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The use of fluoro- and deoxy-substrate analogs to examine binding specificity and catalysis in the enzymes of the sorbitol pathway

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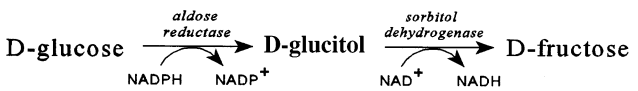
Abstract

The carbohydrate specificity of the two enzymes that catalyze the metabolic interconversions in the sorbitol pathway, aldose reductase and sorbitol dehydrogenase, has been examined through the use of fluoro- and deoxy-substrate analogs. Hydrogen bonding has been shown to be the primary mode of interaction by which these enzymes specifically recognize and bind their respective polyol substrates. Aldose reductase has broad substrate specificity, and all of the fluoro- and deoxysugars that were examined are substrates for this enzyme. Unexpectedly, both 3-fluoro- and 4-fluoro-D-glucose were found to be better substrates, with significantly lower K_m and higher k_{cat}/K_m values than those of D-glucose. A more discriminating pattern of substrate specificity is observed for sorbitol dehydrogenase. Neither the 2-fluoro nor the 2-deoxy analogs of D-glucitol were found to be substrates or inhibitors, suggesting that the 2-hydroxyl group of sorbitol is a hydrogen bond donor. The 4-fluoro and 4-deoxy analogs are poorer substrates than sorbitol, also implying a binding role for this hydroxyl group. In contrast, both 6-fluoro- and 6-deoxy-D-glucitol are very good substrates for sorbitol dehydrogenase, indicating that the primary hydroxyl group at this position is not involved in substrate recognition by this enzyme.

Keywords: Aldose reductase; Sorbitol dehydrogenase; Sorbitol pathway; Substrate specificity; Fluoro sugars; Deoxy sugars

1. Introduction

The sorbitol pathway consists of two enzymes, aldose reductase and sorbitol dehydrogenase:



The physiological role of the sorbitol pathway in most tissues is not completely understood. There is evidence, however, that this pathway functions as a bypass between glycolysis and the pentose phosphate shunt in the metabolism of glucose [1]. Several hypotheses have been proposed which link the increased activity of the sorbitol pathway to tissue injury under conditions of hyperglycemia [2,3], where this pathway can account for more than 30% of glucose utilization [4]. Complications or tissue injury in diabetes can be caused by osmotic stress, by imbalance in *myo*-inositol metabolism, by modification of protein function through glycation, and by alterations in

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redox state [5]. Under these conditions, tissues which do not require insulin-mediated glucose uptake, such as lens, kidney and nerve, are the most susceptible to cellular damage. The accumulation of sorbitol interferes with *myo*-inositol transport, resulting in reduced Na^+/K^+-ATPase activity which is critical for cellular homeostasis [6]. Increases in sorbitol also cause swelling of the epithelial cells of the lens, which has been linked to cataract formation [7]. Increased levels of NAD^+ lead to permeability changes in vessel formation and alterations in glucose and lipid metabolism [1].

Aldose reductase (ALR) is the rate-limiting enzyme in the sorbitol pathway. This enzyme, which converts aldoses to their corresponding alditols, has a broad specificity for a variety of aromatic and aliphatic aldehydes [8], including three-carbon aldehydes and ketones [9]. ALR has been shown to exist in an unactivated (low activity) form [8] that can be converted to an activated (high activity) form by the oxidation of a specific cysteine [10]. Structural studies on human ALR have revealed an α/β-barrel structure, with the coenzyme binding domain located near the carboxyl terminal [11,12]. Tyrosine-48 has been proposed to be the proton donor to the aldehyde substrate, with the participation of a lysine (lys-77) to lower the pK_a of the phenolic hydroxide [13,14]. An active site histidine (his-110) has been assigned a substrate binding and orientation role [13], and chemical modification, peptide mapping, and site-directed mutagenesis studies have identified a lysine and several arginines that are involved in coenzyme binding [15,16].

The second enzyme in the sorbitol pathway is sorbitol dehydrogenase (SDH), which oxidizes D-glucitol to D-fructose. Like aldose reductase, sorbitol dehydrogenase has broad specificity; D-xylitol is a substrate with a lower K_m than that of D-glucitol, but D-galactitol is not a substrate of this enzyme [4]. Aromatic polyols [17] and 3-carbon alcohols [18] have also been found to be substrates for sorbitol dehydrogenase. SDH contains a single zinc per subunit that is essential for catalytic activity [19]. There is no high-resolution structure of SDH, however, a model of the three-dimensional structure of SDH has been constructed from the structure of horse liver alcohol dehydrogenase and an alignment of the amino acid sequences [20]. From this model, the ligands to zinc have been identified as a histidine, cysteine, and glutamate, and these assignments have been supported by X-ray absorption fine structure (EXAFS) studies [21], affinity labeling [22], metal chelation studies [23], and by site-directed mutagenesis [24].

There has been progress on a determination of the mechanism of catalysis of the enzymes in the sorbitol pathway, however, several aspects of these mechanisms remain unresolved. This paper describes the use of alternative substrates, specifically fluorosugars and deoxy-sugars, to probe the active site binding and catalytic requirements of these sorbitol pathway enzymes.

2. Experimental

Materials.—Purified aldose reductase from bovine kidney (high activity form, 3.5 U mg^{-1}) [8] was provided by Dr. Charles Grimshaw (Igen International) and sheep liver sorbitol dehydrogenase (26–28 U mg^{-1}) was purchased from Sigma Chemical Co. (St. Louis, MO). The 2-, 3-, 4-, and 6-fluorodeoxy-D-glucoses were obtained from Dr. Stephen Withers (University of British Columbia). Additional fluoro- and deoxy-D-glucose compounds used for synthesis of the corresponding D-glucitol derivatives were obtained from Lancaster-PCR, Inc. (Gainsville, FL) or from Sigma. The corresponding sorbitol analogs were synthesized by chemical reduction with sodium borohydride by a standard procedure [25], that briefly involves dissolving the various fluoro- and deoxyglucoses in water at 4 °C followed by the dropwise addition of a several fold molar excess of sodium borohydride over a period of 30 min. The reaction mixtures were then allowed to warm to room temperature and were incubated overnight. Aliquots of the reaction mixture were tested for the presence of the aldose starting material by using Fehling's solution or by *o*-toluidine determination, and the isolated yield of products ranged from 60 to 90%. The identities of the products were confirmed by proton and carbon NMR spec-

troscopy. In all cases the expected carbon-13 upfield shift was observed for the deoxy carbon atom, and there was no evidence of any remaining starting material. The purity of the products was confirmed by comparison of the melting points, where crystalline, to reported values, and by thin-layer chromatography (EM silica plate, EtOH/water/ammonium hydroxide, 21/4/1). Detection was achieved by anisidine–HCl spray [26] with 10% sulfuric acid in methanol. All of the compounds yielded a single spot by TLC analysis, except for 6-deoxy-D-glucitol. The impurity that was detected in this compound was nonreactive to *p*-anisidine, and therefore was not a reducing sugar. This product mixture was purified on a AG-50WX8 equilibrated with 1 M $CaCl_2$. TLC analysis after chromatography revealed a single, non-reducing product whose identity was confirmed by NMR spectroscopy.

Enzyme assays.—Aldose reductase kinetics were examined in 100 mM phosphate buffer, pH 7.2, 27 °C, with 160 μM NADPH ($K_m = 1$ μM) by following the decrease in absorbance at 340 nm resulting from the oxidation of NADPH. Sorbitol dehydrogenase kinetics were determined in 100 mM HEPES buffer, pH 8, 30 °C, with 2 mM NAD ($K_m = 11$ μM) by following the increase in absorbance at 340 nm resulting from the formation of NADH. One unit of activity is defined as 1 μmol of product produced min^{-1} mg^{-1} of protein under the standard reaction conditions. The kinetic parameters for each substrate were determined by varying the substrate concentration at saturating concentration (> 20 times K_m) of the cofactor and fitting the measured rates to the equation for Michaelis–Menten kinetics. Kinetic studies with a variety of substrates for each enzyme have shown that the affinity for the coenzyme is not significantly affected by the nature of the substrate [4,27]. When substrate inhibition was observed, the data were modeled by Eq. (1):

$$v = \frac{V_{max}[A]}{K_m + [A] + [A]^2/K_i} \quad (1)$$

where v is the measured rate, and K_m and K_i are the Michaelis and the substrate inhibition constants, respectively. The kinetic data were fit by using BASIC versions (Enzyme Kinetics Package, SciTech International, Chicago, IL) of the kinetics programs originally written by Cleland [28].

Spectroscopic studies.—Circular dichroism studies were conducted on a JASCO J600 CD spectrometer. Samples were monitored near the maximum carbonyl ellipticity peak (285–290 nm) following a method for the quantitation of the proportion of acyclic form [29]. Temperature studies were performed with a thermally heated cuvette block. Spectra were recorded when samples were equilibrated at each temperature, as indicated by no further changes in the measured ellipticities. The ellipticities (in mdeg) were reproducible, leading to less than a 10% error in the calculated percent acyclic form.

3. Results

Substrate specificity of aldose reductase.—ALR catalyzes the reduction of the physiological substrate, D-glucose, to D-glucitol. Structural analogs of D-glucose, in which one of the sugar hydroxyl groups was replaced with either a fluorine or a hydrogen, were examined as possible alternative substrates for the reaction catalyzed by ALR. ALR binds only the acyclic form of glucose and does not catalyze ring-opening [30]. Therefore it is necessary to correct the substrate concentrations for the amount of acyclic form present in order to compare the relative kinetic parameters of these alternative substrates. Table 1 gives the percent acyclic form of these substrates, as determined by circular dichroism measurements. The 4-fluoro analog contains 2.5 times the amount of acyclic form, and the 3-fluoro analog about the same percentage as glucose. The 6-deoxy-D-glucose has been determined to have 0.002% acyclic form [29], while 2-deoxy-D-glucose has about 2.5 times this amount.

Given the broad specificity of ALR, it was not surprising that all of the fluoro- and deoxysugar analogs that were tested were found to be substrates for the enzyme. However, unexpectedly, 3-fluoro- and 4-fluoro-D-glucose were found to be better substrates, with significantly lower K_m values and higher k_{cat}/K_m

Table 1
Kinetic parameters for aldose reductase[a]

Substrate	k_{cat} (min^{-1})	K_m (mM)	% acyclic	K_m^{adj} (μM)[b]	k_{cat}/K_m^{adj} (mM^{-1} min^{-1})[b]	$\Delta\Delta G^{\ddagger}$ (kcal mol^{-1})
D-Glucose	15.1 ± 5.4	12.9 ± 1.3	0.0025	32.3 ± 3.3	467 ± 36	
2-Fluoro-D-glucose	3.00 ± 0.22	12.5 ± 2.6	n.d.	n.d.	n.d.	
2-Deoxy-D-glucose	11.1 ± 0.4	10.4 ± 0.7	0.0073	75.9 ± 6.5	146 ± 11	0.7
3-Fluoro-D-glucose	16.5 ± 1.8	0.31 ± 0.05	0.0024	0.74 ± 0.12	22300 ± 2900	−2.3
4-Fluoro-D-glucose	15.8 ± 0.4	0.54 ± 0.07	0.0073	3.9 ± 0.5	4050 ± 360	−1.3
5-Thio-D-glucose	12.6 ± 1.4	6.3 ± 1.8	0.005[c]	31.5 ± 9.0	400 ± 70	0.1
6-Fluoro-D-glucose	15.1 ± 0.7	3.1 ± 0.4	n.d.	n.d.	n.d.	
6-Deoxy-D-glucose	11.5 ± 0.4	9.8 ± 1.6	0.002[c]	19.6 ± 3.2	587 ± 72	−0.1

[a] Conditions: 100 mM phosphate buffer, pH 7.2, 160 μM NADPH, 10 μg aldose reductase, 27 °C.
[b] The K_m and k_{cat}/K_m values were adjusted by multiplying the apparent K_m by the percent of acyclic form present.
[c] Ref. [29].

values than that of D-glucose (Table 1). 2-Fluoro-D-glucose has a comparable K_m to that of D-glucose, but the k_{cat} for this substrate analog has decreased by 5-fold, in contrast to the similar k_{cat} for the 2-deoxy analog. The 5-thio-D-glucose analog is also a comparable substrate to D-glucose and, when adjusted for the amount of acyclic form present at equilibrium, has identical kinetic parameters to D-glucose (Table 1). The 1-thio-D-glucose analog is not a substrate for ALR, but was found to be an inhibitor of the enzyme.

Changes in the free energy of activation ($\Delta\Delta G^{\ddagger}$) are observed when an individual substrate hydroxyl group is substituted by either a hydrogen or fluorine [31]. These changes can be calculated from a comparison of the k_{cat}/K_m values for the substrate and the analog according to Eq. (2):

$$\Delta\Delta G^{\ddagger} = RT \ln[(k_{cat}/K_m)_{substrate}/(k_{cat}/K_m)_{analog}] \quad (2)$$

These values show a 1.3–2.3 kcal mol^{-1} decrease in the free energy of activation for the 4-fluoro and 3-fluoro analogs of D-glucose compared to the physiological substrate (Table 1).

Substrate specificity of sorbitol dehydrogenase.—A more discriminating pattern of substrate specificity has been observed for sorbitol dehydrogenase. Neither the 2-fluoro nor the 2-deoxy analogs of D-glucitol are substrates for the enzyme (Table 2). However, surprisingly, these analogs do not inhibit SDH when examined at concentrations up to 50 mM, indicating that interactions between the enzyme and the substrate at this position are important for binding. The 3-fluoro and 4-fluoro analogs are poorer substrates than D-glucitol, with an observed increase in the free energy of activation of 1.5–1.6 kcal mol^{-1}, suggesting that hydrogen bonding at these positions also plays a role in substrate recognition and binding. This is confirmed by the 2.6 kcal mol^{-1} increase in activation energy for 4-deoxy-D-glucitol. However, there is a recovery in K_m and k_{cat}/K_m when the 3-hydroxyl group is removed. The 6-fluoro- and 6-deoxy-D-glucitols are very good substrates for SDH (Table 2), with their free energies of activation lowered by about 0.5 kcal mol^{-1} for each of these analogs. These analogs also exhibit substrate inhibition at higher concentrations, with a K_i value of 65 mM for substrate inhibition by 6-fluoro-D-glucitol, while the K_i for 6-deoxy-D-glucitol is 20 mM. The K_m for 6-deoxysorbitol is about 2-fold lower than that of the 6-fluoro compound, and SDH has a higher affinity for each of these compounds than for D-glucitol.

4. Discussion

Substrate recognition and the role of hydrogen bonding.—Binding and orientation of the substrate at the active site of an enzyme plays an important role in catalysis. Recognition and binding of a substrate requires that the enzyme active site provide a complementary

Table 2
Kinetic parameters for D-glucitol dehydrogenase[a]

Substrate	k_{cat} (min^{-1})	K_m (mM)	k_{cat}/K_m (mM^{-1} min^{-1})	$\Delta\Delta G^{\ddagger}$ (kcal mol^{-1})
D-Glucitol	506 ± 53	1.5 ± 0.7	337 ± 103	
2-Fluoro-D-glucitol	[b]			
2-Deoxy-D-glucitol	[b]			
3-Fluoro-D-glucitol	350 ± 8	15.5 ± 0.8	22.6 ± 0.8	1.6
3-Deoxy-D-glucitol	441 ± 15	1.9 ± 0.3	231 ± 24	0.2
4-Fluoro-D-glucitol	354 ± 30	11.7 ± 3.0	30.3 ± 5.3	1.5
4-Deoxy-D-glucitol	129 ± 11	26.4 ± 5.7	4.9 ± 0.5	2.6
6-Fluoro-D-glucitol	559 ± 4	0.74 ± 0.02	755 ± 4	−0.5
6-Deoxy-D-glucitol	274 ± 8	0.48 ± 0.06	685 ± 20	−0.4

[a] Conditions: 100 mM Hepes buffer, pH 8.0, 0.1 mM EDTA, 1 mM DTT, 2 mM NAD, 0.2 μg SDH, 30 °C.
[b] No activity observed.

environment, which can include hydrogen bonding, electrostatic, and/or hydrophobic interactions, and requires the correct steric placement of the active site groups to interact with the available substrate functional groups. Hydrogen bonding is expected to be the primary mode by which carbohydrate-utilizing enzymes specifically recognize and bind their polyol substrates. Each sugar hydroxyl group can potentially participate in three hydrogen bonds, as acceptor of two hydrogen bonds through the lone pairs on oxygen, and as a donor of one hydrogen bond. Hydrogen bonds are highly directional, and are stable enough to contribute significantly to substrate affinity. At the same time hydrogen bonds are transient enough to allow rapid dissociation of product. The hydrogen bonds that are formed with enzyme functional groups are essential for active site binding since these interactions must compete with and replace the hydrogen bonds that are lost upon removal of the sugar substrates from aqueous solution.

Alternative deoxy- and fluorosugar substrates.—Kinetic studies of alternative substrates can examine the role of individual hydroxyl groups in binding and in catalysis. The use of deoxygenated and fluorinated carbohydrate substrate analogs to probe substrate binding is based on the different capacities of these analogs to form hydrogen bonds, while maintaining similar steric interactions. A deoxysugar cannot be involved in a hydrogen bond at the modified position. However, a fluorine substituent in this position can act as a potential proton acceptor due to the electronegativity of fluorine and the inherent polarity of the C–F bond. Reductions in the catalytic rate and in the substrate affinity provide an estimate of the cost of removing specific interactions at each hydroxyl group, and therefore an estimate of the extent to which those specific site interactions stabilize the transition state for the normal substrate.

Substrate recognition by aldose reductase.—ALR is a broad-spectrum reductase with substrates that include a variety of carbohydrates as well as hydrophobic aromatic compounds. In contrast to the binding sites of most carbohydrate-binding proteins, in which an intricate network of hydrogen bonds form the basis for carbohydrate recognition [32], the active site of ALR is quite hydrophobic [11]. Only the polar nature of histidine-110 is involved in the binding and orientation of the polar carbohydrate substrates [13]. Despite the paucity of well-defined hydrogen bonding interactions, ALR still shows a preference for aldoses with both 2-D and 3-L stereochemistry [27].

The 2- and 6-deoxy-D-glucoses are only slightly poorer substrates with ALR than D-glucose, suggesting that any interactions that may occur at these positions are not critical binding determinants. The 6-fluoro analog is a better substrate than the 6-deoxy sugar, suggesting that the 6-hydroxyl group is involved in substrate binding as a hydrogen bond acceptor. The 3- and 4-fluoro analogs are very good substrates, possessing some 40- and 20-fold better k_{cat}/K_m values than D-glucose, respectively. Fluorine substitution at these positions can participate as a potential hydro-

gen bond acceptor, but fluorine substitution also increases the acidity of the adjacent hydroxyl groups. The 2-fluoro compound is a poor substrate for aldose reductase, but the 2-deoxy compound is as good as D-glucose. This result argues that hydrogen bonding at this position is not critical, and points to an important inductive effect in acyclic substrate formation. The 1-thio-D-glucose has little tendency to mutarotate, since the sulfur anion does not favor electron release to the ring oxygen to form the acyclic compound. Thus this analog is not expected to be a substrate, but the binding of this cyclic inhibitor is consistent with the lack of binding discrimination in substrate recognition by ALR.

Substrate recognition by sorbitol dehydrogenase.—The overall conformation of sorbitol in water is not a common zigzag conformation, but is a non-linear bent chain conformation [33] resulting from the steric interaction between substituents on alternate carbon atoms and interactions with solvent water molecules. This conformation is also observed in the crystal structure, where C1 is 1.06 Å out of plane from the other five coplanar carbons [34]. Substitution by either fluorine or hydrogen at the 2- or 4-positions would alleviate some of the steric hindrance in this structure, and could therefore lead to enhanced binding to SDH.

Neither the 2-deoxy nor the 2-fluoro analogs of D-glucitol are substrates or inhibitors, implying that this position is an important substrate binding determinant and that the 2-hydroxyl group of sorbitol is a hydrogen bond donor to an active site group of SDH. The 10-fold increase in K_m for the 3-fluoro- and 4-fluoro-D-glucitols also implies a binding role for the hydroxyl groups at these positions. The results with the corresponding deoxysorbitols show a dramatic effect on the kinetic parameters upon removal of the 4-hydroxyl group, confirming a binding role for this position. However, the recovery of activity with the 3-deoxy analog eliminates this position from involvement in substrate binding, and suggests some additional effects from fluorine substitution at this position. The K_m value for 6-fluoro-D-glucitol is a factor of two lower, and that for the 6-deoxy analog decreases by 3-fold compared to D-glucitol. These results suggest that the presence of a primary hydroxyl group at carbon-6 is not important for substrate binding and orientation.

The free energies of activation for catalysis of the fluoro- and deoxy-substrate analogs of ALR and SDH show significant differences when compared to the physiological substrates. These changes have helped in the assignment of the roles of the individual substrate hydroxyl groups in binding and catalysis. This approach has been used to examine substrate hydrogen bonding interactions in other enzyme systems. In those cases, the activation energies of the fluoro- and deoxy-analogs were all found to be substantially higher than that of the native substrate. These values ranged from 2.7 to 4.5 kcal mol^{-1} for analogs of glucose-1-phosphate interacting with phosphoglucomutase [35], to as high as 8 kcal mol^{-1} for the same substrate analogs interacting with glycogen phosphorylase [31]. The smaller changes in activation energies observed with the enzymes of the sorbitol pathway reflect the broader substrate specificities of these enzymes, as a consequence of the smaller number of strong and specific hydrogen bonding interactions with the substrates.

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