GALACTOSYLTRANSFERASE: CONFIRMATION OF EQUILIBRIUM-ORDERED MECHANISM*

C.R. Geren, L.M. Geren and K.E. Ebner

Department of Biochemistry
University of Kansas Medical Center, Kansas City, KS 66103

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$MnCl_2$ purified with diphenylthiocarbazone and used as a substrate for bovine milk galactosyltransferase resulted in kinetic patterns indicative of an equilibrium-ordered mechanism for the addition of Mn^{2+}. The use of analytical reagent $MnCl_2$ without purification produced kinetic plots intersecting to the left of the vertical axis, indicating sequential addition of Mn^{2+}. Addition of a trace amount of $PbCl_2$ to the purified $MnCl_2$ also resulted in patterns intersecting to the left of the vertical axis.

INTRODUCTION

Based on the results of initial velocity and dead-end inhibition studies with N-acetylglucosamine as the galactosyl acceptor, Morrison and Ebner (1) proposed an ordered mechanism for bovine milk galactosyltransferase (UDP-galactose: D-glucose 1-galactosyltransferase; EC 2.4.1.22) with reactants adding in the order: Mn^{2+}, UDP-galactose and N-acetylglucosamine. A further conclusion was that Mn^{2+} reacted with the free enzyme under conditions of thermodynamic equilibrium and did not dissociate after each turn of the catalytic cycle (i.e. an equilibrium-ordered mechanism).

Recently Powell and Brew (2) and Khatra et al. (3) have reported the addition of Mn^{2+} was not at thermodynamic equilibrium with bovine and human galactosyltransferase. This conclusion was based primarily on the intersection of lines to the left of the vertical axis of a double reciprocal plot of $(velocity)^{-1}$ versus $(UDP\text{-}galactose)^{-1}$ with Mn^{2+} as the fixed variable substrate and constant N-acetylglucosamine concentration. These workers (2,3) proposed that a Mn^{2+}-UDP complex is the final product to dissociate from the enzyme in the catalytic scheme.

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In this communication a kinetic re-examination of the interaction of Mn^{2+} with bovine milk galactosyltransferase is reported. In addition, an explanation for the different results is proposed, mainly that analytical reagent grade $MnCl_2$ contains heavy metal impurities such as Pb^{2+} which are inhibitory to the galactosyltransferase.

MATERIALS AND METHODS

Pyruvate kinase (type I containing 30 units of lactic acid dehydrogenase per mg of protein), NADH and phosphoenolpyruvate were purchased from Sigma. N-acetyl-D-glucosamine and the potassium salt of UDP-galactose were from Calbiochem. The concentrations of UDP-galactose solutions were determined enzymatically using galactosyltransferase. $MnCl_2$ was Mallinckrodt analytical reagent and was further purified with diphenylthiocarbazone (Sigma) as described by Morrison and Uhr (4). N-ethylmorpholine was from Aldrich, EDTA from Baker, $PbCl_2$ from Merck and $MgSO_4$, $ZnCl_2$ and KCl from Mallinckrodt.

Galactosyltransferase was isolated from bovine skim milk by hydrophobic and affinity chromatography (5) and was stored in 20 mM Tris-HCl, 1 mM β-mercaptoethanol, pH 7.5 at -20° C. Activity was measured by the method of Morrison and Ebner (1) and each assay contained 6 μg of galactosyltransferase. All assays were run in duplicate.

RESULTS

The effect of the concentration of UDP-galactose on the initial velocity of the reaction at various fixed concentrations of purified $MnCl_2$ and a fixed concentration of N-acetylglucosamine is illustrated (Fig. 1) in the form of a double reciprocal plot. The data are representative of five separate experiments. The family of lines intersect on the vertical axis. If these same data are plotted as $(\text{velocity})^{-1}$ versus $(Mn^{2+})^{-1}$ at various fixed concentrations of UDP-galactose, the lines intersect to the left of the vertical axis. Slopes of these lines plotted against $(\text{UDP-galactose})^{-1}$ are shown in Fig. 2. The intersection of the lines on the vertical axis in Fig. 1 and the fact that the plot of the slopes pass through the origin in Fig. 2 are indicative of an equilibrium-ordered mechanism for the addition of Mn^{2+} which is consistent with the results obtained by Morrison and Ebner (1) and inconsistent with the results obtained previously by Powell and Brew (2).

An explanation for these differing results appears to reside in the purity of the $MnCl_2$. Fig. 3 illustrates the effect of the concentration of UDP-galac-

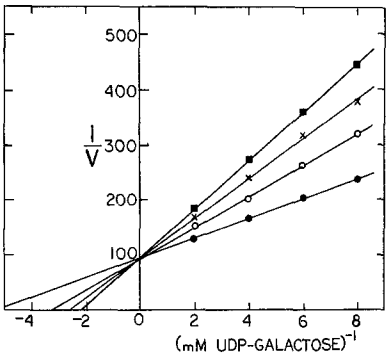

Figure 1. Effect of UDP-galactose on the initial velocity of the reaction at different fixed concentrations of purified $MnCl_2$ and with N-acetylglucosamine held constant at a concentration of 10.0 mM. The concentrations of $MnCl_2$ were: ●, 1.0 mM; O, 0.50 mM; ×, 0.33 mM; ■, 0.25 mM. Velocities are expressed as μmoles of product per minute.

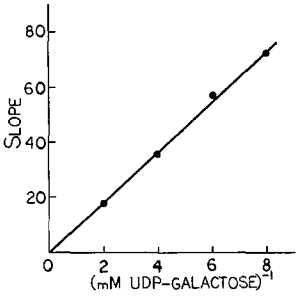

Figure 2. A secondary plot of slopes of lines obtained by replotting the data of Fig. 1 with Mn^{2+} as the variable substrate and UDP-galactose as the fixed variable substrate against UDP-galactose concentration.

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tose on the initial velocity of the reaction at various fixed concentrations of analytical reagent $MnCl_2$ and a fixed concentration of N-acetylglucosamine. The lines intersect to the left of the vertical axis which is similar to the results reported by Powell and Brew (2). Since diphenythiocarbazone reacts with Pb^{2+} (6) and lead was listed by the supplier (Mallinckrodt) of the analytical reagent $MnCl_2$ as a possible contaminant, the effect of trace amounts of $PbCl_2$ on initial velocity was examined. Addition of 0.001% (M/M) $PbCl_2$ to purified $MnCl_2$ produced a result (Fig. 4) similar to that obtained with un-

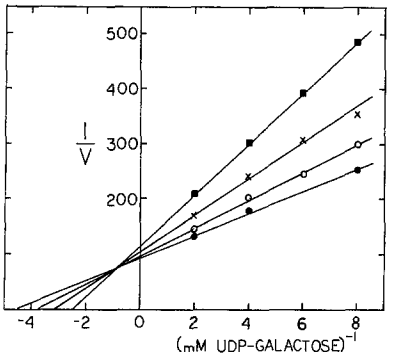

Figure 3. Effect of UDP-galactose on the initial velocity of the reaction at different fixed concentrations of analytical reagent $MnCl_2$ and with N-acetylglucosamine held constant at a concentration of 10.0 mM. The concentrations of $MnCl_2$ were: ● , 1.0 mM; O , 0.50 mM; X , 0.33 mM; ■ , 0.25 mM.

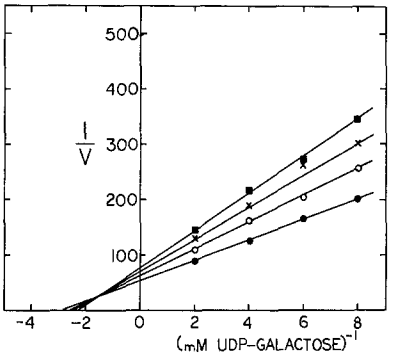

Figure 4. Effect of UDP-galactose on the initial velocity of the reaction at different fixed concentrations of purified $MnCl_2$ containing 0.001% $PbCl_2$ (M/M) and with N-acetylglucosamine held constant at a concentration of 10 mM. The concentrations of $MnCl_2$ were: ● , 1.0 mM; O , 0.50 mM; X , 0.33 mM; ■ , 0.25 mM.

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purified $MnCl_2$. Addition of 0.02% (M/M) $ZnCl_2$, the major contaminant of $MnCl_2$, to the purified $MnCl_2$ did not affect the intersection of the lines on the vertical axis.

DISCUSSION

The above results emphasize the importance and necessity of having very

well defined reagents for the investigation of kinetic parameters of an enzyme especially when contaminants such as heavy metals may be inhibitory. Galactosyltransferase has a very sensitive sulfhydryl group and is inhibited readily by sulfhydryl reagents (7). Diphenylthiocarbazone removes Pb^{2+} from $MnCl_2$ and thus the purified $MnCl_2$ showed no inhibition (Fig. 1) unless small quantities of $PbCl_2$ were added to the purified reagent (Fig. 4). Galactosyltransferase contains one free sulfhydryl and the amount of lead in the unpurified $MnCl_2$ is about 2-5 fold in excess of the enzyme in the assay. This inhibition would remove reactive enzyme from the reaction resulting in the observed shift of the intersection of lines to the left of the vertical axis (Fig. 3). Furthermore, most Mn^{2+} catalyzed enzymic reactions are of the types where Mn^{2+} is an integral part of the enzyme or Mn^{2+} is at thermodynamic equilibrium with the enzyme and does not dissociate at each turn of the catalytic cycle. Such mechanisms are consistent with the extremely low concentration (low μM range) manganese found in milk (8) and other body tissues.

The data presented confirm the equilibrium-ordered mechanism for bovine galactosyltransferase as proposed by Morrison and Ebner (1) and provide an explanation for the results obtained by Powell and Brew (2) and Khatra *et al.* (3) since these authors did not report purification of $MnCl_2$ prior to their extensive kinetic studies.

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