per mole of enzyme in this particular experiment was 1.6:1 while other titrations yielded values which averaged 2.0 ± 0.3. Of significance here was a stoichiometry greater than unity, which indicates the presence of two Mn(II) binding sites on the enzyme. The apparent equivalence of the two sites can be a result of the manner in which the titrations were run and does not conflict with two different binding sites.

The results of three UDP-Gal titrations with different $MnCl_2$/GT concentrations (data not shown) were used to determine the equilibrium constants in the general equilibrium scheme shown in Scheme I. Of the four equilibrium constants involving enzyme, only three are independent ($K_{EM}K_{EMU} = K_{EU}K_{EUM}$). K_{UM} was determined in a separate experiment as noted earlier.

Calculated values for the free Mn^{2+} concentrations were fitted to the experimental results by using a least-squares minimization program described by Bevington (1969) to find the best values for the equilibrium constants K_{EM}, K_{EU}, and K_{EMU}. The theoretical free manganese concentrations were calculated assuming a given set of equilibrium constants and total concentrations of components by using a multiequilibrium iteration program similar to that described in Dwek (1973). Table I gives the best fit equilibrium parameters.

Table I: Values for the Dissociation Constants in the Galactosyltransferase·UDP-Galactose·Manganese System Determined by Various Methods

	ESR[a] (mM)	kinetics (mM)	inactivation protection (mM)
K_{EM}	5	1.3[b]; 0.95, 3.0[c]; 0.002[d]	
K_{EU}	10		4.2[f]; 0.31[g]
K_{EMU}	0.025	0.065[b]; 0.013[e]	0.014[f]; 0.016[g]
K_{EUM}	0.013		

[a] Calculated constants (±30%) are from Scheme I for M/E and U/E at 2:1. Results for U/E = 1 did not differ significantly. [b] Morrison & Ebner (1971). [c] Powell & Brew (1974). First value for colostrum enzyme; second value for trypsin-treated milk enzyme. [d] Powell & Brew (1976). Value for (stronger) site I. [e] Bell et al. (1976). [f] Magee & Ebner (1974). Values derived from thiol protection against *N*-ethylinaleimide inactivation. [g] Kitchen & Andrews (1974). From the thiol protection against *p*-(hydroxymercuri)benzoate (human milk enzyme).

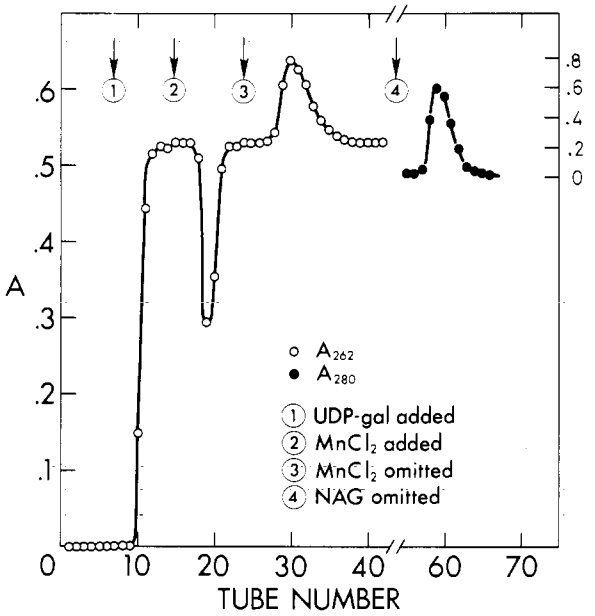


FIGURE 2: UDP-Gal binding to GT on α-lactalbumin–Sepharose. GT with 25 mM GlcNAc was applied to α-lactalbumin–Sepharose until the column was saturated with enzyme and then washed with 25 mM GlcNAc. (1) UDP-Gal (0.062 mM) applied in 25 mM GlcNAc. (2) $MnCl_2$ (1.5 mM) included in the buffer in (1). (3) $MnCl_2$ omitted, i.e., step 1 repeated. (4) Wash with 25 mM GlcNAc only, followed by buffer alone (without GlcNAc). Open circles represent absorbance of UDP-Gal at 262 nm while filled circles represent released GT absorbance at 280 nm. Buffer was 0.05 M Tris-HCl, pH 7.4, 0.1 M $(NH_4)_2SO_4$, and 2 mM ε-aminocaproic acid. Experiments were run at 4 °C.

The model above was used with the assumption of two identical binding sites for both Mn(II) and UDP-Gal. Other possibilities like the existence of only one UDP-Gal site or a nonequivalence of the two Mn(II) sites are not excluded by the data.

While the assumption of one or two UDP-Gal binding sites per enzyme molecule does not drastically change the determined constants, it was of considerable interest in itself whether there were actually one or two sites. Some preliminary evidence for UDP-Gal binding stoichiometry was obtained in an experiment on an α-lactalbumin–Sepharose affinity column (see Figure 2). The column was saturated with bound GT by applying an excess of the enzyme in the presence of 25 mM GlcNAc. After being washed, 0.062 mM UDP-Gal was included in the applied buffer and its presence in the eluate was followed at 262 nm. Subsequent addition of 1.5 mM $MnCl_2$ resulted in a trough in the eluate absorbance showing that

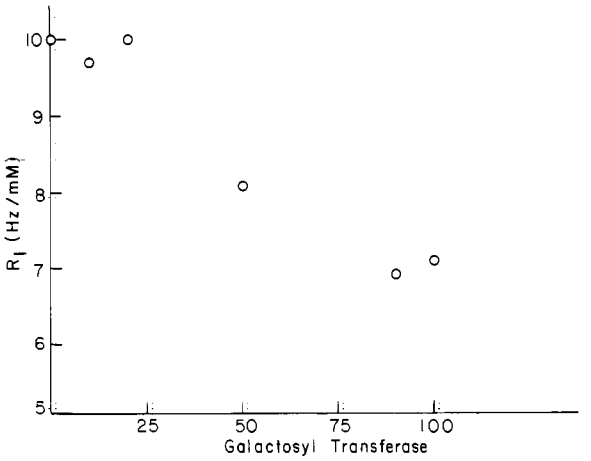


FIGURE 3: NMR "E" titration at 90 MHz of the observed water proton longitudinal relaxation rate at fixed UDP-Gal (28 mM) and $MnCl_2$ (0.1 mM). Conditions were 0.05 M Tris-HCl, pH_{obsd} 7.4, 20% D_2O, 0.1 M $(NH_4)_2SO_4$, 2 mM ε-aminocaproic acid, and 30 ± 1 °C. Enzyme specific activity was ~20 units/mg.

UDP-Gal was retained on the column.[4] It was released when $MnCl_2$ was omitted. In the control experiment, no binding was detected if GT was absent from the column. The amounts of UDP-Gal first retained and later released were calculated from the measured tube volumes and absorbance differences. The amount of GT bound to the column was determined after washing the column with buffer containing no UDP-Gal and subsequently releasing the enzyme by omitting GlcNAc. In another control experiment this amount was also determined where the UDP-Gal binding step was omitted to ensure that no enzyme leakage had occurred during the course of elutions. The molar amounts of UDP-Gal retained and released in the experiment were 2.0 times the molar amount of enzyme on the column.[5]

Nuclear Magnetic Resonance Studies. Our NMR results, while yielding additional information about the dynamics of GT·Mn(II) complexes, were also consistent with the stoichiometry of metal ion binding as determined by ESR. Figure 3 shows the net observed paramagnetic molar relaxation rate R_M of water protons for a $MnCl_2$·UDP-Gal solution (0.1 and 28 mM, respectively) with increasing GT (0–100 μM). The observed deenhancement, characteristic of most of our T_1 studies with Mn(II)·GT complexes, seems to level off between Mn(II)/GT = 1.5 and Mn(II)/GT = 2.0 at an R_M value which was approximately the same as that for free aqueous Mn(II). Thus, water proton relaxation rate behavior changes in a manner consistent with Mn(II) saturation of GT at a stoichiometry of 2:1 (see also Figure 1).

Figure 4 shows water proton T_1 relaxation data in a Mn(II) titration at fixed GT (0.044 mM) and UDP-Gal (3.0 mM). The similarity to the water proton T_1 values in free $MnCl_2$ (dotted line) of the ternary complex [Mn(II)·GT·UDP-Gal] was purely a coincidence in that the two species compared differ, in general, in their chemical composition, coordination number, and correlation time. The evidence for actual ternary complex formation came from the strong deenhancement in the transverse relaxation rates ($1/T_2$) and from parallel ESR

[4] Identical results were obtained at $MnCl_2$ concentrations threefold higher.

[5] There remain at least two possible models to explain this stoichiometry. Since UDP-Gal alone retards GT in α-lactalbumin–Sepharose (Mawal et al., 1971), the second UDP-Gal may have bound at a carbohydrate site. On the other hand, the second metal site may also bind nucleotide. In either case these results are specifically for the complex with α-lactalbumin and may not be significant at physiological levels.

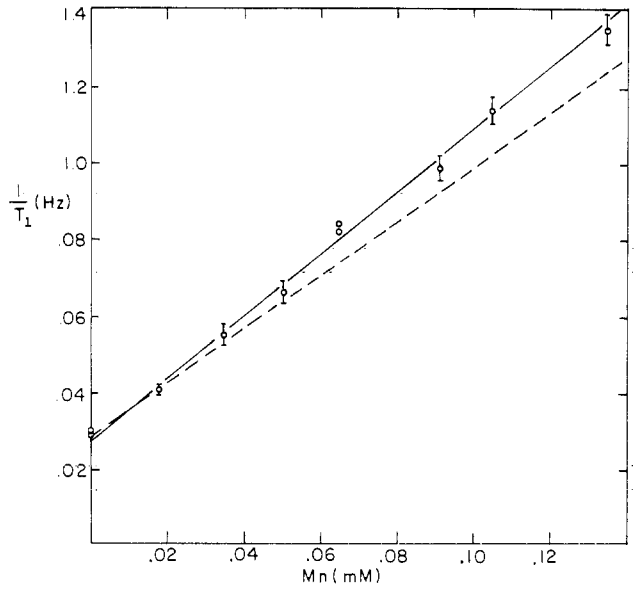


FIGURE 4: Mn titration at 90 MHz of the ternary complex water proton longitudinal molar relaxation rate at fixed UDP-Gal (3.0 mM) and GT (0.044 mM). The data are corrected for binary complex [Mn(II)·UDP-Gal] relaxation contributions. The dotted line represents free aqueous Mn(II). All other conditions were as described in Figure 3.

Table II: Water Proton Molar Relaxation Rates[a]

complex[b]	T_1^{-1}	T_2^{-1}	ϵ_1	ϵ_2
M	7.0	75	1.0	1.0
UM	14.0	39	2.0	0.52
EUM	7.0	9.2	1.0	0.12

[a] Units are s^{-1} mM^{-1}. Values given are paramagnetic relaxation rates for a 1 mM concentration of the indicated complex. Calculated uncertainties are 5–10%. [b] In the abbreviated notation for each complex M refers to manganese, U refers to UDP-galactose, and E refers to the enzyme. Conditions were as indicated in Figure 3.

measurement on these same samples.

Relaxation parameters for the binary Mn(II)·UDP-Gal complex were determined for several samples in which the amount of bound Mn(II) was separately determined by ESR. The specific molar relaxation rate for Mn(II)·UDP-Gal (14.0 s^{-1} mM^{-1}) was in good agreement with the published value for Mn(II)·UDP-Glc (Dwek, 1973). The specific molar relaxation rates for the ternary complex were calculated from experimental data from eq 5, by using concentrations of the various Mn(II) complexes calculated from the results in Table I. The calculated concentrations of free Mn(II) were always in good agreement with the ESR-measured data. The contribution of the binary Mn(II)·GT complex was assumed to be zero since no enhancement was ever detected in enzyme·Mn(II) mixtures alone. The results for the specific molar relaxation rates are given in Table II as averages over several experiments using different enzyme preparations.

In order to further characterize the ternary GT·Mn(II)·UDP-Gal complex, we measured proton relaxation times (T_1 and T_2) for UDP-Gal protons in 100% D_2O. Accurate values for the specific intrinsic relaxation times for the binary Mn(II)·UDP-Gal complex were easily determined from formula 6 by using high UDP-Gal concentrations that were saturating manganese.

Ternary complex measurements were obtained under conditions in which the ternary complex was in excess over the binary. The specific intrinsic relaxation rates were calculated from the data by using formula 6, by substituting complex

Table III: Specific Intrinsic Paramagnetic Relaxation Rates for UDP-Galactose·Manganese Complexes

	T_1^{-1} [a]			T_2^{-1}, [a]
	uracil		galactosyl	uracil
complex	H-6	H-5	H-1′	H-6
UM	3.4	2.0	0.2	4.8
EUM	0.2	0.3	0.05	0.8

[a] Units are kHz. Conditions were as described in Table I. Assignment of resonances was according to Lee & Sarma (1976).

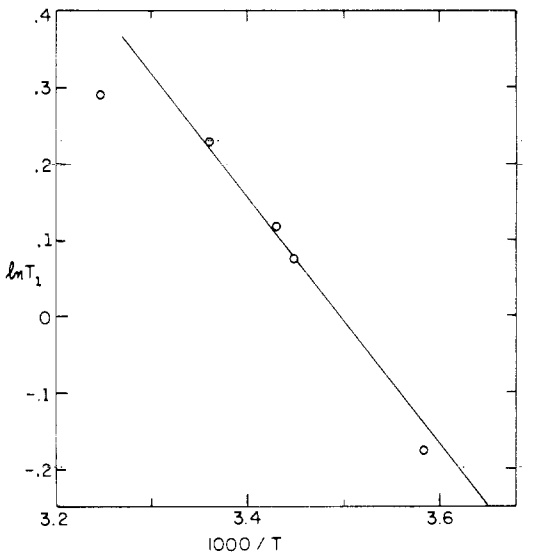


FIGURE 5: Dependence of observed water proton T_1 with temperature. Conditions were GT (80 μM), $MnCl_2$ (68.6 μM), and UDP-Gal (1.34 mM). All other conditions were as described in Figure 3.

concentrations calculated from the ESR data. The values have a large relative error because the largest contribution to the relaxation rate is made by the binary complex even if the ternary complex is in excess.

The results are listed in Table III. Note again here the *deenhancement* of both uridyl and galactosyl proton T_1^{-1} and T_2^{-1} rates upon ternary complex formation.

Variable-Temperature Studies. The most straightforward explanation for the observed deenhancements upon ternary complex formation would be that chemical exchange was the limiting factor in the relaxation process. T_1^{-1} rates were studied with increasing temperature in order to distinguish this further.

Figure 5 shows a plot of ln T_1 vs. $1/T$ for water protons under conditions favoring ternary complex formation. The results show increasing relaxation times at higher temperatures as expected for free manganese,[6] under conditions where most of the relaxation was caused by manganese bound in the ternary complex. This result was inconsistent with a simple one-step binding model, where increasing relaxation rates were expected at higher temperature; a fractional coordination number for fast-exchanging water ligands on Mn offers one simple model;[7] however, there are other more complex binding mechanisms which might account for the data (see Discussion). Accurate nucleotide sugar temperature-dependent relaxation rate measurements were complicated by the small contribution

[6] The enthalpy of activation calculated from Figure 5 is 3.2 kcal/mol.

[7] The additional coordination to Mn(II) arises from (divalent) sulfate ion binding to the ternary enzyme·Mn(II)·UDP-Gal complex if, indeed, Mn(II) binding to the enzyme significantly increased its affinity for sulfate ion.

of the ternary complex to the overall relaxation and by disproportionation side reactions at elevated temperatures (Nunez & Barker, 1976), although the general trend seemed consistent with the water proton results.

Discussion

Both the T_1 and T_2 data for water or UDP-Gal substrate protons showed decreased relaxation upon binding of GT to the Mn(II)·UDP-Gal complex. In view of the expected increase in rotational tumbling time of the complex which usually results in an *increased* relaxation rate, these results would seem to support a model in which decreased chemical exchange limits the relaxation (Sykes et al., 1970). This is even more remarkable when one considers that two Mn(II)'s are bound per macromolecule. At first glance, our temperature-dependent measurements contradict this behavior since increasing the temperature should increase the rate of chemical exchange, hence increasing the relaxation rate. However, this is true only if the dissociation of the ligand (water or UDP-Gal) from the complex is a simple one-step process. In a more complicated system the relaxation might be exchange limited while the temperature dependence of this relaxation appears similar to a fast-exchange situation. A model which would fit this above behavior is depicted as

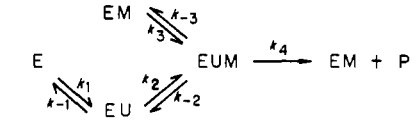
$$\mathrm{E} + \mathrm{MU} \overset{\text{fast}}{\rightleftharpoons} \mathrm{EMU} \overset{\text{slow}}{\rightleftharpoons} \mathrm{EMU}^* \qquad (9)$$

where the intermediate ternary complex undergoes a slow conformational transition to the major ternary complex denoted with an asterisk. This intermediate ternary complex, which is in relatively low concentration, is that complex in fast exchange with the ligand(s), while the major complex, formed by the slow interconversion, does not contribute much, if at all, to the relaxation. The slow transition proposed here might correspond with structural changes in the enzyme upon ternary complex formation as detected by circular dichroism (Geren et al., 1975) and protection against photoinactivation (Clymer et al., 1976) and trypsinolysis (Magee & Ebner, 1974).

Other, perhaps simpler, explanations can be forwarded to account for the observed deenhancements. The low solvent relaxation rate can be the result of a low coordination number ($q = 1$ for the first coordination sphere of manganese) or might only result from outer sphere contributions. The UDP-Gal proton relaxation rate decrease on binding of the Mn(II)·UDP-Gal complex to the enzyme could be the result of an actual separation of Mn(II) and UDP-Gal on the enzyme. The mechanism depicted above, however, can account for both sets of results. It is in general difficult to rule out a mechanism as shown in eq 9, which may also play a role in other paramagnetic relaxation studies on other enzymes. This behavior was also proposed for substrate binding to glutamate dehydrogenase (Andree, 1978).

Recent kinetic studies of metal ion binding had suggested that for Mn(II), Zn(II), and Co(II) two binding sites were present, while other metals could bind only to a second activation site (Powell & Brew, 1976; E. T. O'Keefe, R. L. Hill, and J. E. Bell, unpublished experiments). Furthermore, these kinetics on the colostrum form of this enzyme were analyzed in terms of two distinctly different affinity sites. The results presented here on the milk enzyme species clearly show the presence of two Mn(II) sites with an overall dissociation constant in the micromolar range in the presence of UDP-Gal. Table I presents a comparison between our results and those obtained from other studies. The agreement is generally good, in particular in the indication of strong heterotropic positive interactions between the cofactor, Mn^{2+}, and the substrate UDP-Gal. The major discrepancy concerns the binary enzyme–manganese complex where the physically determined dissociation constant is consistently higher than the kinetic ones.

When this discrepancy is considered, it first should be noted that the kinetically determined dissociation constant is not necessarily correct if the wrong kinetic mechanism was applied. Rudolph & Fromm (1971) have considered the full nonlinear steady-state rate equation for a two-substrate mechanism. In resolving a similar discrepancy for hexokinase, they pointed out that apparent linearity in an experimental situation can lead to errors of several-fold in the determined dissociation constants. Even for mechanisms that give rise to linear double-reciprocal plots, K_{ia} does not always equal the dissociation constant. As a possible pertinent example, we have considered the following mechanism, which, while not the most ubiquitous case with weakly binding metal ligands, cannot be entirely ruled out



where manganese does not dissociate from the enzyme after a catalytic cycle and the E + M ⇌ EM equilibrium is too slow to be kinetically relevant. The rate equation for this mechanism is

$$\frac{V_{max}}{v} = 1 + \frac{K_a}{[A]} + \frac{K_b}{[B]} + \frac{K_{ia}K_b}{[A][B]}$$

where

$$K_{ia} = K_{EM}\frac{k_{-3}}{k_{-3} + k_4}$$

Such a mechanism would explain the higher dissociation constant detected by ESR and could also give rise to a very low value for K_a (as observed for galactosyltransferase) if K_{EUM} is sufficiently small. It is quite interesting by itself that the mechanism depicted above with $K_a = K_{EUM} \to 0$ produces the kinetic pattern that is commonly interpreted as indicating ordered addition with manganese binding first to the enzyme while in fact the direct formation of this complex does not occur at all. While the fractional coordination/outer sphere relaxation model discussed above is certainly simpler and cannot be categorically ruled out, it seemed pertinent to discuss this latter model as a viable consideration, especially in light of multistep equilibrium phenomena detected in several systems by cryoenzymological techniques. The present discussion shows once more that one has to exercise caution in deriving thermodynamic constants from kinetic data.

While we have not yet carried out extensive analogous kinetic experiments on the milk enzyme to compare with those results from the colostrum form, the physical results presented here, together with the available kinetic data, present a complementary picture of GT cation binding and its effects on structure and function.

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Interaction of Ribulosebisphosphate Carboxylase/Oxygenase with Transition-State Analogues†

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ABSTRACT: 2-*C*-Carboxy-D-ribitol 1,5-bisphosphate and 2-*C*-carboxy-D-arabinitol 1,5-bisphosphate have been synthesized, purified, and characterized. In the presence of Mg^{2+}, 2-*C*-carboxy-D-arabinitol 1,5-bisphosphate binds to ribulose-1,5-bisphosphate carboxylase/oxygenase by a two-step mechanism. The first, rapid step is similar to the binding of ribulose 1,5-bisphosphate or its structural analogues. The second step is a slower process ($k = 0.04\ s^{-1}$) and accounts for the tighter binding of 2-*C*-carboxy-D-arabinitol 1,5-bisphosphate ($K_d \lesssim 10^{-11}$ M) than of 2-*C*-carboxy-D-ribitol 1,5-bisphosphate ($K_d = 1.5 \times 10^{-6}$ M). Both carboxypentitol bisphosphates exhibit competitive inhibition with respect to ribulose 1,5-bisphosphate. 2-*C*-(Hydroxymethyl)-D-ribitol 1,5-bisphosphate and 2-*C*-(hydroxymethyl)-D-arabinitol 1,5-bisphosphate were also synthesized; both are competitive inhibitors with respect to ribulose 1,5-bisphosphate with $K_i = 8.0 \times 10^{-5}$ M and $K_i = 5.0 \times 10^{-6}$ M, respectively. Thus, the carboxyl group of 2-*C*-carboxy-D-arabinitol 1,5-bisphosphate is necessary for maximal interaction with the enzyme. Additionally, Mg^{2+} is essential for the tight binding of 2-*C*-carboxy-D-arabinitol 1,5-bisphosphate. A model for catalysis of ribulose 1,5-bisphosphate carboxylation is discussed which includes a functional role for Mg^{2+} in the stabilization of the intermediate 2-*C*-carboxy-3-keto-D-arabinitol 1,5-bisphosphate. Mechanistic implications that arise from the stereochemistry of this intermediate are also discussed.

An essential reaction for CO_2 fixation in all photosynthetic organisms is catalyzed by ribulose-P_2 carboxylase/oxygenase.[1] Interest in this enzyme has been stimulated by the discovery of the oxygenase reaction (Ogren & Bowes, 1971; Andrews et al., 1973) and its role in the glycolate pathway of photorespiration (Tolbert & Ryan, 1976).

An outline for the chemical mechanism of the carboxylation reaction was predicted by Calvin (1954) even before the discovery of the enzyme (Scheme I). The initial step is the enolization of ribulose-P_2 (**1**) to form **2**, which is attacked by CO_2 to form a 2-*C*-carboxy-3-ketopentitol bisphosphate, **3**. Addition of H_2O across the bond at C-2 and C-3 of **3** yields two molecules of D-glycerate-3-P (Fiedler et al., 1967; Cooper

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[1] Abbreviations used: ribulose-P_2, D-*erythro*-pentulose 1,5-bisphosphate; P, phosphate; P_2, 1,5-bisphosphate; ^{13}C NMR, ^{13}C nuclear magnetic resonance spectroscopy; carboxypentitol-P_2, an unresolved mixture of 2-*C*-(phosphohydroxymethyl)-D-ribonic acid 5-phosphate and 2-*C*-(phosphohydroxymethyl)-D-arabinonic acid 5-phosphate.

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