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BINDING STUDIES WITH BOVINE LIVER UDP-D-GLUCOSE DEHYDROGENASE*†

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ABSTRACT

The direct binding of uridine 5′-(α-D-glucopyranosyluronic acid pyrophosphate) (UDP-D-glucuronic acid) and of uridine 5′-(α-D-xylopyranosyl pyrophosphate) (UDP-D-xylose) to bovine liver UDP-D-glucose:NAD oxidoreductase (EC 1.1.1.22) has been measured by equilibrium dialysis. At saturation, the hexameric enzyme binds six molecules of UDP-D-glucuronic acid to noninteracting sites. UDP-D-xylose binds to 2 distinct classes of sites, each class binding six molecules of ligand. UDP-D-xylose is able to displace either UDP-D-glucose or UDP-D-glucuronic acid and UDP-D-glucose is able to displace UDP-D-glucuronic acid from the enzyme. It is proposed that the enzyme displays half-of-the-sites reactivity toward both substrate (UDP-D-glucose) and cosubstrate (NAD) and all-of-the-sites reactivity toward UDP-D-glucuronic acid. UDP-D-xylose is considered to bind cooperatively with high affinity to six regulatory sites and independently with lower affinity to six catalytic sites on the enzyme. Active-enzyme centrifugation studies show that UDP-D-glucose: NAD oxidoreductase is hexameric at concentrations corresponding to those used in steady-state kinetic measurements.

INTRODUCTION

UDP-D-glucose dehydrogenase (UDP-D-glucose:NAD oxidoreductase, EC 1.1.1.22), first demonstrated in mammalian liver by Strominger *et al.*[1], has subsequently been shown to be widely distributed among both pro- and eu-caryotic organisms[2–5]. Neufeld and Hall[6] showed that partially purified UDP-D-glucose

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dehydrogenase from beef liver, chick cartilage, and pea seedlings is strongly and specifically inhibited by uridine 5′-(α-D-xylopyranosyl pyrophosphate) (UDP-D-Xyl). Similar effects of UDP-D-Xyl have been shown with UDP-D-glucose dehydrogenases from other eucaryotic sources[3,7–10]. In these organisms, uridine 5′-(α-D-glucopyranosyluronic acid pyrophosphate) (UDP-D-GlcA) is the precursor of UDP-D-Xyl, which, in turn, functions as donor of the D-xylosyl residue. Therefore, UDP-D-Xyl acts as a specific feedback inhibitor of UDP-D-glucose dehydrogenase, serving to regulate the level of UDP-D-GlcA and also to regulate to some extent its own concentration. Since UDP-D-GlcA and UDP-D-Xyl act as the terminal precursors of polysaccharide synthesis in these organisms, this inhibitory mechanism probably functions to maintain the various sugar nucleotide precursors at the optimum relative concentrations.

Many bacteria contain D-glucuronic acid but are devoid of D-xylose and also of UDP-D-glucuronic acid carboxy-lyase (EC 4.1.1.35), which catalyzes UDP-D-Xyl formation. In these procaryots, UDP-D-glucose dehydrogenase does not show the type of feedback inhibition found in organisms that contain D-xylose. UDP-D-Xyl is a non cooperative mixed-type inhibitor of the dehydrogenase from *Aerobacter aerogenes*[4] and an inhibitor strictly competitive with uridine 5′-(α-D-glucopyranosyl pyrophosphate) (UDP-D-Glc) for UDP-D-glucose dehydrogenase from *Escherichia coli*[5]. Furthermore, the bacterial enzymes differ from the beef liver dehydrogenase in their physical properties. Whereas beef liver UDP-D-glucose dehydrogenase consists of six identical polypeptide chains having a molecular weight of 50,000 daltons[11,12], the molecular weight of the *A. aerogenes* enzymes is in the order of 100,000 daltons[4], and the *E. coli* enzyme consists of two polypeptide chains having a molecular weight of 45,000 daltons[13]. These differences probably reflect the less complex regulatory mechanisms operative with the bacterial enzymes.

Pure beef-liver UDP-D-glucose dehydrogenase, at saturating substrate concentration, binds three moles each of the substrate, UDP-D-Glc, and cosubstrate, NAD, per mole of enzyme[14]. Since the enzyme is constituted of six identical polypeptide chains, this stoichiometry suggests that this enzyme can be added to the growing list of enzymes showing so-called "half-of-the-sites" reactivity[15]. Any one of several possible "half-of-the-sites" reactivity mechanisms described for other enzymes might be invoked for UDP-D-glucose dehydrogenase[15–17]. Further elucidation of the mechanistic details of bovine liver UDP-D-glucose dehydrogenase depends, in part, upon a knowledge of the binding relationships for the reaction products and inhibitors, both singly and in combination with each other and with substrates. In this report we extend our studies on ligand binding to include interactions of the product, UDP-D-GlcA, and the negative effector, UDP-D-Xyl, with the enzyme.

In addition, to verify the legitimacy of applying binding data obtained at high protein concentrations to the interpretation of enzyme catalysis measured at very low protein concentrations, we will present results of the studies of active enzyme centrifugation (AEC)[18].

EXPERIMENTAL

Materials. — The enzyme was isolated as previously described[19]. Enzyme having a specific activity of 2.8 international units per mg of protein was used. Non-radiolabeled UDP-D-Glc, UDP-D-Xyl, and UDP-D-GlcA were obtained from Sigma Chemical Co. (St. Louis, Mo. 63178). UDP-D-Xyl and UDP-D-GlcA uniformly labeled with ^{14}C in the glycosyl moiety were obtained from New England Nuclear (Boston, Mass. 02118) at specific activities of 150 and 240 Ci/mole, respectively. These samples were used without further purification, and the results were not corrected for the very minor amounts of impurity indicated with the sample specifications provided by the supplier.

Equilibrium dialysis. — The procedure used previously, as described by Franzen *et al.*[14], was used without additional modification. All measurements were performed in 0.1M glycylglycine buffer, pH 8.5.

Active-enzyme centrifugation. — These centrifugations were performed by the method of Cohen and Mire[18]. The enzyme (140 ng) in 15 μl of solution was layered on top of 0.5 ml of substrate in 0.1M glycylglycine buffer, by use of a type I band-forming centerpiece. UDP-D-Glc and NAD were present at concentrations of 0.98 and 0.59mM, respectively. Sedimentation at 36,000 r.p.m., 20°, was monitored by the assessment of densitometer tracings of photographic recordings of the transmitted light from the Hg emission lines at 366 nm. A type UG-1 filter from Carl Zeiss, Inc. (New York, N.Y. 10018) was used to isolate these emission lines. The u.v. optics system of the Spinco Model E analytical ultracentrifuge was aligned for light source radiation in this spectral region, achieving proper focusing of the camera lens by employing a 17-inch focal-length camera lens, obtained from Spinco.

Circular dichroism. — Circular dichroism spectra were obtained with a Cary Model 60 spectropolarimeter equipped with a 6001 CD attachment. Spectra were recorded at room temperature at 1.5 nm band-width.

RESULTS

Figures 1 and 2 present Scatchard plots of the results of measurements of the binding of UDP-D-GlcA and UDP-D-Xyl. For comparison purposes, the isotherms describing the association of UDP-D-Glc and NAD, as evaluated previously[14], are included in Fig. 1. The uniformly linear nature of the plot for UDP-D-GlcA binding is consistent with the presence of six independent sites for attachment of this ligand, as determined from the abscissa intercept of Fig. 1. The saturation value for UDP-D-Xyl fixation, on the other hand, is more questionable. To make a judgment on the maximum number of sites available for UDP-D-Xyl binding, it is necessary to have some measure of the quality of the data in Fig. 2. Table I presents the values of the measurements used for generating some representative points contained in Fig. 2. To illustrate the magnitude of the uncertainty to be associated with the values in the last two columns of Table I, the accompanying errors were estimated by standard propagation of error techniques[20]. These errors are indicated by the dimensions of

TABLE I

RESULTS OF MEASUREMENTS OF UDP-D-Xyl BINDING TO UDP-D-GLUCOSE DEHYDROGENASE[a]

Experiment No.	*Specific activity of ligand (c.p.m./pmole)*	*Net radioactivity at equilibrium of 5-μl aliquots (c.p.m.)*		*UDP-D-Xyl concentration (μM)*		*Protein concentration (μM)[b]*	*Binding ratio ($\bar{v}$) (mole of UDP-D-Xyl/mole of protein)*
		Solvent side	*Protein side*	*Free*	*Bound*		
1	280	458	4236	0.327	2.70	4.03	0.67
2	37.5	171	841	0.912	3.57	2.35	1.52
3	77.0	904	3757	2.35	7.41	2.50	2.96
		1551	5513	4.03	10.3		4.12
4	35.8	1839	4466	10.3	14.7	2.58	5.68
5	6.87	619	1803	18.0	34.5	5.32	6.48
		1246	2761	36.3	44.1		8.29
		2438	4164	70.9	50.2		9.45
6	3.46	2678	3565	155	51.3	4.92	10.4
7	1.84	3078	3600	335	56.7	4.88	11.6

[a]Each chamber contained a total volume of 20 μl. The solvent in all cases was 0.1M glycylglycine buffer, pH 8.5. Equilibration was achieved in 6 h at 2°.
[b]Based on a molecular weight of 312,000 daltons.

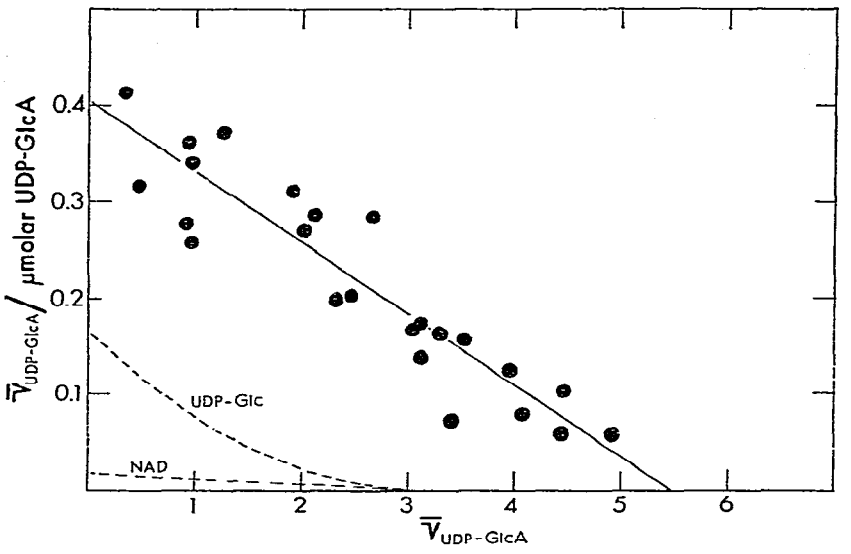

Fig. 1. Scatchard plot of UDP-D-GlcA binding. The solid line represents the result of an unweighted linear least-squares analysis. The lines representing UDP-D-Glc and NAD binding are derived from earlier data[14].

the rectangles surrounding the corresponding points in Fig. 2. The illustrated errors are consistent with the error envelope to be expected in a Scatchard presentation of binding data, as shown by Deranleau[21]. It is clear that binding ratios as large as 9 are probably genuine, whereas larger ratios are of more doubtful validity. If the data of Fig. 2 for UDP-D-Xyl binding-ratios of eight or greater are recast in the form of reciprocal binding ratios as a function of reciprocal concentrations, an unweighted, linear least-squares analysis yields an extrapolated saturation value of 11.1 ±1.4 UDP-D-Xyl molecules per native enzyme molecule. Unfortunately, at this point, we can only say that the data lead us to prefer the conclusion that there are actually twelve, rather than nine, possible binding locations for UDP-xylose on this enzyme. In keeping with the three-fold nature of the quaternary structure of the enzyme, we *assume* that binding sites exist in multiples of three.

It is obvious from Figs. 1 and 2 that though UDP-D-GlcA binds to essentially identical non-interacting sites, UDP-D-Xyl either binds to two or more classes of independent sites or it binds to sites, or groups of sites, which antagonize each other. Table II summarizes the dissociation constants pertaining to the curves of Figs. 1 and 2, and includes the half saturation concentrations for UDP-D-Glc binding and NAD binding reported earlier[14]. The entries for UDP-D-Xyl binding pertain to the consideration that this ligand binds to two independent classes of sites, each class having six sites. The constants were obtained by trial and error adjustment of initial values resulting from following the analysis of Klotz and Hunston[22], the criterion of goodness of fit used being only the apparent centeredness of the resulting function within the data of Fig. 2. This independent model of classes of sites is proposed to

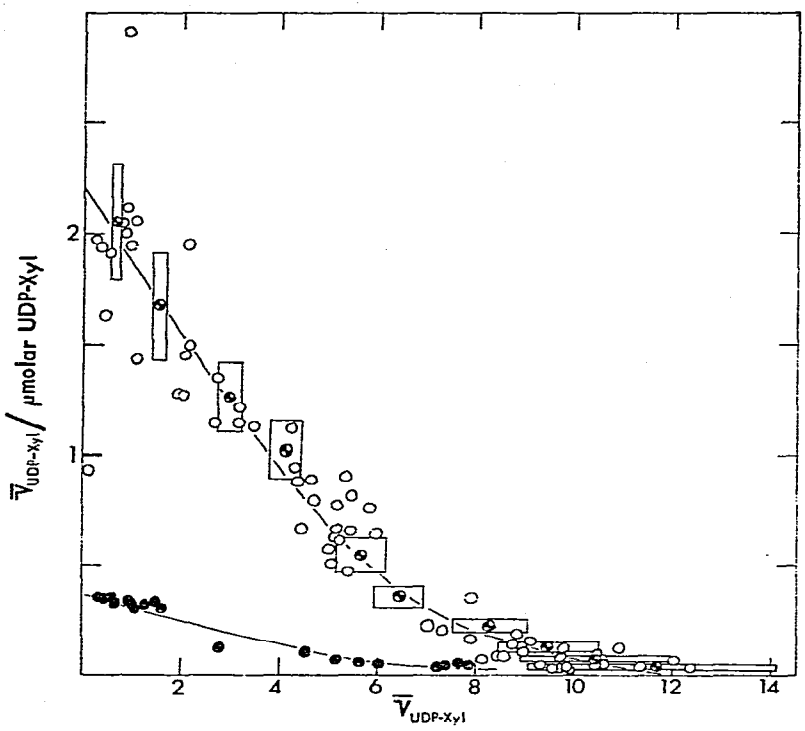

Fig. 2. Scatchard plot of UDP-D-Xyl binding. The upper points (○), represent binding in the absence of other ligands. The outer limits of the rectangles surrounding half-filled circles (◒) correspond to the estimated error in the coordinates of these points. Similar error levels, though not shown, exist with neighboring points. The drawn curve corresponds to the independent classes of sites model for this system employing the dissociation constants listed in Table I. The lower points (●) represent binding in the presence of UDP-D-Glc at a fixed total concentration of 4.6mM.

TABLE II

DISSOCIATION CONSTANTS FOR VARIOUS UDP-D-GLUCOSE DEHYDROGENASE-LIGAND COMPLEXES

Ligand	*Site designation*	*Number of sites*	*Dissociation constant (μM)*	*Ref.*
UDP-D-GlcA	catalytic	5.5±0.2	13.6±1.2	Fig. 1
UDP-D-Xyl	effector	6	2.86	Fig. 2
UDP-D-Xyl	catalytic	6	57.1	Fig. 2
UDP-D-Xyl (with high concentration of UDP-D-Glc)	effector	6	21.4[a]	Fig. 2
UDP-D-Glc	catalytic	3	33[b]	Fig. 1 and Ref. 1
NAD	catalytic	3	172[b]	Fig. 1 and Ref. 1

[a]Evaluated from the limiting slope of the lower line of Fig. 2 at low UDP-D-Xyl concentrations.
[b]These values for UDP-D-Glc and NAD are half-saturation concentrations of these substrates taken from Ref. 14. The corresponding curves appear in Fig. 1 of this paper.

permit convenient and simple calculation of the approximate magnitudes of the affinities associated with the UDP-D-Xyl sites.

Two kinds of competition-binding experiments were performed. In the former, the association of UDP-D-Xyl was examined in the presence of fixed high concentrations of UDP-D-Glc, as shown in Fig. 2. It is apparent that competition indeed does occur. From earlier work, we know that only three molecules of UDP-D-Glc can be bound to the enzyme[14]. Interestingly, the attachment of at most three moles of UDP-D-Glc leads to the loss of six UDP-D-Xyl sites. This number, six, is the X-intercept of the straight line through the low UDP-D-Xyl concentration points of the lower data set of Fig. 2. The UDP-D-Xyl sites available in this case are of substantially lower affinity, 8-fold, than the high affinity UDP-D-Xyl sites on the free enzyme. At high UDP-D-Xyl concentrations, Fig. 2 shows that more than six UDP-D-Xyl molecules are bound. This can be viewed as the result of direct competition with the UDP-D-Glc present.

In the second kind of competition experiment performed, illustrated in Fig. 3, the total concentration of UDP-D-GlcA was kept constant, while increasing amounts of nonradiolabeled competitor, UDP-D-Glc, were added. Though enough UDP-D-GlcA was present to produce a binding ratio substantially greater than three, the addition of UDP-D-Glc sufficient to cause the binding of three UDP-D-Glc molecules brings about the release of more than three molecules of UDP-D-GlcA. This effect is similar to that of UDP-D-Glc on what we call the low affinity UDP-D-Xyl sites just

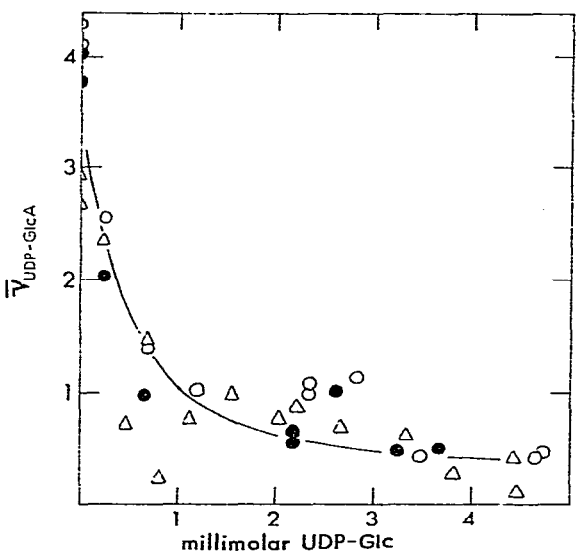

Fig. 3. Displacement of bound UDP-D-GlcA by added UDP-D-Glc. Binding experiments were performed, as previously described[14], in which increasing amounts of nonradioactive UDP-D-Glc were added to three separate series of dialysis cells (designated by △, ○, and ●). All cells contained the same amount of enzyme (2.81 μg) and the same amount of ^{14}C-labeled UDP-D-GlcA (1.92 μmoles). The volume of liquid in each cell chamber was 20 μl.

discussed. Although the data are not shown here, one similar experiment was performed in which UDP-D-Xyl served as the competitor for UDP-D-GlcA binding. Over the antagonist concentration range represented in Fig. 3, UDP-D-GlcA binding was essentially abolished by UDP-D-Xyl. The effects of low concentrations of UDP-D-Xyl were not examined.

The experiments of active enzyme centrifugation yielded a sedimentation coefficient of 11.7 $\pm$0.7 *S*, average and standard deviation of three separate determinations. The sedimentation coefficients obtained at enzyme concentrations corresponding to those employed in the binding experiments described here is[19] 12.8 *S*. It is reasonable to believe, therefore, that the quaternary structure of the enzyme, existing at concentrations employed in binding studies, persists at the low enzyme concentrations used in kinetic studies.

In an attempt to learn something about the secondary structure of bovine liver UDP-D-glucose dehydrogenase and the extent to which conformational adjustments might accompany ligand attachment, the circular dichroism spectrum of the enzyme was obtained in the absence and in the presence of UDP-D-Glc (and UDP-D-Xyl), From Fig. 4, which portrays the deeper u.v. circular-dichroic spectrum of the enzyme.

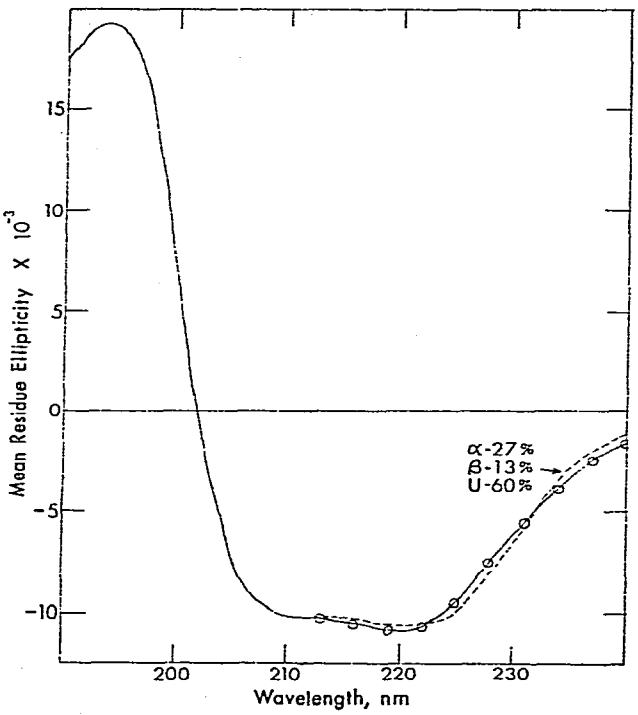

Fig. 4. Circular dichroism spectrum of UDP-D-glucose dehydrogenase. The dashed line is the computed spectrum for a protein having the secondary structure composition indicated in this figure. These values were obtained by the analysis of Chen *et al.*[23], using the experimental points indicated on the solid line.

we observe a typical protein c.d. pattern. Analysis of this spectrum according to the scheme of Chen *et al.*[23] indicates that the protein may have a secondary structure composition of 27% of α-helix, 13% of β-structure, and 60% of nondescript backbone ordering.

Figure 5 shows the region of aromatic-residue Cotton effects. Strict additivity of the ligand and protein spectra in isolation yields a spectrum indistinguishable from that of the mixture of ligand and protein. This indicates that, as far as the side chains

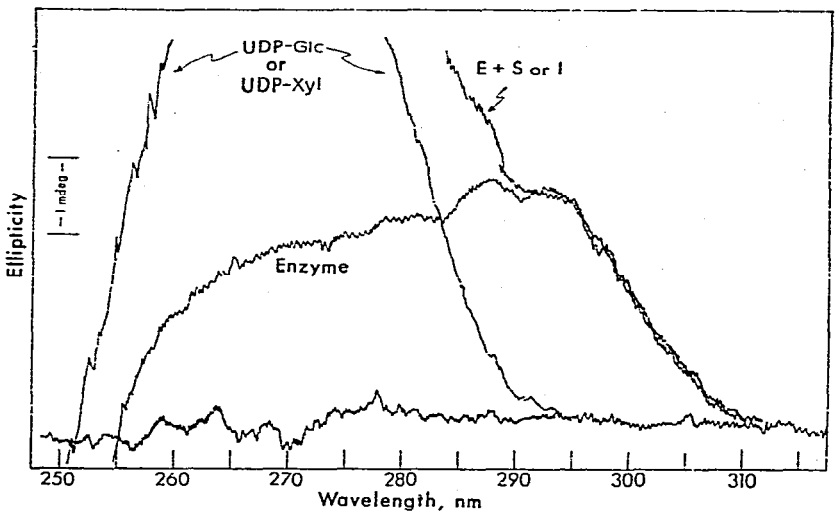

Fig. 5. Circular dichroism spectra in the region of aromatic chromophore residues. The spectra for enzyme alone and for ligand alone were obtained with one of the 1-cm chambers of a tandem cell containing solvent only and the other containing the solution of protein at a concentration of 1.4 mg/ml, or ligand at a concentration of 140μM. With protein in one chamber and ligand in the other chamber, the spectrum indicated E+(S or I) was obtained. When the two equal-volume solutions were withdrawn from the chambers, mixed, and the mixture placed in the two chambers of the tandem cell, a spectrum indistinguishable from that labeled E+(S or I) was obtained. On the basis of the dissociation constants of Table II, it is estimated that the UDP-D-Glc binds to about 2.5 UDP-D-Glc sites at the concentrations employed. UDP-D-Xyl binds to approximately 9 sites at these concentrations. Enzyme, E; UDP-D-Glc, S; UDP-D-Xyl, I.

generating the c.d. signals above 285 nm are concerned, there is no influence of either the substrate, UDP-D-Glc, or the effector, UDP-D-Xyl, on conformation. These nucleotides were present at concentrations sufficient to cause fractional saturations of greater than one-half.

DISCUSSION

UDP-D-glucose dehydrogenase contains[14] three noninteracting sites for NAD and three negatively cooperative sites for UDP-D-Glc. The data presented here show that UDP-D-GlcA binds to six sites which, as is evident from the strict linearity of

the Scatchard plot (Fig. 1), are non-interacting. UDP-D-Glc and UDP-D-GlcA, substrate and product, respectively, of UDP-D-glucose dehydrogenase, can be assumed to bind to the same catalytic sites. This is also consistent with the strict competition between the two ligands observed in kinetic studies[6]. The effect of the ligands on the enzyme is markedly different, however. The successive binding of three molecules of UDP-D-Glc leads to loss of affinity of half of the substrate sites on the hexameric protein, whereas UDP-D-GlcA binds to each of the six sites with essentially identical affinity; in other words, the enzyme displays half-of-the-sites reactivity toward UDP-D-Glc and all-of-the-sites reactivity toward UDP-D-GlcA. The interaction between UDP-D-Glc and UDP-D-GlcA sites is also apparent from the results of the experiments presented in Fig. 3, in which the binding of only three molecules of UDP-D-Glc leads to complete loss of binding sites for UDP-D-GlcA. The kinetically determined inhibition constant[6] for UDP-D-GlcA (50 μM) is in reasonable agreement with the dissociation constant found here, 11μM, especially since the kinetic work was done at 22°, whereas our experiments were all conducted at 2°.

In contrast to UDP-D-Glc, NAD, and UDP-D-GlcA, UDP-D-Xyl binds to the enzyme in a manner that suggests the presence of two distinct classes of sites (Fig. 2). We propose that the binding of UDP-D-Xyl at low concentrations occurs at regulatory or allosteric sites rather than at the catalytic sites, the number of such regulatory sites being six per native enzyme molecule. The data are insufficiently precise to allow us to draw conclusions about cooperativity between the sites that comprise this class. At higher UDP-D-Xyl concentrations, binding occurs at the catalytic sites as well. The stoichiometry of UDP-D-Xyl binding to the catalytic sites is probably similar to that for UDP-D-GlcA binding to these sites. If UDP-D-Xyl binds to the catalytic sites, it ought to be displaceable by UDP-D-Glc. This effect is seen in Fig. 2 where the lower curve indicates that only six sites are available for UDP-D-Xyl attachment at low UDP-D-Xyl concentration and high UDP-D-Glc concentration. At higher UDP-D-Xyl concentrations, Fig. 2 shows that additional sites can be occupied by UDP-D-Xyl, even though UDP-D-Glc is present. At high concentrations of both ligands, true competition is presumed to occur. In addition, the competition observed between UDP-D-Xyl and UDP-D-GlcA further suggests that, at high concentrations, the inhibitor binds to the catalytic site.

Kinetic observations[6] imply that UDP-D-Xyl binding reduces the affinity of the enzyme for UDP-D-Glc. Figure 2 and Table II of this work show that the reciprocal effect also exists, namely, that UDP-D-Glc binding reduces the affinity for UDP-D-Xyl. The dissociation constant for the high affinity UDP-D-Xyl sites increases from 3μM to 57μM upon addition of saturating levels of UDP-D-Glc. It is of interest that the kinetic inhibition constant for UDP-D-Xyl at low UDP-D-Xyl concentrations[6] is 3μM.

Though homotropic and heterotropic interactions occur upon binding of different ligands to UDP-D-glucose dehydrogenase, these effects do not lead to changes in the spectroscopic parameters that we have been able to investigate to date (Fig. 5).

Gainey *et al.*[11] have proposed, on the basis of kinetic and structural considera-

tions, that UDP-D-Xyl is a true competitive inhibitor of UDP-D-glucose dehydrogenase with respect to UDP-D-Glc, and in this manner exerts a cooperative homotropic effect rather than an allosteric effect on the enzyme. Our findings are partly in accord with this view; however, the model we propose assigns secondary importance to inhibitor binding at catalytic sites, and major importance to binding at what we designate as effector sites. Further work is required to resolve these differences.

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