Martin, B. R., Rendell, M., and Berman, M. (1975), *Adv. Cyclic Nucleotide Res. 53* (in press).
Pfeuffer, T., and Helmreich, E. J. M. (1975), *J. Biol. Chem. 250,* 867.
Rendell, M., Salomon, Y., Lin, M. C., Rodbell, M., and Berman, M. (1975), *J. Biol. Chem. 250,* 4253.
Rodbell, M., Birnbaumer, L., Pohl, S. L. and Krans, H. M. J. (1971a), *J. Biol. Chem. 246,* 1877.
Rodbell, M., Krans, H. M. J., Pohl, S. L., and Birnbaumer, L. (1971b), *J. Biol. Chem. 246,* 1872.
Rodbell, M., Lin, M. C., and Salomon, Y. (1974), *J. Biol. Chem. 249,* 59.
Salomon, Y., Lin, M. C., Londos, C., Rendell, M., and Rodbell, M. (1975), *J. Biol. Chem. 250,* 4233.
Salomon, Y., Londos, C., and Rodbell, M. (1974), *Anal. Biochem. 58,* 541.
Schramm, M., and Rodbell, M. (1975), *J. Biol. Chem. 250,* 2232.
Spiegel, A. M., and Aurbach, G. D. (1974), *J. Biol. Chem. 249,* 7630.
Steer, M. L., Atlas, D., and Levitzki, A. (1975), *N. Engl. J. Med. 292,* 409.
Steer, M. L., and Levitzki, A. (1975a), *J. Biol. Chem. 250,* 2080.
Steer, M. L., and Levitzki, A. (1975b), *Arch. Biochem. Biophys. 167,* 371.

Affinity Labeling of Bovine Colostrum Galactosyltransferase with a Uridine 5′-Diphosphate Derivative†

Janet T. Powell and Keith Brew*,‡

ABSTRACT: The dialdehyde produced by the periodate cleavage of the ribose moiety of uridine 5′-diphosphate (UDP) has been used as an affinity label for the UDP-galactose/UDP binding site of galactosyltransferase from bovine colostrum. This derivative causes progressive inactivation of galactosyltransferase at a rate dependent on its concentration, and under certain conditions is a competitive inhibitor with respect to UDP-galactose. The substrate UDP-galactose protects the enzyme from inactivation. The inactivation is also dependent on Mn^{2+} concentration, in a range that implies that the binding of Mn^{2+} at site I is a prerequisite for the binding of the UDP derivative. The inactivation can be progressively reversed by nitrogenous bases, or stabilized by KBH_4 reduction, which is consistent with the hypothesis that a Schiff base has formed with a lysine residue. Galactosyltransferase was inactivated with a [^{3}H]UDP derivative and the predominant labeled peptide, from thermolysin digestion, isolated and characterized as: Ser-Gly-Lys-UDP

UDP-D-galactose–*N*-acetylglucosamine β-4-galactosyltransferase (EC 2.4.1.38), catalyzes two reactions of distinct physiological significance: (1) a step in the serial addition of monosaccharides during the biosynthesis of plasma-type glycoproteins: the transfer of galactose from UDP-galactose to glycoprotein bound GlcNAc[1] to form an *N*-acetyllactosamine moiety. Free GlcNAc is also an acceptor in this reaction. (2) The transfer of galactose from UDP-galactose to glucose to form lactose, a reaction for which, at physiological glucose levels, the regulatory protein α-lactalbumin is also required. Galactosyltransferase and α-lactalbumin together form the lactose synthase enzyme system (EC 2.4.1.22) that catalyzes the terminal, rate-limiting step in the biosynthesis of lactose in the lactating mammary gland. The various aspects of this complex enzyme have been reviewed recently (Brew and Hill, 1975; Hill and Brew, 1975).

Galactosyltransferase can be isolated as a soluble homogeneous glycoprotein, molecular weight 50 000, from bovine colostrum, (Powell and Brew, 1974), while an enzyme form isolated from bovine milk contains variable amounts of partial proteolytic degradation products (Barker et al., 1972; Magee et al., 1974; Powell and Brew, 1974). Galactosyltransferase in these secretions appears to originate from a membrane-bound enzyme form present in the golgi apparatus of the mammary gland, and it is used as a marker enzyme for the golgi membranes from the mammary gland and other tissues (Fleischer et al., 1969; Schachter et al., 1970). Divalent metal ions are essential for the activity of galactosyltransferase. While the enzyme was previously supposed to possess a single Mn^{2+} specific binding site (see Ebner, 1973, Brew and Hill, 1975; Hill and Brew, 1975) more recent studies have revealed the presence of two activating metal-binding sites. The first, designated site I can accept Mn^{2+} (dissociation constant of 2.3×10^{-6} M), and must be occupied before binding of UDP-galactose, regulatory protein or acceptor substrate can occur. The second site, designated site II, is a lower affinity site that can accept either Mn^{2+} or Ca^{2+} (dissociation constant of $1-2 \times 10^{-3}$ M) and has a specific kinetic interconnection with UDP-galactose, indicative of ordered equilibrium binding, but

† From the Department of Biochemistry, University of Miami School of Medicine, Miami, Florida 33152. *Received February 11, 1976.* This work was supported by a grant from the National Institutes of Health (GM 21363). A preliminary account of some of these studies has been previously reported (Powell and Brew, 1975b).

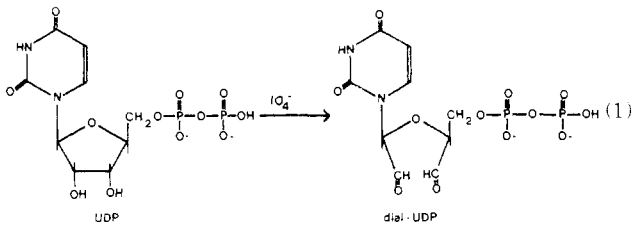
‡ Recipient of a NIH Research Career Development Award.

[1] Abbreviations used are: GlcNAc, *N*-acetylglucosamine; dial-UDP, the dialdehyde formed by periodate cleavage of the ribose moiety of UDP, as described in reaction 1; DNS, dansyl, 5-dimethylaminonaphthalene-1-sulfonyl; UDP, CDP, ADP, uridine, cytidine, and adenosine 5′-triphosphates; Tris, 2-amino-2-hydroxymethyl-1,3-propanediol; EDTA, (ethylenedinitrilo)tetraacetic acid; BSA, bovine serum albumin.

has no effect on the binding of acceptor or regulatory protein (Powell and Brew, 1976a,b).[2]

A multiplicity of binding sites are therefore present on galactosyltransferase: two metal binding sites, a site for UDP-derivatives, an acceptor binding site, (for GlcNAc, glucose, or glycoprotein) and a site for regulatory protein. Various types of functional interaction between these sites have been observed by kinetic and binding studies (see Powell and Brew, 1976b for discussion). Besides those previously mentioned are the potentiation of the binding of acceptor or regulatory protein by UDP derivatives, strongly synergistic binding of regulatory protein and monosaccharides and mutually exclusive binding of glycoproteins and regulatory protein.

As an approach to studying the topography of these binding sites and the structural basis of the catalytic activities of galactosyltransferase, we are seeking affinity labels for those binding sites that are accessible to such chemical probes. A suitable derivative for the UDP site has been found in a dialdehyde, dial-UDP, produced by periodate cleavage of UDP (reaction 1). Total inactivation of galactosyltransferase is



(1)

achieved by a 1:1 reaction between inhibitor and enzyme, through Schiff base formation between an aldehyde group and the ϵ-NH_2 group of a lysine in the UDP-binding site. Low concentrations of Mn^{2+} are required for inactivation, and the enzyme can be reactivated after complete inactivation with dial-UDP, by treatment with Tris buffer. UDP-galactose or high concentrations of Mn^{2+} greatly decrease both the rate and extent of inactivation. Inactivation is rendered irreversible by borohydride reduction of enzyme treated with dial-UDP, and the sequence of a small peptide containing the site of labeling is reported.

The affinity label has advantages in terms of ease of preparation, potential reversibility, and chemical nature that may render it and similar derivatives of other nucleoside phosphates of unique value in the study of nucleoside phosphate binding enzymes, including membrane-bound glycosyltransferases. Recently the use of a similar derivative of ATP to label the ATP binding site of pyruvate carboxylase has been reported (Easterbrook-Smith et al., 1976).

Materials and Methods

Materials

Bio-Gels P2 and P4, AG1X8 (200–400 mesh) and AG1X2 (−400 mesh) were purchased from Bio-Rad. UDP-galactose, UDP-glucose, UDP-*N*-acetylglucosamine, UDP, UMP, CDP, KBH_4, sodium cacodylate, and GlcNAc were from Sigma. UDP-[^{14}C]galactose and [5-^{3}H]UDP were purchased from Amersham-Searle. Other UDP derivatives were synthesized as described by Barker et al., 1972. Thermolysin was purchased from Calbiochem.

Bovine colostrum was supplied by the Department of Agriculture, University of Florida, Gainesville, and the galactosyltransferase was purified from the colostrum as described previously (Powell and Brew, 1974).

Methods

Preparation of Dial-UDP. Aqueous solutions of UDP (2–5 mM) were treated with equimolar quantities of $NaIO_4$ in the dark at room temperature (22 °C) for 30 min, at which time the absorbance at 223 nm had reached a maximum value. Galactose (0.2 mol/mol of UDP) was added to remove any unreacted periodate. Further studies have shown that this is unnecessary as galactosyltransferase is not affected by these concentrations of periodate and that all the periodate is consumed by the UDP before reaction with the enzyme. Treatment of UDP with excess periodate and back-titration showed that during the reaction 1.04 mol of periodate were consumed per mol of UDP.

Inactivation Experiments. Aliquots of dial-UDP solution were added to tubes containing galactosyltransferase in cacodylate buffer (0.05–0.1 M), pH 7.4, containing 0.1% bovine serum albumin to enhance enzyme stability (Khatra et al. 1974) and variable concentrations of Mn^{2+}, GlcNAc, and UDP-galactose. Controls containing separately UDP, EDTA, $NaIO_4$, or no additions were used in some experiments. A proportion of galactosyltransferase activity (about 10%) was lost during longer incubations (1 h) of the colostral enzyme. Incubations were carried out for various time intervals in the dark at 22 °C, the precise details for each experiment being given in the appropriate figure and table legends. The time course of inactivation or activation was followed by removing aliquots and assaying immediately. Assays of *N*-acetyllactosamine synthase activity were performed as described previously (Khatra et al., 1974; Powell and Brew, 1974) for time intervals of 10 to 15 min at 37 °C. The concentration of Mn^{2+} used in the assays (10 mM), together with the dilution of the reaction mixture, would be expected to prevent further inactivation during the assay period (see Figure 3).

The procedures used to prepare and to test the dial-derivatives of other nucleotides were similar.

Borohydride Reduction. Galactosyltransferase, inactivated by treatment with dial-UDP to less than 0.6% of the original activity was reduced with 2.5 equivalents of KBH_4/mol of UDP derivative (generally 2.5 mM KBH_4, 1 mM dial-UDP) in 0.2 M cacodylate buffer, pH 7.4, for 2 h. As a control, enzyme not treated with dial-UDP was incubated with the same concentration of KBH_4. Excess reagents were removed, when required, by dialysis against 50 mM cacodylate buffer, pH 7.4.

Peptide Separation. Peptides from a thermolysin digest of labeled enzyme were initially fractionated by gel filtration with a column of Bio-Gel P4 (2.5 × 90 cm) equilibrated with 0.1 M ammonium bicarbonate at room temperature. Peptide fractions (4 ml), pooled as indicated (Figure 5a) were freeze-dried. The main labeled peptide (Th-2) was dissolved in 1 mM HCl (3 ml) and applied to a column (1 ml) of AG1X2 (Cl^-). After washing with 1 mM HCl (5 ml), stepwise elution was performed with 2 × 1 ml aliquots of 0.1, 0.2, and 0.4 M LiCl in 1 mM HCl. A small proportion of the radioactivity was eluted with the 0.2 M LiCl, but most (90%) with the 0.4 M LiCl. This main fraction was desalted with a column of Bio-Gel P2 (1 × 25 cm) in 0.1 M ammonium bicarbonate, and freeze-dried. As it was still clearly heterogeneous, this fraction

[2] The two metal binding sites described by Powell and Brew (1976a) are distinct in their binding constants from those proposed by Berliner and Wong (1975). These workers used the heterogeneous galactosyltransferase from bovine milk, and the functional significance of their metal binding sites remains to be elucidated.

TABLE I: Dissociation Constants of Some Competitive Inhibitors of UDP-galactose for Bovine Colostrum Galactosyltransferase.[a]

	K_i (μM)[b]
UDP	32
UDP-glucose	65
UDP-*N*-acetylglucosamine	640
UDP-$(CH_2)_2NH_2$	270
UDP-$(CH_2)_2NHCOCF_3$	490
UDP-$(CH_2)_6NH_2$	220
Dial-UDP	194
UMP	450
CDP	350

[a] The dissociation constants were determined from steady-state kinetic data (Powell and Brew, 1974). [b] The K_i for UDP-galactose, determined kinetically, is zero.

was dissolved in 1 mM HCl (3 ml) and applied to a column of AG1X2 (1 × 6 cm, Cl^- cycle). Elution was performed with a linear gradient composed from equal volumes (50 ml) of 1 mM HCl and 0.4 M LiCl in 1 mM HCl, (Figure 5b). The pooled radioactive peptides, Th-2a and Th-2b, were again desalted with a column of Bio-Gel P2.

Peptide Characterization. For amino acid analysis, samples (about 1 nmol) were hydrolyzed with 6 M HCl containing 1% phenol at 110 °C in vacuo for 24 h, and analyzed with a Durrum D 500 amino acid analyzer. The spectra of peptides were determined with a Cary 118 spectrophotometer and were found to be dominated by the absorption spectrum of the uracil ring. Sequences were determined by the dansyl chloride–Edman degradation procedure as described previously (Findlay and Brew, 1972).

Experimental Results

Reversible Inhibition of Galactosyltransferase. Uridine 5′-diphosphate derivatives are, in general, good reversible inhibitors of galactosyltransferase, being competitive in nature with respect to UDP-galactose. The K_i values (dissociation constants of inhibitor from E-$Mn^{2+}_{I,II}$-inhibitor complexes) of a number of such compounds are given in Table I, together with that for CDP. It can be deduced from these values that the phosphate groups are important for binding and that modification of the uracil ring has a weakening effect on binding. The presence of a more bulky moiety than galactose also decreases binding, for example, UDP-*N*-acetylglucosamine is a tenfold poorer inhibitor than UDP-glucose. For this reason, phosphate-substituted and pyrimidine-ring-substituted compounds would appear to be poor candidates as labels for galactosyltransferase, as well as, in the former case, being possible labels of groups distant from the UDP binding site. Some inactivation was achieved using a diazo derivative prepared from CDP, but maximum inactivation levels of only 50% were achieved (J. T. Powell and K. Brew, unpublished results).

Inactivation by Dial-UDP. Incubation of galactosyltransferase with dial-UDP (0.125–1 mM) results in a progressive loss of enzyme activity, at a rate dependent on the concentration of the reagent (Figure 1). After longer incubations (1 h) with 1 mM dial-UDP-*N*-acetyllactosamine synthase activity was not detectable by our assay procedure. If the extent of inactivation is plotted on a logarithmic scale, only the initial phase of the inactivation is found to be first order.

The presence of the substrate UDP-galactose decreases the

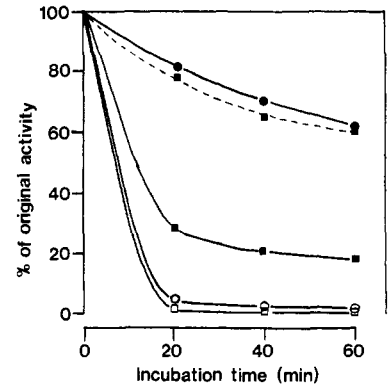


FIGURE 1: The time course of the inactivation of galactosyltransferase by different concentrations of dial-UDP. Galactosyltransferase (2 μg/ml) was incubated at 22 °C, in the dark, with the following concentrations of dial-UDP: (●) 0.12 mM, (■) 0.25 mM, (○) 0.5 mM, (□) 1.0 mM in the presence of 50 μM $MnCl_2$, 0.1 M cacodylate buffer, pH 7.4, 5 mM GlcNAc, and 0.1% BSA. The broken line (-■- - -■-) shows the protection offered by 0.25 mM UDP-galactose at 0.25 mM dial-UDP. Aliquots (20 μl) were removed at intervals and assayed for *N*-acetyllactosamine synthase activity.

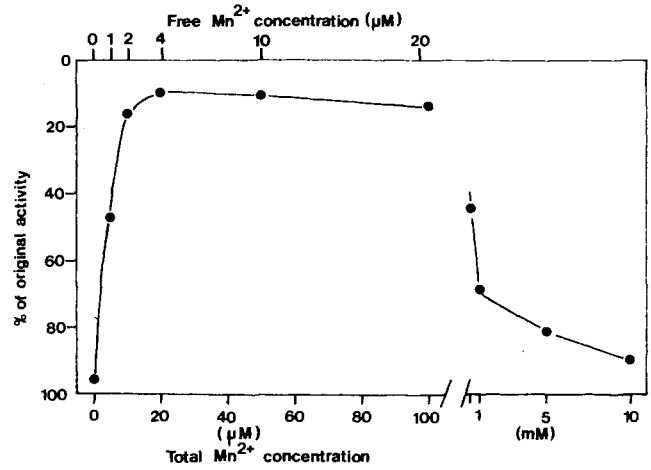


FIGURE 2: The effect of Mn^{2+} concentration on the inactivation of galactosyltransferase by dial-UDP (0.5 mM), in the presence of 25 mM GlcNAc, 0.1 M cacodylate buffer, pH 7.4, 0.01% BSA, and different concentrations of $MnCl_2$ for 15 min. Assays were commenced by the addition of a mixture of $MnCl_2$ (to 20 mM) and UDP-galactose (to 0.3 mM). The lower scale shows total Mn^{2+} and the upper one free Mn^{2+}; the galactosyltransferase had been treated with EDTA to remove any traces of Mn^{2+}.

rate of inactivation in a manner that resembles the effect of lowering the concentration of dial-UDP (Figure 1).

Galactosyltransferase, prepared from bovine colostrum by our procedure, contains a low intrinsic level of Mn^{2+}, which can be removed on treatment with EDTA (0.2 M). A low concentration of Mn^{2+} is required for inactivation, as EDTA prevents inactivation (data not shown). On the other hand, high concentrations (10 mM) of divalent metal ions, Mn^{2+} and Ca^{2+}, markedly reduce the inactivation (data for Mg^{2+} and Ca^{2+} not shown). As recent studies have shown that the metal ion activation of galactosyltransferase is complex and involves two metal binding sites on the enzyme (Powell and Brew, 1976a), the dependance of inactivation by dial-UDP on the concentration of Mn^{2+} might be expected to be relevant to the functional effects of these sites. The dependance of the inactivation by dial-UDP (0.5 mM) on the total Mn^{2+} concentration is shown in Figure 2. The inactivation rises rapidly at low Mn^{2+} concentrations (<20 μM), reaches a maximum at

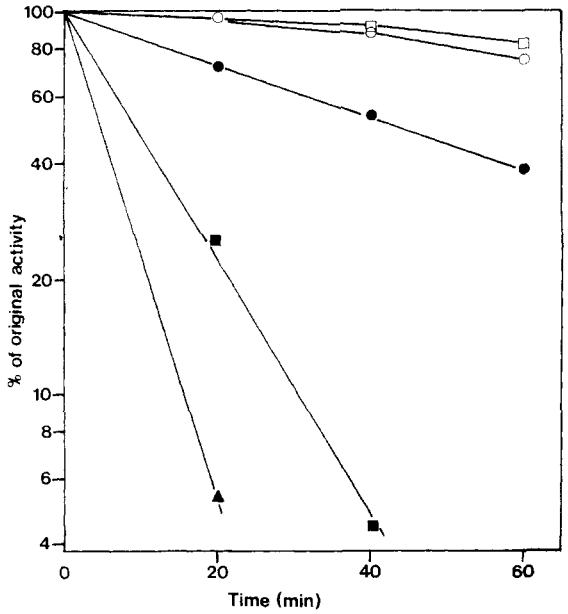


FIGURE 3: A comparison of the rates of inactivation by different dial-UDP derivatives. In each case the concentration of UDP, or dial-UDP derivative, is 2 mM, other conditions are as for Figure 1; UDP alone; (○) dial-UDP-*N*-acetylglucosamine, (●) dial-UMP, (■) dial-UDP, (▲) dial-UDP-galactose.

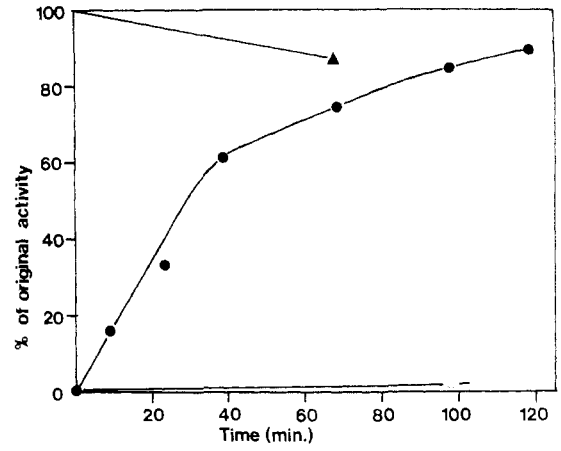


FIGURE 4: Reactivation of galactosyltransferase, inactivated by dial-UDP, with Tris buffer. Galactosyltransferase was inactivated with dial-UDP (1.0 mM), at 22 °C in the dark, in the presence of 0.1% BSA, 0.1 M cacodylate buffer, pH 7.4, 5 mM GlcNAc, and 50 μM $MnCl_2$ for 1 h (0.8% of original enzyme activity, time 0 min on graph). Aliquots (20 μl) were then incubated with 0.5 M Tris buffer, pH 7.4; the time course of reactivation is given by the filled circles, the open circle shows the activity for an aliquot after reduction with KBH_4, and the filled triangle shows enzyme activity in the presence of KBH_4 (2 mM) alone.

20 μM and decreases only slightly up to 100 μM. At higher concentrations of Mn^{2+}, the inactivation falls off, reaching a low level at 10 mM Mn^{2+}. The interpretation of this study is complicated by the binding of Mn^{2+} to the nucleoside diphosphate. ADP and other diphosphates bind Mn^{2+} with an association constant of 8750 M^{-1} under similar conditions to those used here (Bock, 1960). Assuming that the same association constant applies to the binding of Mn^{2+} with dial-UDP, the concentration of free Mn^{2+} can be calculated, which is shown on the upper scale in Figure 3. Below 20 μM Mn^{2+}, the concentration of free dial-UDP will be affected only slightly by complex formation, but the decreased inactivation above 100 μM Mn^{2+} (total) can be accounted for by the conversion of dial-UDP to its Mn complex, if this complex is ineffective in the inactivation process. The Mn^{2+} dependance of the inactivation at low metal ion concentrations is consistent with the binding of Mn^{2+} to galactosyltransferase with a K_d of 1 to 2 μM to produce an enzyme form to which free dial-UDP can bind. This is in close agreement with the properties previously deduced from kinetics for metal binding at site I (K_d of 2.3 μM), occupation of which is required for the binding of any substrate to galactosyltransferase.

Specificity for the UDP-Galactose Binding Site. A steady-state kinetic study in which the Mn^{2+} complex of dial-UDP was utilized as a reversible inhibitor of galactosyltransferase, by assaying shortly after the addition of inhibitor showed that the Mn^{2+} complex is a linear competitive inhibitor with respect to UDP-galactose. The K_i (194 μM) is sixfold higher than the corresponding value for MnUDP (see Table I).

In Figure 3 the rates of inactivation of galactosyltransferase by the dialdehydes generated from various nucleotide derivatives are compared. The effectiveness of the derivatives decreases in the order UDP-galactose, UDP, UMP, UDP-*N*-acetylglucosamine, which correlates well with the inhibition constants of the parent compounds as reversible inhibitors of galactosyltransferase (Table I).

Characteristics of Inactivated Enzyme. After incubation of galactosyltransferase with dial-UDP (1 mM) for 60 min to produce complete inactivation, the addition of 0.5 M Tris buffer, pH 7.4, or imidazole buffer, caused a progressive reactivation (Figure 4). Prior treatment with KBH_4 (2.5-fold excess over dial-UDP) prevents reactivation, while KBH_4 alone causes only slight inactivation of galactosyltransferase (13%) over 1 h. (see Figure 4).

An investigation of the effect of Tris concentration on the extent of reactivation over a fixed time interval (1 h), showed that little increase in reactivation is obtained between 0.3 and 0.5 M. Imidazole buffer also catalyzes the reactivation of labeled galactosyltransferase, at rates greater than those obtained with Tris (data not shown), but this effect is complicated by the fact that imidazole appears to have an activating effect on galactosyltransferase. After inactivation, galactosyltransferase can be separated from excess dial-UDP by dialysis against cacodylate buffer, pH 7.4 (25 mM), or by absorption onto a small column of α-lactalbumin–Sepharose in the presence of GlcNAc (20 mM) and elution, after washing with buffer containing GlcNAc, with buffer devoid of monosaccharide. During dialysis the enzyme remained almost completely inactive (less than 3% of the original activity), and could be reactivated with Tris buffer (to a maximum of 84%). Slight reactivation (15%) occurred during reisolation on α-lactalbumin–Sepharose.

Stoichiometry and Site of Labeling. Galactosyltransferase (6.25 mg, 125 nmol) in 0.1 M cacodylate buffer, pH 7.4 (25 ml), containing 25 μM $MnCl_2$ and 1 mM mercaptoethanol was reacted with 15.6 μmol of dial-[^{3}H]UDP (20×10^3 counts min^{-1} $nmol^{-1}$) in the dark at 20 °C. *N*-Acetyllactosamine synthase activity was measured at hourly intervals. After 3 h the activity had decreased to 0.6% of the starting level. KBH_4 (2.5 mg, 50 μmol) was added and the solution was left for a further 2 h. The irreversibly inactivated protein was dialyzed against dilute cacodylate buffer (20 mM), (2×1 l.) and then against distilled water (4×1 l.), over the course of two days. After this time no further radioactivity was detectable in the dialysate. The protein was then freeze-dried, and the ^{3}H content was determined. A level of radioactivity corresponding

to the incorporation of 113 nmol of UDP was found, which gives a stoichiometry of 0.91 mol of bound UDP/mol of protein, based on a protein molecular weight of 50 000.

The labeled protein was reduced with β-mercaptoethanol (0.1 M) in urea (8 M), containing Tris buffer (0.2 M), pH 8.5, and the cysteinyl residues converted to aminoethylcysteines by reaction with ethyleneimine. Following exhaustive dialysis against distilled water, the protein was freeze-dried, dissolved in a buffer (4 ml) containing cacodylate (50 mM, pH 7.4) and calcium chloride (2 mM), and digested with thermolysin (150 μg) at 45 °C for 2 h. The digest was fractionated by gel filtration with a column of Bio-Gel P4 (Figure 5a), labeled peptides being detected by their ^3H content. The main ^3H-labeled peak (Th 2) was refractionated by ion-exchange chromatography with AG1X2, using stepwise and gradient elution, as described in methods. The major peaks from the final gradient separation (Figure 5b) were desalted with Bio-Gel P2 and subjected to amino acid analysis. Their compositions were found to be: Th 2a $(Asp_{0.50})Ser_{0.93}(Glu_{0.73})$-$Gly_{1.04}(UDP)_{1.00}$; Th 2b $Ser_{0.97}(Glu_{0.45})Gly_{1.00}(UDP)_{1.00}$ in yields of 26 and 45 nmol, respectively (21%, 36%). The molar ratios are based on the UDP content, and amino acids given in parentheses are assumed to be contaminants.

As Th-2b was the less contaminated, it was subjected to the sequence analysis by the dansyl-Edman degradation procedure, with the result:

$$\overrightarrow{\text{Ser}}\text{-}\overrightarrow{\text{Gly}}\text{-}\overrightarrow{\text{X}}$$

where DNS-X cochromatographed with DNS-Arg and DNS-His in solvent I, but moved ahead of both derivatives in solvents III and IV. The characteristics of the affinity label suggest that the third residue might be a modified lysine, produced by reduction of a Schiff base formed between the ϵ-NH_2 group and an aldehyde group on the dial-UDP. From such a derivative ϵ-*N*-alkylsubstituted lysines and other derivatives could be formed on acid hydrolysis, when the ribose ring is cleaved. The α-DNS derivative, on acid hydrolysis, might be expected to have chromatographic properties more similar to DNS-Arg than to bis(DNS-Lys). Unfortunately, more definitive evidence for the identification of the third residue as lysine is not presently available, as galactosyltransferase is obtained in only small amounts, insufficient for the full chemical characterization of the third residue. On the basis of the characteristics of the affinity label, the third residue is tentatively identified as a modified lysine, giving a sequence: Ser-Gly-Lys-UDP.

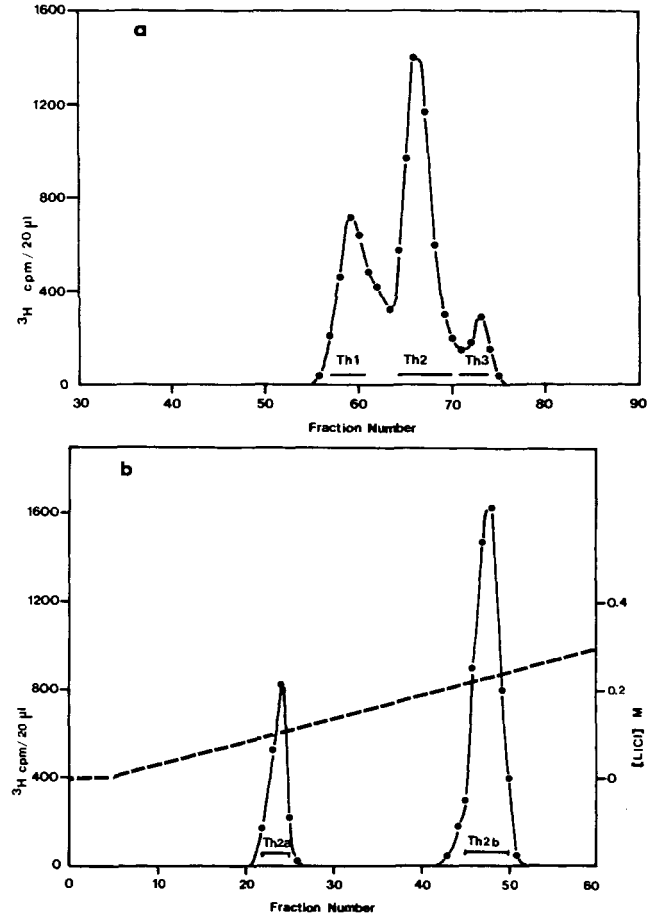


FIGURE 5: The separation of dial-UDP labeled peptides from inactivated galactosyltransferase. (a) Fractionation of the thermolysin digest of inactivated galactosyltransferase on Bio-Gel P4 (2.5 × 90 cm). Peptides were eluted with 0.1 M ammonium bicarbonate and pooled as shown; fraction size was 4 ml. (b) Fractionation of Th 2 on AG 1X2 (1 × 6 cm), eluted with a linear gradient of LiCl (0–0.4 M) in HCl (1 mM), total volume 100 ml; the fraction size was 1.25 ml.

Discussion

Dial-UDP can be classed as an affinity label of bovine galactosyltransferase by several generally accepted criteria (see Singer, 1967; Shaw, 1970). The reagent produces progressive inactivation of the enzyme at a rate dependent on its concentration. Under appropriate conditions, complete inactivation is achieved, which, although it is not in general a necessary criterion for a site-specific label, is to be expected in the present case where the binding site for an essential substrate is being blocked. The labeling is progressively reversed by incubation with Tris buffer, but the labeled complex is sufficiently stable to remain inactive during prolonged dialysis, while reduction with borohydride renders the labeled enzyme stable to Tris, as well as to urea (8 M). Although we have not obtained enough material to satisfactorily characterize the product of the labeling, the circumstantial evidence cited above, together with the similar behavior of pyridoxal phosphate as an affinity label (Fischer et al., 1958) indicates that an aldehyde group on dial-UDP is forming a Schiff base with an amino group on the protein. Such a derivative will break down on treatment with Tris and other nitrogenous bases, and be stabilized by borohydride reduction.

The inactive, borohydride reduced enzyme contained 0.91 mol of UDP/mol of protein, indicating that inactivation results from a 1:1 binding of dial-UDP with galactosyltransferase. This is consistent with equilibrium dialysis studies that have shown the presence of a single UDP-galactose binding site on the galactosyltransferase (Powell and Brew, 1975a). The specificity of the label for part of the UDP-galactose binding site is supported by a number of observations: (1) the behavior of uridine 5′-phosphate derivatives as competitive inhibitors with respect to UDP-galactose, including dial-UDP under conditions where it is acting essentially as a reversible inhibitor (10 mM Mn^{2+}, short-time intervals); (2) inactivation by the dial-derivatives of other uridine compounds at rates depending on the effectiveness of the parent compounds as inhibitors of galactosyltransferase (Figure 3 and Table 1); (3) the reduction of the rate of inactivation in the presence of the substrate UDP-galactose; (4) the Mn^{2+} dependance of the inactivation, which suggests that the binding of dial-UDP to galactosyltransferase depends on the previous attachment of Mn^{2+} to a binding site with a dissociation constant in the micromolar

concentration range. A comparison with previous kinetic and binding studies shows that this must be the metal activation site 1, occupation of which is an essential prerequisite for the binding of substrates to the enzyme (Powell and Brew, 1976a,b). The attachment of a UDP derivative to enzyme molecules in which site II is not occupied suggests that the binding of metal ion at site II and of UDP-galactose may be random and synergistic rather than ordered. This can only be clarified by further binding studies.

The protection achieved with 10 mM concentrations of Mn^{2+}, Mg^{2+}, or Ca^{2+} cannot be attributed specifically to the effects of metal binding to site II on galactosyltransferase, as 10 mM Mg^{2+} neither activates nor inhibits the enzyme (Powell and Brew, 1976a). This protection may reflect the binding of metal ion to UDP, if the M^{2+} UDP complexes are unable for steric reasons to react with the appropriate amino group, or could be attributed to the catalysis by the metal ions of the breakdown of the Schiff base.

Although the peptide of sequence Ser-Gly-Lys(UDP)(Th 2b) isolated from the thermolysin digest of the UDP-labeled protein contained only 36% of the label originally incorporated, it can be argued that this represents the major and perhaps only site of labeling in the primary structure of galactosyltransferase. The peptide pool from the original Bio-Gel P4 separation of the thermolysin digest from which it was purified (Th 2) contained about 70% of the original label, the remainder being present in the minor peaks Th-1 and Th-3, which may be different proteolysis products from the same region of sequence. Reseparation of Th 2 by ion-exchange chromatography with AG 1 X 2 by stepwise elution and gradient elution (to give Th-2a and Th-2b) leads to splitting each time into minor components of which only the major peptide Th-2b was used for sequence analysis. However, the peptide Th-2a had a similar amino acid composition to Th-2b, with a slightly higher level of contaminants, suggesting that the labeled components separated by ion-exchange chromatography may contain the same tripeptide with a partially dephosphorylated label. Inclusion of Th-2a in the estimate of yield will bring it to 58% which, given the losses expected for peptide separation by ion exchange, is consistent with this representing the only site of labeling. The residue of lysine is not identified directly, no amino acid being detectable in the basic region in analyses of acid hydrolysates of the peptide. However, the characteristics of labeling by the derivative point clearly to Schiff base formation with an amino group, and the isolation of the label in a peptide with a free α-NH_2 group excludes the α-amino terminus of the protein as the site of labeling, which must therefore involve the ϵ-NH_2 group of a lysine. The presence of an amino group in the UDP binding site is not surprising, as electrostatic interactions between a phosphate on the substrate and such a basic group on the protein would help to explain the tight binding of most UDP derivatives by galactosyltransferase. The other two amino acids in the labeled tripeptide, serine and glycine, have minimal side chains, and it is interesting to speculate that they may form part of an area on the enzyme surface where the bulky pyrimidine ring is accommodated. The seryl side chain hydroxyl group could possibly form a hydrogen bond with a carbonyl group on the uracil ring. Model building studies have shown that such an arrangement is possible. Studies are currently in progress aimed at determining a larger region of sequence around the labeled lysine and at developing UDP derivatives with reactive groups in different positions.

The presence of aldehyde groups together with phosphate on the affinity label renders it chemically similar to pyridoxal phosphate, which has been used extensively in the affinity labeling of enzymes, but the stereochemistry of the UDP-derivative is completely different. As potential affinity labels for nucleoside mono-, di- and triphosphate binding enzymes, the dial-nucleoside phosphates have considerable strategic advantages. Thus, there is a reasonable chance that an amino group may be present in the binding sites for the purposes of electrostatic stabilization and that will give a site for reaction with the aldehyde. In addition, there is a tendency for enzymes to have lower specificities for the ribose ring over other parts of the substrate (for example, see Yount, 1975) and modification of this part of the molecule is less likely to hinder binding than would modification of the base or phosphates. The presence of two aldehyde groups gives a large scope for reaction of the derivative with groups in the binding site. Therefore, comparative studies of the nucleotide binding sites of different enzymes with similar dial-derivatives are possible. The ease of preparation of the derivatives by periodate cleavage is a considerable advantage, and the procedure we describe could possibly be used for immobilizing nucleotides with reporter groups on the surface of enzymes.

The regeneration of active enzymes after inactivation with dial-derivatives offers the additional possibility of labeling a specific enzyme in a complex mixture, such as a membrane, and using the label to locate the enzyme during separation. Provided nitrogenous bases and heavy metals are excluded during the separation, the label should remain attached, but could be removed with concomitant reactivation, following separation. Preliminary studies have shown that dial-[^{3}H]UDP can be used to label specific proteins in golgi membranes isolated from rat liver and lactating guinea pig mammary gland. Dial-UDP could therefore possibly be used to study the orientation of galactosyltransferase and other glycosyltransferases in biomembranes. This is currently under investigation.

References

Barker, R., Olsen, K., Shaper, J. H., and Hill, R. L. (1972), *J. Biol. Chem. 247,* 7135–7147.

Berliner, L. J., and Wong, S. S. (1975), *Biochemistry 14,* 4977–4982.

Bock, R. M. (1960), *Enzymes 2nd Ed. 2,* 3–38.

Brew, K., and Hill, R. L. (1975) *Rev. Physiol. Biochem. Exp. Pharmacol. 72,* 105–108.

Easterbrook-Smith, S. B., Wallace, J. C., and Keech, D. B. (1976), *Eur. J. Biochem. 62,* 125–130.

Ebner, K. E. (1973), *Enzymes 3rd Ed. 9* B, 368–377.

Findlay, J. B. C., and Brew, K. (1972), *Eur. J. Biochem. 27,* 65–86.

Fischer, E. H., Kent, A. B., Snyder, E. R., and Krebs, E. G. (1958), *J. Am. Chem. Soc. 30,* 1180–1186.

Fleischer, B., Fleischer, S., and Ozawa, H. (1969), *J. Cell Biol. 43,* 59–79.

Hill, R. L., and Brew, K. (1975), *Adv. Enzymol. 43,* 411–490.

Khatra, B. S., Herries, D. G., and Brew, K. (1974), *Eur. J. Biochem. 44,* 537–560.

Magee, S. E., Mawal, R., and Ebner, K. E. (1974), *Biochemistry 13,* 99–102.

Powell, J. T., and Brew, K. (1974), *Eur. J. Biochem. 48,* 217–228.

Powell, J. T., and Brew, K. (1975a), *J. Biol. Chem. 250,* 6337–6343.

Powell, J. T., and Brew, K. (1975b), *Trans. Biochem. Soc. London* (in press).

Powell, J. T., and Brew, K. (1976a), *J. Biol. Chem.* (in

press).
Powell, J. T., and Brew, K. (1976b), *J. Biol. Chem.* (in press).
Schachter, H., Jabbal, I., Hudgin, R. L., and Pinteric, L. (1970), *J. Biol. Chem. 245,* 1090-1100.
Shaw, E. (1970), *Enzymes, 3rd Ed. 1,* 99-148.
Singer, S. J. (1967), *Adv. Protein Chem. 22,* 1-54.
Yount, R. G. (1975), *Adv. Enzymol. 43,* 1-56

Essential Arginyl Residues in Fructose-1,6-bisphosphatase†

Frank Marcus

ABSTRACT: Modification of pig kidney fructose-1,6-bisphosphatase with 2,3-butanedione in borate buffer (pH 7.8) leads to the loss of the activation of the enzyme by monovalent cations, as well as to the loss of allosteric adenosine 5′-monophosphate (AMP) inhibition. In agreement with the results obtained for the butanedione modification of arginyl residues in other enzymes, the effects of modification can be reversed upon removal of excess butanedione and borate. Significant protection to the loss of K^+ activation was afforded by the presence of the substrate fructose 1,6-bisphosphate, whereas AMP preferentially protected against the loss of AMP inhibition. The combination of both fructose 1,6-bisphosphate and AMP fully protected against the changes in enzyme properties on butanedione treatment. Under the latter conditions, one arginyl residue per mole of enzyme subunit was modified, whereas three arginyl residues were modified by butanedione under conditions leading to the loss of both potassium activation and AMP inhibition. Thus, the modification of two arginyl residues per subunit would appear to be responsible for the change in enzyme properties. The present results, as well as those of a previous report on the subject (Marcus, F. (1975), *Biochemistry 14,* 3916-3921) support the conclusion that one arginyl residue per subunit is essential for monovalent cation activation, and another arginyl residue is essential for AMP inhibition. A likely role of the latter residue could be its involvement in the binding of the phosphate group of AMP.

Mammalian liver and kidney fructose-1,6-bisphosphatase (EC 3.1.3.11, D-fructose-1,6-bisphosphate 1-phosphohydrolase) are tetrameric enzymes composed of four presumably identical subunits with molecular weights of approximately 35 000 (Mendicino et al., 1972; Tashima et al., 1972; Traniello et al., 1972). Each enzyme subunit possesses at neutral pH a single substrate binding site (Pontremoli et al., 1968a; Sarngadharan et al., 1969; Kratowich and Mendicino, 1974), and a divalent metal ion binding site (Pontremoli et al., 1969). The enzyme is also activated by monovalent cations (Hubert et al., 1970). In addition, an allosteric site for the highly specific inhibitor AMP[1] is also present per enzyme subunit (Pontremoli et al., 1968b; Sarngadharan et al., 1969; Kratowich and Mendicino, 1974). Based on recent structural studies (Abrams et al., 1975), liver and kidney fructose-1,6-bisphosphatase appear to have similar primary structures.

From chemical modification studies it has been established that arginyl residues play an essential role in many enzymes by participating in the recognition of anionic ligands. Examples include the participation of enzyme arginyl residues at the binding sites of phosphorylated substrates (Daemen and Riordan, 1974; Borders and Riordan, 1975; Lobb et al., 1975; Powers and Riordan, 1975), pyridine nucleotides (Yang and Schwert, 1972; Lange et al., 1974; Bleile et al., 1975; Blumenthal and Smith, 1975), and carboxylic substrates (Riordan, 1973; Riordan and Scandurra, 1975; Werber et al., 1975). With this background, I initiated a study on the modification of pig kidney fructose-1,6-bisphosphatase with the arginine specific reagent, 2,3-butanedione. The existence of positively charged recognition sites on the enzyme appeared a priori likely, since both the substrate fructose 1,6-bisphosphate and the allosteric inhibitor AMP, are negatively charged. Indeed, an ϵ-amino group of lysine has already been identified at the C-6 phosphate binding site of the substrate by modification studies of pig kidney fructose-1,6-bisphosphatase with pyridoxal-P (Colombo and Marcus, 1974). Unexpectedly, the first results of modification of fructose-1,6-bisphosphatase with butanedione led to the recognition of highly reactive arginyl residues that are essential for the monovalent cation activation of the enzyme (Marcus, 1975). However, it was also noticed that significant losses of AMP inhibition can also occur upon modification. The latter finding prompted a continuation of the study of the modification of fructose-1,6-bisphosphatase by butanedione. The experiments reported herein demonstrate that arginyl residues are also essential for AMP inhibition of fructose-1,6-bisphosphatase.

Materials and Methods

Pig kidney fructose-1,6-bisphosphatase with optimal activity at neutral pH was purified as previously described (Colombo and Marcus, 1973). Its protein concentration was determined spectrophotometrically at 280 nm using the extinction coefficient $E_{1cm}^{1\%}$, of 7.55 (Marcus and Hubert, 1968). Based on a subunit molecular weight of 35 000 (Mendicino et al., 1972), the molecular weight of the enzyme tetramer was taken as 140 000 for all calculations. Rabbit liver fructose-1,6-bisphosphatase was partially purified as previously described (Marcus, 1975).

Fructose-1,6-bisphosphatase activity was measured as described (Marcus, 1975). The assays were carried out at 30 °C

† From the Institute for Enzyme Research, University of Wisconsin, Madison, Wisconsin 53706. *Received March 12, 1976.* This work was supported by National Institutes of Health Research Grant AM 10334 and by American Cancer Society Grant BC149.

[1] Abbreviations used are: AMP, adenosine 5′-monophosphate; EDTA, (ethylenedinitrilo)tetraacetic acid; NADP, nicotinamide adenine dinucleotide phosphate.