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Structure–Function Relationships in Lactose Synthase. Structural Requirements of the Uridine 5′-Diphosphate Galactose Binding Site†

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ABSTRACT: The structural requirements for the donor pyranosyl moiety of UDP-Gal in either galactosyl transfer or "lactose" biosynthesis have been determined. The 4″-deoxy analogue, UDP-4″-deoxyglucose, was synthesized and fully characterized as a donor substrate for galactosyltransferase. The relative rate of deoxyglucosyl transfer to glucose or GlcNAc acceptors was 5.5 ± 0.6% of that of UDP-Gal as the substrate, with K_m values in the same range as that for UDP-Gal or UDP-Glc. Several conclusions may be drawn as to the detailed structural requirements of the UDP-Gal binding site: an *axial* 4″-hydroxyl group on the pyranosyl moiety is necessary for precise substrate alignment as is also an *equatorial* 6″-CH_2OH moiety. Where one or the other moiety was lacking (UDP-dGlc or UDP-Arab), the maximal rate of glycosyl transfer was ca. $^1/_{20}$th that of UDP-Gal.

Galactosyltransferase (uridine diphosphate D-galactose:D-glucose 1-galactosyltransferase, EC 2.4.1.22) catalyzes the lactose synthase reaction

$$\text{UDP-Gal} + \text{Glc} \xrightarrow[\alpha\text{LA}]{\text{Mn(II)}} \text{UDP} + \text{lactose} \qquad (1)$$

where αLA[1] is the modifier protein α-lactalbumin. It also catalyzes a general glycosyl transferase reaction, not restricted to lactating mammary glands

$$\text{UDP-Gal} + \text{GlcNAc} \xrightarrow{\text{Mn(II)}} \text{UDP} + \text{LacNAc} \qquad (2)$$

where the acceptor, GlcNAc, is a (nonreducing end) residue in complex carbohydrate biosynthesis. The metal ion specificity is broad, although the optimal cation is Mn(II) which binds to two metal ion sites per galactosyltransferase molecule (O'Keefe et al., 1980; Andree & Berliner, 1980). Until Andree & Berliner (1978) first showed that UDP-Glc was an acceptable (yet marginally active) substrate in both reactions 1 and 2, UDP-Gal was believed to be the only (donor) substrate with the correct pyranosyl stereochemistry to bind properly for productive glycosyl transfer. It had also been ascertained that the uridylyl moiety was most important in binding UDP-Gal substrate and inhibitor analogues (Andree & Berliner, 1978; Berliner & Wong, 1975).

Since the small, yet observable activity of UDP-Glc vs. UDP-Gal (0.3% vs. 100%) suggested that an equatorial hydroxyl moiety in the 4″ position of the pyranosyl position was (barely) tolerable for productive binding, we have examined the consequences of the absence or presence (axial or equatorial) of hydroxyl substituents at this position.

Materials and Methods

UDP-Gal, UDP-Glc, uridine 5′-phosphomorpholidate, phosphoenolpyruvate, NADH, pyruvate kinase, and α-lactalbumin were from Sigma Chemical Co. Pyridine and *N*,*N*-dimethylformamide (reagent grade) were dried over CaH_2 and distilled immediately before use.

TLC was performed on Baker-Flex (IB-F) silica gel with UV or H_2SO_4 charring for visualization. Descending paper chromatography (Whatman No. 1) utilized periodate–$AgNO_3$ (Trevelyan et al., 1950) or phosphate spray (Hanes & Isherwood, 1949) for sugar and nucleotide visualization, respectively. The solvent systems used were (A) 7% (v/v) ethyl

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[1] Abbreviations: UDP-Gal, uridine 5′-diphosphate galactose; UDP-Glc, uridine 5′-diphosphate glucose; UDP-dGlc, uridine 5′-diphosphate 4″-deoxyglucose; UDP-Arab, uridine 5′-diphosphate L-arabinopyranose; αLA, α-lactalbumin; Glc, glucose; GlcNAc, *N*-acetylglucosamine; LacNAc, *N*-acetyllactosamine; dGlc, 4-deoxyglucose or 4-deoxyxylohexose; $R_{UDP\text{-}Gal}$ or R_{ref}, the R_f value relative to a UDP-Gal or reference standard; TLC, thin-layer chromatography.

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Table I: Paper Chromatographic Results of Various Galactosyltransferase Reactions[a]

compounds	R_{ref}	R_{GlcNAc}
standards		
4-deoxyxylohexose (dGlc)	0.59 ± 0.04	1.10 ± 0.04
glucose (Glc)	0.39 ± 0.05	0.71 ± 0.05
GlcNAc	0.55 ± 0.05	1.00 ± 0.05
galactose	0.41 ± 0.05	0.77 ± 0.05
lactose	0.31 ± 0.04	0.57 ± 0.04
LacNAc		0.65 ± 0.03[b]
glucose reaction		
UDP-dGlc + glucose (4-deoxylactose)	0.34 ± 0.03	0.62 ± 0.03
hydrolysate[c] from 4-deoxylactose	0.39 ± 0.03 0.59 ± 0.03	0.71 ± 0.03 1.10 ± 0.03
GlcNAc reaction		
UDP-dGlc + GlcNAc (4-deoxy-LacNAc)	0.42 ± 0.03	0.74 ± 0.03
hydrolysate[c] from 4-deoxy-LacNAc	0.59 ± 0.03 0.55 ± 0.03	1.10 ± 0.03 1.00 ± 0.03

[a] Conditions: Whatman No. 1 paper; descending in solvent E [1-butanol–pyridine–0.01 M sodium borate, 2:2:1 (v/v/v)]; visualization by $AgNO_3$. [b] Andree & Berliner (1978), for comparative purposes only. [c] Prepared by cutting out a nonstained duplicate spot of the reaction product and eluting from the paper into 3 M HCl for 1 h at 100 °C, followed by lyophilization and rechromatographing.

ether in benzene, (B) ethyl acetate–ethanol (5:2), (C) ethanol–0.5 M ammonium acetate (pH 3.2, 5:2), (D) ethanol–1.0 M ammonium acetate (pH 7.0, 5:2), and (E) 1-butanol–pyridine–0.01 M sodium borate (2:2:1).

Galactosyltransferase was purified from bovine milk to an average specific activity of 18–20 units/mg at 30 °C (Andree & Berliner, 1978). Enzyme kinetic measurements utilized the coupled spectrophotometric assay procedure of Fitzgerald et al. (1970) for reactions 1 or 2 above.

Proton, ^{13}C, and ^{31}P NMR spectra were measured on Bruker WP-200 and WM-300 spectrometers. IR and polarimetric measurements were made on Perkin-Elmer Models 1200 and 241, respectively.

Synthesis of Uridine 5'-Diphosphate 4''-Deoxy-α-D-xylo-hexopyranose (UDP-dGlc). The synthetic procedures, available as supplementary material (see paragraph at end of paper regarding supplementary material), were a modification of the syntheses of Reist et al. (1965), Kochetkov et al. (1970), Franchiomont (1879), MacDonald (1962), and Moffatt & Khorana (1958). NMR and mass spectral data are included.

Results

Evidence for 4-Deoxyglucose Transfer. The enzyme galactosyltransferase was found to utilize UDP-dGlc as a substrate in either of its two basic reactions. Galactosyltransferase (2 μM) was incubated with 237 μM UDP-dGlc, 16.5 mM GlcNAc, and 4 mM $MnCl_2$ or with 16.4 mM Glc plus 6 μM αLA instead of GlcNAc for 2 h at room temperature and 4 °C overnight. All charged materials were removed by passing the reaction mixture down an RG501-X8 mixed-bed column, the eluate lyophilized and concentrated for paper chromatography in solvent E. The resultant mono- and disaccharide products as well as several standards were visualized with $AgNO_3$ stain. Table I lists R_f values for several standards and the products of galactosyltransferase reactions. The disaccharide spots resulting from the two galactosyltransferase reactions 1 and 2 were subsequently hydrolyzed in 3 M HCl at 100 °C for 1 h and then rechromatographed. In both of the galactosyltransferase reactions 1 and 2, when UDP-dGlc was substituted for UDP-Gal, a new disaccharide product was formed which had a characteristically higher R_f value than

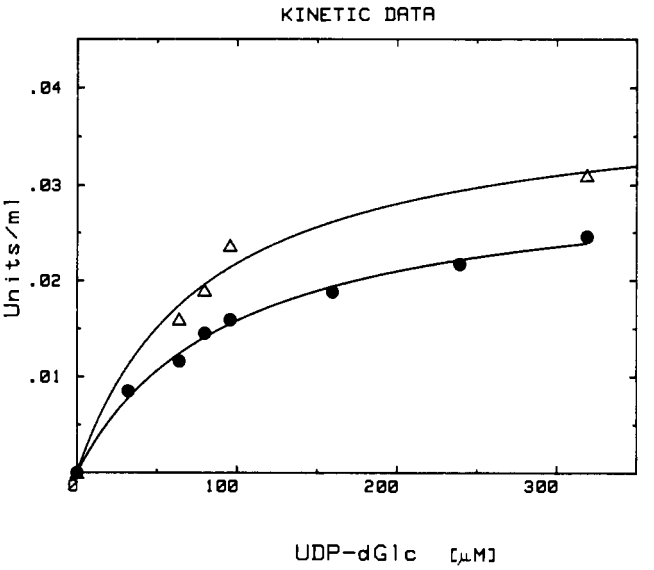

FIGURE 1: Velocity vs. substrate (UDP-dGlc) plots for the glucose reaction (1) (△) and GlcNAc reaction (2) (●), respectively. The solid curves are the theoretical (nonlinear regression) fits to the K_m (apparent) values noted in the text. The enzyme samples differed slightly in total units for the two reactions above, thus the noncoincidence at the highest substrate concentrations. Conditions were 4 mM $MnCl_2$, 0.1 M *N*-methylmorpholine–KCl, pH 7.4, 30 °C, and 15.8 mM Glc and 6.1 μM αLA or 15.8 mM GlcNAc for reactions 1 and 2, respectively.

Table II: Rate and Michaelis Parameters for Various Donor Substrates of Galactosyltransferase[a]

substrate	V_{max} (%)	K_m (μM)	structural differences from UDP-Gal
UDP-Gal	100	13.7[b]	
UDP-dGlc	5.5	26[c]	lacks 4''-OH (no axial substituent)
UDP-Arab	4.0	~14[d]	lacks 6''-CH_2OH moiety but contains an axial 4''-OH group
UDP-Glc	0.3[e]	23[f]	4''-OH is equatorial instead of axial

[a] Data shown above refer to reaction 2 where GlcNAc is the acceptor. [b] Bell et al. (1976). [c] Calculated from Bell et al. (1976) assuming Mn was not totally saturating; K_m for reaction 1 was 19.4 μM. [d] J. C. Paulsen and J. E. Bell, private communication of unpublished results. [e] Andree & Berliner (1978). [f] From thiol protection experiments (Magee & Ebner, 1974).

that of the corresponding galactosyl disaccharide (see lactose vs. 4-deoxylactose and LacNAc vs. 4-deoxy-LacNAc, respectively, in Table I) due to the somewhat more nonpolar nature of the 4-deoxyglucose moiety which has one less hydroxyl group. Furthermore, each new product, 4-deoxylactose or 4-deoxy-LacNAc, were both shown to contain 4-deoxyglucose and glucose or GlcNAc, respectively.

Kinetic Measurements with UDP-dGlc. Enzyme kinetic studies of both reactions 1 and 2 using UDP-dGlc as the substrate were measured at 30 °C, pH 7.4 (0.1 M *N*-methylmorpholine–KCl), at a fixed concentration of $MnCl_2$ and acceptors, Glc–α-lactalbumin or GlcNAc, in each case, respectively. Rate saturation plots are shown in Figure 1. For both reactions 1 and 2 the V_{max} with UDP-dGlc as the donor substrate was 5.5 ± 0.6% of that for UDP-Gal under the same conditions. The apparent Michaelis constants under these conditions for UDP-dGlc were 78 ± 14 μM and 95 ± 20 μM for reactions 1 and 2, respectively. These data, when fit to the kinetic model of Bell et al. (1976), yielded true K_m values, which are listed in Table II.

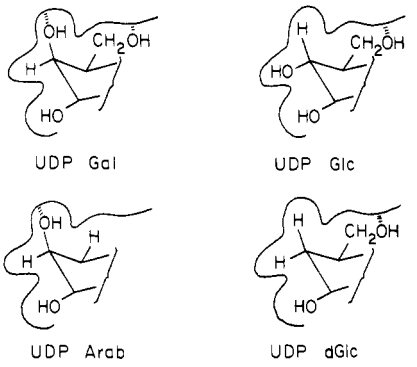

FIGURE 2: Binding model for various pyranosyl moieties in the UDP-Gal binding site of galactosyltransferase. The binding locus near the 4″-pyranosyl position exactly fits a galactosyl moiety (UDP-Gal) with a possible hydrogen bond to its axial hydroxyl group for correct alignment. In contrast, those pyranosyl moieties which lack a axial 4″-hydroxyl group (UDP-dGlc, UDP-Glc) are poorer substrates due to a "wobbly" or strained misalignment, respectively. Also important is an equatorial 6″-CH_2OH group for proper alignment, since substrates which lack this potential hydrogen-bonding interaction are poorer substrates (e.g., UDP-Arab).

Discussion

The experimental results of this work and that of Andree & Berliner (1978) suggest an unusual degree of adaptivity in the donor site of galactosyltransferase. Figure 2 depicts the structural differences in the donor glycosyl moieties under discussion. Inversion of configuration at the axial 4″-hydroxyl group of UDP-Gal yields UDP-Glc which binds as well as the normal substrate and is turned over, albeit at a quite diminished rate. While the consequences of a bulky equatorial 4″-hydroxyl group (UDP-Glc) were to cause a severe misalignment of the UDP-glycosyl moiety for efficient catalysis, the complete lack of a 4″-hydroxyl substituent on the glycosyl pyranose ring (i.e., UDP-dGlc) should alleviate all steric stress *and* consequently accelerate the catalytic rate to a value comparable to that of UDP-Gal, *unless* there was a critical hydrogen-bonding interaction between specific amino acid residue(s) and an axial 4″-hydroxyl group. The kinetic data, which are compiled in Table II, indicate that for UDP-dGlc the V_{max} was only ca. 6% that for UDP-Gal as the donor substrate. Further evidence for another crucial (hydrogen-bonding) interaction comes from the work of J. C. Paulsen and J. E. Bell (unpublished results) with UDP-arabinose as a donor substrate. Since the pyranose conformation of the arabinosyl moiety in this substrate resembles UDP-Gal, except for the lack of an equatorial 6″-CH_2OH group (see Figure 2), the critical enzyme–substrate interaction with an axial 4″-hydroxyl group (UDP-Gal, UDP-Arab) should be intact. However, the loss of a specific interaction with the 6″-CH_2OH group in UDP-Arab results in a much reduced catalytic rate, V_{max} = 4% vs. that of UDP-Gal, under the same conditions.

It is clear from the K_m values in Table II that all of the substrates depicted in Figure 2 have similar K_m values. While the substrate specificity of galactosyltransferase has been shown to be somewhat broader than that of UDP-Gal alone, it is clear that the glycosyl binding site is nonetheless quite specific. For example, upon substituting a piperidine nitroxide for the glycosyl moiety of the uridine nucleotide sugar (Berliner & Wong, 1975), Andree & Berliner (1978) showed that this spin-labeled analogue (UDP-R) binds to the enzyme via the uridylyl portion while the piperidine nitroxide moiety was not very strongly associated to the enzyme and enjoyed substantial freedom of rotation. Furthermore, they showed that the amino acid group which "interacted" with the 4″-OH of UDP-Gal was neither a thiol nor a very reactive nucleophile by the lack of reactivity of galactosyltransferase with *p*-(bromoacetamido)phenyldiphosphouridine (Berliner & Andree, 1978). While uridine was the only naturally occurring nucleotide base in the donor substrate to support binding and catalysis with galactosyltransferase [see Ebner (1973)], the substitution of a mercuri group at the 5 position of UTP did not substantially affect the efficacy of this modified nucleotide as a competitive inhibitor of galactosyltransferase vs. that of UTP or UDP (B. G. Conery and L. J. Berliner, unpublished results).

The glycosyl donor specificity of galactosyltransferase (or lactose synthase) may be described, at least in part, from the results of the studies reported above. The 4″-pyranosyl position can tolerate either an axial or equatorial hydroxyl group or none. The 6″ position can also tolerate the lack of a primary -CH_2OH group. However, both axial 4″-hydroxyl and equatorial 6″-CH_2OH groups contribute to specific enzyme–substrate interactions (probably hydrogen bonds) which are critical to optimal catalytic turnover rates. Whether these interactions are required simply for proper substrate alignment to avoid "substrate wobble" or for an allosteric protein conformational change remains an intriguing question for further work. Some support comes from circular dichroism (CD) studies by Geren et al. (1975), who found that addition of Mn (II)–UDP-Gal to galactosyltransferase caused changes in the near-UV CD spectrum. On the other hand, the product UDP, which lacks a glycosyl moiety, caused no changes upon binding nor did the acceptor substrate, glucose.

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Supplementary Material Available

The detailed synthetic procedures and characterization of all intermediates for the synthesis of UDP-dGlc (11 pages). Ordering information is given on any current masthead page.

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Exploration of the Nucleotide Binding Sites of the Isolated ADP/ATP Carrier Protein from Beef Heart Mitochondria. 1. Probing of the Nucleotide Sites by Naphthoyl-ATP, a Fluorescent Nontransportable Analogue of ATP†

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ABSTRACT: The ADP/ATP carrier protein was extracted and purified from beef heart mitochondria, and its binding parameters with respect to 3′-*O*-naphthoyladenosine 5′-triphosphate (N-ATP), a fluorescent nontransportable analogue of ATP, were studied. The binding of N-ATP to the isolated carrier protein was accompanied by a decrease in fluorescence. Conversely, the release of bound N-ATP upon addition of carboxyatractyloside (CATR) or ATP resulted in a fluorescence increase. The bound N-ATP that was released upon addition of an excess of CATR or ATP was referred to as specifically bound N-ATP, i.e., N-ATP bound to the nucleotide sites of the carrier protein. Two classes of binding sites for N-ATP could be identified; the number of high-affinity sites (K_d < 10 nM) was equal to the number of low-affinity sites (K_d = 0.45 μM). CATR behaved apparently as a noncompetitive inhibitor of the binding of N-ATP. The amount of N-ATP released increased linearly with the amount of CATR added, indicating an extremely high affinity of the carrier protein for CATR. The number of CATR binding sites was equal to half the total number of N-ATP binding sites (high- and low-affinity sites); at saturating concentrations of N-ATP, the binding of 1 mol of CATR resulted in the release of 2 mol of bound N-ATP, one from the high-affinity site and the other from the low-affinity site, showing unambiguously that each CATR site is interacting with a pair of probably interdependent N-ATP sites. A clear competition between N-ATP and ATP for binding to the carrier protein was demonstrated. The K_d values of the high- and low-affinity sites for ATP were <50 nM and 5 μM, respectively. In the presence of high concentrations of ATP, the two classes of N-ATP binding sites became indistinguishable, suggesting interconversion. It is proposed that the asymmetry in affinity for N-ATP binding is induced by the binding step itself, the carrier protein exhibiting a negative cooperativity for N-ATP binding.

Upon addition of substrates or inhibitors, the mitochondrial ADP/ATP carrier exhibits conformational changes which are reflected by unmasking of SH groups (Leblanc & Clauser, 1972; Vignais & Vignais, 1972) or antigenic determinants (Buchanan et al., 1976) and by modification of the intrinsic fluorescence of tryptophanyl residues (Brandolin et al., 1981). These conformational changes can be observed not only with the ADP/ATP carrier in the mitochondrial membrane but also with the isolated ADP/ATP carrier protein (Brandolin et al., 1981; Aquila & Klingenberg, 1982); they are probably related to the transport mechanism of ADP and ATP. To obtain more information about the molecular mechanism of ADP/ATP transport, we have studied nucleotide binding to the isolated carrier protein, by using fluorescent analogues of ATP. The binding of these fluorescent analogues to the nucleotide sites of the carrier can be readily assessed in fluorometric assays by chase with specific inhibitors, e.g., carboxyatractyloside (CATR)[1] and bongkrekic acid (BA).

In this paper we describe the binding properties of 3′-*O*-naphthoyladenosine 5′-triphosphate (N-ATP) to the isolated ADP/ATP carrier protein. In preliminary experiments (unpublished results), it was found that N-ATP is not transported in mitochondria or in inside-out submitochondrial particles but is able to bind to the carrier protein with high affinity. A similar study was performed with formycin triphosphate, another fluorescent analogue which differs from N-ATP in that it is transported (Brandolin et al., 1982). The data are interpreted in terms of interaction between several nucleotide binding sites located on the carrier.

Experimental Procedures

Materials. Nucleotides and carboxyatractyloside (CATR)[1] were purchased from Boehringer, 1-naphthoic acid was from

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[1] Abbreviations: N-ATP, 3′-*O*-naphthoyladenosine 5′-triphosphate; CATR, carboxyatractyloside; BA, bongkrekic acid; LAPAO, lauryl-amido-*N*,*N*-dimethylpropylamine oxide; Mops, 3-(*N*-morpholino)-propanesulfonic acid; EDTA, ethylenediaminetetraacetic acid.

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