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Fine print in isotope effects: the glucose anomeric equilibrium and binding of glucose to human brain hexokinase

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Abstract Binding isotope effects are a sensitive measure of changes in molecular vibrational character that occur during ligand-receptor binding. In this study, we have measured isotope effects on the binding of glucose to human brain hexokinase using the ultrafiltration method, with the following results: 0.991±0.001, 0.908±0.003, 1.010±0.001, 0.974±0.002, 1.022±0.002 for [^{14}C]-glucose mixed with [1-^{3}H]-, [2-^{3}H]-, [3-^{3}H]-, [5-^{3}H]-, [6,6-$^{3}H_2$]-glucose, respectively. Comparing the observed data with isotope effects on the anomeric equilibrium in glucose reported previously [3] proves the existence of binding isotope effects in this system. Preliminary computational results are presented to explain the observed binding isotope effects in terms of hydrogen bond patterns and molecular crowding found in the binary complex of sugar and enzyme.

Key words anomeric equilibrium • binding isotope effects • computational chemistry • conformational equilibrium isotope effects • glucose • hexokinase

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Introduction

Isotope effects reveal differences in the vibrational properties of a molecule as it passes between two states. They have been used extensively in chemistry and enzymology to probe structure changes in transition state formation. More recently, equilibrium isotope effects have been used to investigate substrate-enzyme binding reactions. We have undertaken the measurement of binding isotope effects for glucose binding to human brain hexokinase in order to: 1) estimate the impact of binding isotope effects on kinetic isotope effect studies and transition-state determination, and 2) obtain information on the structural changes carbohydrates undergo upon binding their receptors. We have found that isotope effects exist for both the binding of glucose to human brain hexokinase and for the anomeric equilibrium of glucose in water. The data were taken using radioisotope competition studies for the binding studies, or by ^{13}C-NMR spectroscopy for studies of the anomeric equilibrium. *Ab initio* electronic modeling is necessary to understand the underlying processes. The results of these computations and studies are presented herein.

The binding reaction and binding isotope effects

Physiologically, hexokinase binds glucose first and MgATP second, although high concentrations of nucleotide can reverse the order. In the absence of MgATP, glucose forms a catalytically competent binary complex with the enzyme [4]. Combining ^{3}H- and ^{14}C-labeled glucose with enzyme results in isotopic competition for enzyme binding

in a dynamic equilibrium. The unbound glucose can be separated from the equilibrium binding reaction by filtration through a semipermeable membrane and the glucose--bound and glucose-free components are analyzed for isotopic ratio. A change in radiolabel ratio indicates which of the labeled glucose molecules prefers to bind to the enzyme. In this way, we measured the following binding isotope effects in this system: 0.991±0.001, 0.908±0.003, 1.010±0.001, 0.974±0.002, 1.022±0.002 for [^{14}C]-glucose mixed with [1-^{3}H]-, [2-^{3}H]-, [3-^{3}H]-, [5-^{3}H]-, [6,6-$^{3}H_2$]-glucose, respectively (unpublished observations). Values greater than one are called "normal effects", and indicate that the C-H bond to the radiolabeled hydrogen is more vibrationally loose when glucose is bound to the enzyme. "Inverse" isotope effects (values less than one) indicate a vibrationally constrained environment when bound, compared to the solution structure.

Binding isotope effects have typically been analyzed in terms of ground state destabilization, so that these isotope effects would represent the enzymatic attempt to get a head start toward chemical activation as early as the formation of the binary complex, without nucleotide. However, the data is remarkable for the presence of the largest effect at H2, far from the site of chemistry at the 6-hydroxyl. This "inverse" isotope effect of over 9% indicates a strongly tightened CH bond at the site most distant from the reactive nucleophile O6. It is unlikely that the enzyme achieves chemical activation of O6 through interactions it might have at the C2 center. What then does this isotope effect reflect? One purpose of this work is to explain this unusual binding isotope effect.

Anomeric equilibrium: a complicating factor?

Hexokinase will utilize either α- or β-glucose as phosphoryl acceptor from ATP and does not catalyze the anomeric equilibrium. Therefore it must be possible to bind either anomer. In investigating the reasons behind binding isotope effects, one must consider the entire binding reaction (Fig. 1). It would be possible for the observed binding isotope effect to result from the combination of an anomeric equilibrium isotope effect combined with different dissociation constants for the two anomers. For example, a "normal" isotope effect on conversion of α-glucose to β-glucose would enrich the α-glucose pool with tritium. If the α-sugar exhibited a tighter dissociation constant for the enzyme,

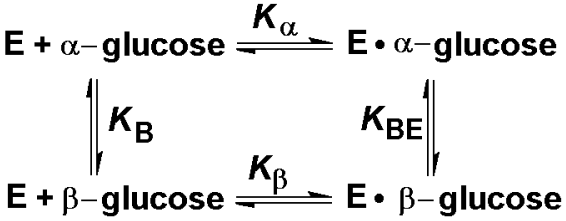

Fig. 1. The binding of glucose to human brain hexokinase. Each anomer binds hexokinase with a slightly different affinity in other systems, this could lead an anomeric equilibrium isotope effect to be interpreted as a binding isotope effect. The constants represent the anomeric equilibrium (K_B), dissociation constants for the binary complexes of hexokinsase with α or β-D-glucose (K_α and K_β) and for the anomeric equilibrium while bound to the enzyme (K_{BE}).

one would observe that the enzyme-bound glucose would be enriched in tritium. This experiment could be interpreted as a binding isotope effect, but in fact it would simply be the manifestation of a solution equilibrium isotope effect. Proving the existence of binding isotope effects in this system requires a more careful analysis.

Dissociation constants and equilibrium isotope effects

Fluorescence titration was performed with the individually recrystallized anomers of glucose, and the independent K_d values were determined to be 15 μM and 23 μM for α- and β-glucose, respectively. Using inverse-degated H-decoupled ^{13}C NMR spectroscopy; we found isotope effects of 1.043±0.004, 1.027±0.005, 1.027±0.004, 1.001±0.003, 1.035±0.004, and 0.998±0.004 for the 1-d, 2-d, 3-d, 4-d, 5-d, 6-d_2 substitutions, respectively [3]. The convention used is for the equilibrium conversion of α-glucose to β-glucose, ($^{D}K_{\beta/\alpha} = {}^{1}K_{\beta/\alpha} / {}^{2}K_{\beta/\alpha}$).

If the binding isotope effects were due only to the anomeric isotope effects, then the relative magnitudes of the anomeric effects would be conserved in the binding isotope effects, and this is clearly not the case. Furthermore, the difference in affinity for the two sugars in binding hexokinase is not great enough to transform any anomeric equilibrium isotope effect into a significant contributor to the observed binding isotope effect (Table 1). Therefore, we conclude that the observed binding isotope effects represent an expression of the actual binding reactions for the two separate sugars, independent of the anomeric equilibrium between the free sugars.

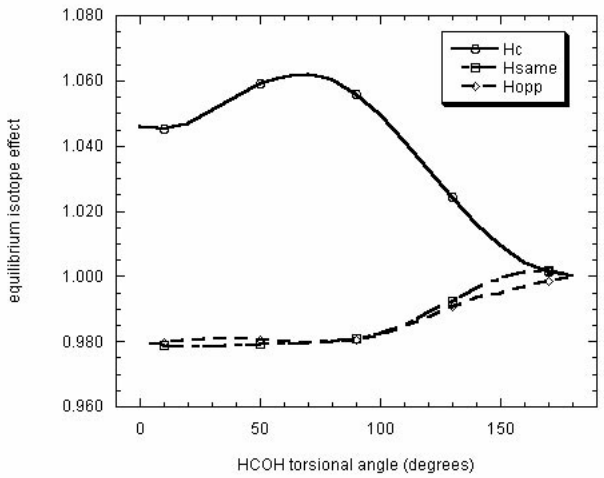

Fig. 2. Angular variation of CH bond strengths in 2-propanol. Density functional theory calculations in 2-propanol predict an angular dependence in bond strength for the CH bond geminal to the hydroxyl (Hc). The vibrational force constants governing this bond are greatest when the hydroxyl torsional angle is 180 degrees and least at approximately 70 degrees, the angle that corresponds roughly to the point of maximal geometric overlap between the oxygen *p*-type electron lone pair and the CH bond antibonding σ* orbital. As the angle is changed to 0 degrees, this overlap is lost but the force constants do not regain the strength of the 180 degree geometry. This is due in part to the stiffening of the bending modes at 180° in the presence of the bulky *sp*-type main lobe orbital. The antiperiplanar CH bonds are also shown here. H_{same} refers to the CH bond that is antiperiplanar to the central CH bond and proximal to the OH proton in the asymmetrical geometries. H_{opp} refers to the other antiperiplanar CH bond. These bonds show the opposite trend, and this is probably due to variation in their interaction with the central CH bond as its length changes.

Table 1. Contribution of anomeric EIE to isotope effects on Glu binding to brain hexokinase

		K_α/K_β**				
^{3}H-substitution	$^TK_{\alpha-\beta}$*	0.15	1.00	1.50	15.00	^TK_A
[1-^{3}H]-glucose	1.063	0.975	1.000	1.006	1.022	0.991
[2-^{3}H]-glucose	1.039	0.984	1.000	1.003	1.013	0.908
[3-^{3}H]-glucose	1.039	0.984	1.000	1.003	1.013	1.010
[4-^{3}H]-glucose	1.001	1.000	1.000	1.000	1.000	–
[5-^{3}H]-glucose	1.053	0.979	1.000	1.005	1.018	0.974
[6,6-$^3H_2$]-glucose	0.997	1.001	1.000	1.000	0.999	1.022

* Extrapolated from deuterium isotope effects measured in Ref. [2] by the Swain-Schaad relationship.
** Ratio of dissociation constants for α- and β-anomer with human brain hexokinase.

Modeling the anomeric equilibrium isotope effects

Gas phase isopropanol

Using isopropanol as a cutoff model for the secondary hydroxyls of glucose, we have found that the ternary CH bond strength varies with hydroxyl torsional angle (tightest at 180 degrees and smoothly varying to a normal isotope effect of almost 1.05 at 0 degrees); furthermore, the methyl hydrogens antiperiplanar to this central CH bond vary with an opposite trend (Fig. 2). These antiperiplanar hydrogens are analogous to neighboring hydrogens in glucose.

An ensemble of glucose rotamers

We minimized and calculated cartesian force constants for a 12-membered ensemble of glucose rotamers (6 alpha, 6 beta) at Hartree-Fock and B3PW91 (density functional) levels of theory with the 6-31G** basis set. These calculations reflected the anomeric equilibrium isotope effect data remarkably well. The gas phase demonstrates several structural features that correlate with calculated isotope effects from the same models. Foremost is the isotope effect for the 2-d molecule. Gas phase α-sugars have a 2-OH torsional angle of about 160 degrees, but for the β-sugars, the same angle is approximately 60 degrees (Fig. 3). Thus for each anomer, the 2-OH group tends to donate its proton to a hydrogen bond formed with one pair electrons of the 1-OH group. This angular change can be demonstrated to give rise to a normal effect of the correct magnitude, as demonstrated via the calculations on isopropanol (Fig. 2).

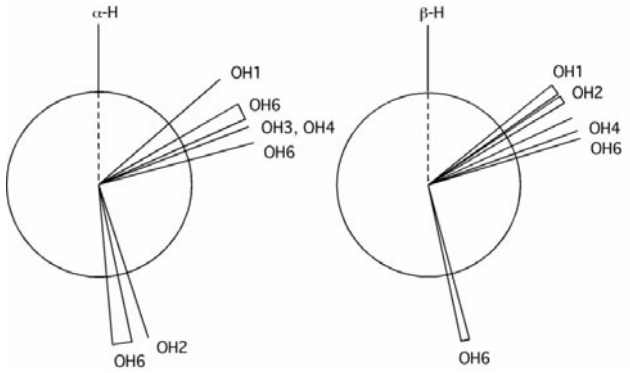

Fig. 3. Hydroxyl torsional angle summary from glucose gas-phase calculations. For clarity, the backbone hydrogens are all represented in the north position and the ends of similarly labeled lines are connected. Only the 2-OH undergoes a major angular change between anomers. (Reprinted by permission from Ref. [4], Copyright Am Chem Soc 2000.)

The isotope effect on H1 can be explained by examining the intramolecular hyperconjugative energies for the *p*-type lone pair of ring oxygen O5. In α-glucose, this lone pair overlaps with the antibonding σ* orbital of C1-O1, giving rise to the famous anomeric effect [2]. However, in β-glucose, C1-H1 now occupies the correct position for maximal overlap from this lone pair, and the antibonding density decreases its bond order and loosens it vibrationally with respect to C1-H1 in α-glucose (Fig. 4). Finally, the models demonstrated no real hyperconjugative differences for H3 and H5, but the models reveal that in α-glucose, the axial O1 atom is bulky enough to impinge sterically on both H3 and H5 (the internuclear distances are 2.64 angstroms and 2.63 angstroms respectively, and the van der Waals distance expected for an oxygen-hydrogen pair is 2.72 angstroms). The models demonstrate no real effect at H4 or H6, in agreement with experiment. Thus, it appears that these equilibrium isotope effects, even though occurring in aqueous solution, may be explained through strictly intramolecular arguments.

Modeling the binding isotope effects

The most obvious caveat to the last statement is that a strictly aqueous environment provides a homogenous set of hydrogen bonding partners, whereas enzyme binding involves acquiring contacts with the enzyme's active site

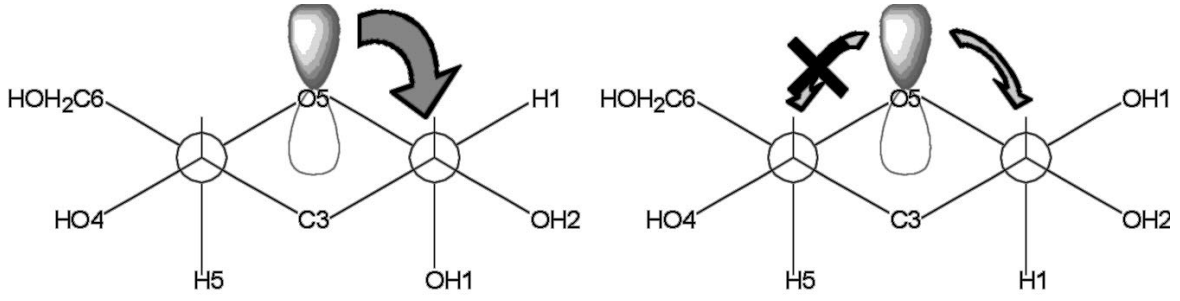

Fig. 4. The anomeric effect in α-glucose (left) contributes to anomer stabilization, but in β-glucose (right) the same orbital interaction serves to loosen the CH1 bond; the CH5 bond experiences no real hyperconjugative change between anomers. (Reprinted by permission from Ref. [4], Copyright Am Chem Soc 2000.)

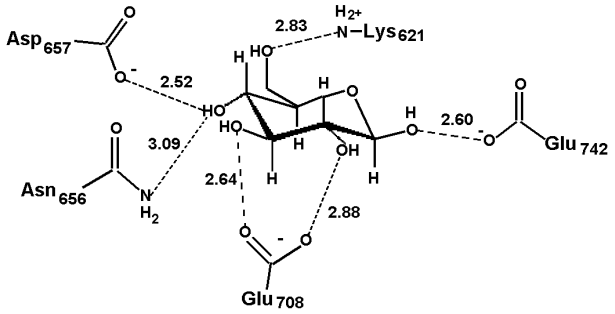

Fig. 5. Active site contacts in glucose-brain hexokinase crystal structure [1]. Each glucose hydroxyl participates in a hydrogen bond with an active site side chain. Amino acids Asp_{657}, Glu_{707}, and Glu_{742} are assumed to be ionized, while Lys_{621} is assumed to be protonated. The actual ionization states are unknown.

residues. The crystal structures of hexokinase from several sources have been solved. In the active site, the 1-, 2-, 3-, and 4-hydroxyls of glucose are in hydrogen bond interactions with glutamate, aspartate, and asparagine residues (Fig. 5), and the 6-hydroxyl is in hydrogen bond distance to an active site lysine residue. Therefore, in modeling the effect at H2, we have studied both 1) the approach of formate into proper hydrogen bonding configuration with the hydroxyl of an isopropanol model with H-C2-O-H torsional angle of 180 degrees (Fig. 6, Panels a and b), and 2) the approach of formic acid into a hydrogen bond with an isopropanol in which the same angle is fixed at 0 degrees (Fig. 6, Panels c and d). It is clear that the approach of a hydrogen donor (as in formic acid) induces an inverse effect, while the approach of an electron donor (as in formate) causes the opposite, a normal effect. This is due to internal hyperconjugative changes involving one or both of the isopropanol oxygen's lone pairs (Fig. 7). An electron donor will pull the hydroxyl proton away from its covalent partner, leaving more electron density behind to loosen the central CH bond through a $n_O \rightarrow \sigma^*_{CH}$ orbital-orbital interaction. Conversely, a proton donor will occupy these oxygen lone pairs to make them less available for such an interaction.

Curiously, even the attack on a hydroxyl by formic acid does not produce an inverse isotope effect as large as the observed -9.0% binding isotope effect. Hartree-Fock calculations of the collision between methane and the CH bond of 2-propanol demonstrate a large inverse deuterium isotope effect which depends on proximity of the two molecules (Fig. 8). As the binding isotope effects were measured using tritium-labeled sugars, it is likely that such a proximity effect could be invoked to explain the binding isotope effect at H2. In fact, adding sugar backbone hydrogens to the crystal structure of Aleshin *et al*. [1] reveals a close contact (2.67 angstroms) between the carbonyl oxygen of Ser-603 and H2 itself (Figure not shown). It is quite possible that this interaction is responsible for the inverse binding isotope effect. Similar close contacts are found between H5 and Arg-683 and may be responsible in part for the -2.5% binding isotope effect at that position. We are compiling a summary of contact distances for each backbone proton in each of the approximately 30 solved crystal structure active sites available.

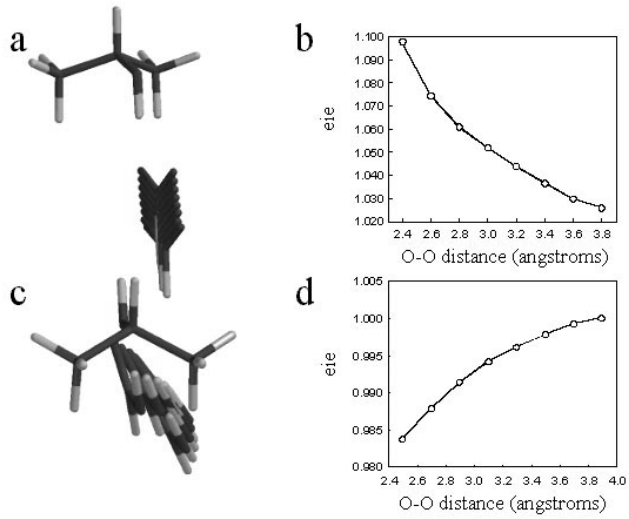

Fig. 6. Distance to hydrogen-bond partner affects CH bond strength. Panels a and b show the approach of formate into a hydrogen bond with 2-propanol, where the hydroxyl torsional angle of the latter is constrained to 180 degrees. As the O-O distance is decreased, increasing hydrogen-bond strength, the central CH bond is weakened. Strengthening the hydrogen bond leaves more electron density on the isopropanol oxygen and increases $n \rightarrow \sigma^*$ hyperconjugation, lowering CH bond order. The opposite effect is seen as formic acid is brought closer to 2-propanol constrained instead at 0 degrees (reverse view, Panels c and d). The closer proximity of the formic acid proton to the 2-propanol oxygen preoccupies the latter's electron lone pairs and decreases the $n \rightarrow \sigma^*$ overlap, increasing bond order and force constant magnitude.

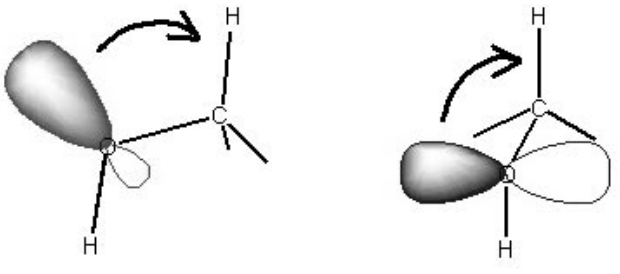

Fig. 7. Hyperconjugative overlap in 2-propanol. Each of the two oxygen lone pairs gives a nonzero overlap with the central CH bond only when oriented parallel to the target σ^* orbital. The *sp*-type lone pair (left) demonstrates maximum overlap when the hydroxyl torsional angle is 0 or 180 degrees, while the *p*-type orbital, perpendicular to the *sp*-type lone pair, demonstrates maximal overlap at 90 degrees.

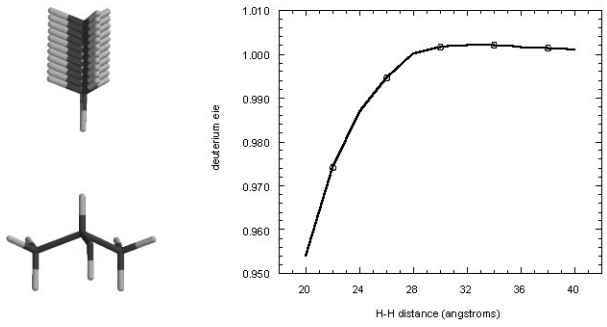

Fig. 8. Calculation of isotope effects induced by the collision between methane and 2-propanol. Contacts closer than 3.2 angstroms give rise to inverse equilibrium isotope effects (right). This is probably due to increasing the force constant for the CH stretching vibrational mode.

Conclusion

Binding isotope effects are large enough to be measured for glucose and human brain hexokinase. Isotope effects on the equilibrium constant between glucose anomers also exist. While such prebinding isotope effects may perturb binding studies, this is not the case for glucose binding to human brain hexokinase. The anomeric data independently represent an opportunity to understand more deeply both the behavior of aqueous glucose and conformational equilibrium isotope effects.

Computational studies reflect the anomeric isotope effects and suggest that intramolecular orbital-orbital hyperconjugative interactions and intramolecular steric interactions play the largest role in anomeric vibrational differences. We also observe that there exist preferred hydroxyl orientations for glucose in water, despite the myriad available hydrogen bonding partners. Naturally, water lattice structure may also play a role, and this lattice-substrate interaction remains open to further investigation.

Binding isotope effects reveal information about the charge states of active site residues and the preferred hydroxyl orientations when glucose is bound. We also find it likely that the binding isotope effects reflect close contacts between sugar backbone hydrogens and active site residue hydrogens or heavy atoms. It is also possible that the sum of the binding interactions between hexokinase and glucose cause distortion of bond angles at sp^3 carbon centers and also contribute to the isotope effects. Studies are underway to distinguish these effects. This represents information of the highest resolution yet available for protein-carbohydrate interactions.

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