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COMMUNICATIONS

Published on Web 03/07/2003

Glucose Binding Isotope Effects in the Ternary Complex of Brain Hexokinase Demonstrate Partial Relief of Ground-State Destabilization

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The average adult human's 1.4 kg brain will consume 77 mg of glucose and 46 mL of O_2 per minute while resting.[1,2] To be funneled into brain glycolysis, glucose is transported across the plasma membrane and rapidly phosphorylated by hexokinase. This step is intricately controlled and represents a necessary structural modification to prevent diffusion of the molecule back to the blood supply.

The mechanism of brain hexokinase is well understood, including binding order,[3] ATP transition state structure,[4] and identification of Asp657 as the general base responsible for deprotonating OH6.[5,6] However, the extent to which the nucleophilic O6 is activated in the ternary Michaelis complex is unknown. Previous work has reported evidence for ground-state destabilization of glucose in the binary complex due to close contact with this general base.[7] Recently, a high-resolution crystal structure of human hexokinase I containing glucose and ADP bound in the active site has been solved and used to construct a model for the ternary Michaelis complex.[8] The goal of our work has been to study the ternary complex from the perspective of binding isotope effects for glucose.

We have used isotope effects on the equilibrium constant of association for glucose binding to hexokinase in the absence or presence of Mg^{2+} and β-γ,CH_2-ATP (at $15 \times K_i = 30$ mM) via the ultrafiltration method[7,9] of simultaneous isotope competition. We have previously found isotope effects for the binary complex between glucose and enzyme (Table 1), and the present data show how sugar binding contacts change in the ternary complex of sugar, enzyme, and $MgATP^{2-}$ analogue.

We observe in Table 1 that three isotope effects change upon addition of nucleotide: H1, H5, and H6. Phenomena that cause binding isotope effects are well understood. The steric imposition of an active site group directly upon a CH bond causes an inverse isotope effect on the binding equilibrium constant. Normal effects occur on full or partial deprotonation of neighboring groups.[10−13] The latter result is due to increased potential energy of heteroatom electron lone-pairs, which become more free to donate density to the antibonding orbital of the CH of interest through an increase in n−σ* hyperconjugation. A similar mechanism has been used to explain CH bond effects due to hydroxyl torsional angle changes in 2-propanol.[14]

The isotope effect at H6 was found to change from 1.065 to 1.032 upon nucleotide binding. On the basis of the various crystal structures showing glucose bound to the active site, it was proposed that the large isotope effect in the binary complex was caused by partial deprotonation of OH6 by Asp657.[7] The model proposed by Aleshin et al. for ATP binding in the ternary complex retains Asp657 in hydrogen-bonding orientation with OH6 but not with OH4, shifts Lys621 from hydrogen bonding to the sugar instead to the terminal phosphate, and places that terminal phosphate in approximation to the nucleophilic O6[8] (see Figure 1).

The decrease in isotope effect at CH6 implies that n−σ* hyperconjugation has been attenuated in the ternary complex. This could result from weakened hydrogen bonding between O6 and Asp657, partial protonation of O6 from another source, or partial satisfaction of its Lewis base character in another way. The first two of these represent diminished activation of O6, are anticatalytic, and are therefore unlikely. Instead, we concur with Aleshin's model that O6 is positioned for in-line nucleophilic attack on the γ-phosphate in the ternary complex and further assert that O6 has become partially satisfied as a Lewis base due to this approach (see Figure 2).

The isotope effect at H4 is unchanged between binary and ternary complexes and makes the possibility of a conformational change involving Asp657 unlikely. This point stands in contrast to the model by Aleshin et al., who propose that in the ternary complex,

Table 1. Equilibrium Binding IE for Glc to HK·β-γ,CH_2-ATP

competitive labels	binary[a]	ternary[b]
[1-^{3}H]- + [2- or 6-^{14}C]glucose	1.027 ± 0.002	1.013 ± 0.001
[2-^{3}H]- + [2- or 6-^{14}C]glucose	0.927 ± 0.0003	0.929 ± 0.002
[3-^{3}H]- + [2- or 6-^{14}C]glucose	1.027 ± 0.004	1.031 ± 0.0009
[4-^{3}H]- + [2- or 6-^{14}C]glucose	1.051 ± 0.001	1.052 ± 0.003
[5-^{3}H]- + [2- or 6-^{14}C]glucose	0.988 ± 0.001	0.997 ± 0.0009
[6,6-$^3H_2$]- + [2- ^{14}C]glucose	1.065 ± 0.003	1.034 ± 0.004

[a] Reference 7. [b] $K_i = 1.8 \pm 0.5$ mM for β-γ,CH_2-ATP determined competitively against ATP in competitive assay. Conditions for the present experiments were identical to those for the binary complex,[7b] with the addition of 30 mM nucleotide analogue and 31 mM Mg^{2+}.

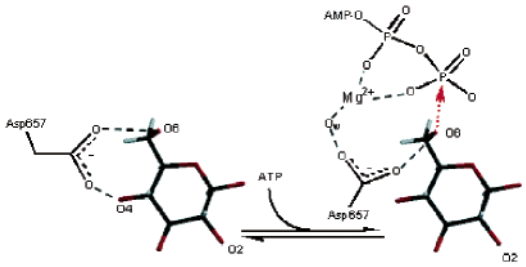

Figure 1. In the model for trinucleotide binding proposed by Aleshin et al.[8] (schematically depicted here), O6 is positioned to attack the γ-PO_4 of ATP, but OH4 loses Asp657 as the hydrogen-bond partner. The conformation of glucose is taken from 1DGK.

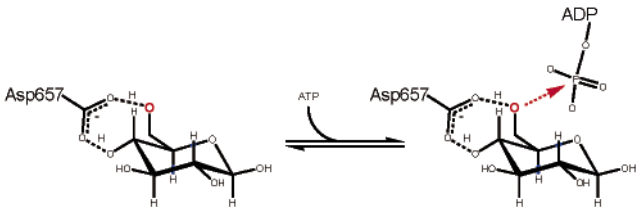

Figure 2. Binding isotope effects indicate that Asp657 remains hydrogen-bonded to both OH4 and OH6, activating the latter for attack on the terminal phosphoryl group of ATP.

10.1021/ja029852k CCC: $25.00

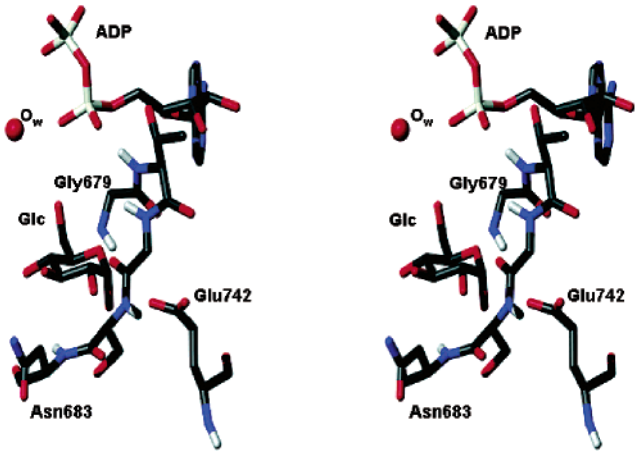

Figure 3. Stereoview of glucose and ADP bound in the enzyme active site shows that nucleotide binding can transmit conformational change to Glu742 and Asn683. Structure taken from 1DKG (Aleshin et al.[8]).

Asp657 is hydrogen-bonded to a water molecule of a hydrated Mg^{2+} ion instead of to OH4.

Constant isotope effects at H2, H3, and H4 strongly imply that the active site contacts to OH2, OH3, and OH4, as well as to CH2, remain unaltered on formation of the ternary complex. The isotope effects at these positions have been interpreted as resulting from a steric conflict at H2 with Ser603 and from partial deprotonation by active site Glu708 and Asp657 at OH3 and OH4, respectively.[7]

In light of the changed effects at H5 and H1, we can consider that the plane of glucose bound in the ternary complex may be rotated clockwise about an imaginary O3−O4 axis with respect to the binary position. Such a motion would preserve interactions at OH2 and OH4, and approximately so at OH3, while relieving the steric contact between H5 and Asn683 and altering (potentially weakening) the hydrogen bond between OH1 and Glu742. However, this explanation seems unlikely, especially because the steric inverse isotope effect at H2 is unchanged in the ternary complex. Considering the active-site loop conformational changes proposed for formation of the ternary complex,[8] it is remarkable that the isotope effects at H2, H3, and H4 do not change in any systematic way and instead support minimal changes in glucose contacts on forming the ternary complex other than at H1, H5, and H6.

Instead, the altered effect at H5 together with the unchanged effect at H2 suggest that ATP binding shifts the positions of Asn683, relieving its steric imposition on CH5, and of Glu742, weakening that residue's hydrogen bond with OH1. From the structures obtained by Aleshin, it appears possible that nucleotide binding transmits a conformational change to both residues via Thr680 (shown to bind to α-PO_4 of ADP), and this could affect the strength of hydrogen bonding between Glu742 and OH1 (see Figure 3) as well as the steric contact at H5 with Asn683.

In summary, the binding isotope effects reported here are consistent with the model by Aleshin et al., except for the position of Asp657 in the ternary complex, and support the idea that hexokinase utilizes ground-state destabilization of glucose alone and accomplishes partial nucleophilic attack by O6 on the bound nucleotide in the Michaelis complex. The changed isotope effects between binary and ternary complexes are attributed to steric and hydrogen-bonding/hyperconjugative causes (see Table 2).

Table 2. Factors Affecting Change in Tritium BIE between Binary and Ternary Complexes

label	binary[a]	ternary[b]	steric	hyperconj	total (%)
1	1.027	1.012		−1.5	−1.5
2	0.927	0.929			
3	1.027	1.031			
4	1.051	1.052			
5	0.988	0.998	+1.0		+1.0
6	1.065	1.032		−3.3	−3.3

[a] Reference 7. [b] Extrapolated as 94% contributor to observed ternary BIE in Table 1 (because [I] = 15 × K_i).

The present interpretations rely heavily upon an accurate picture of active-site residues and conformations. There are no crystal structures of a hexokinase-glucose-ATP analogue complex. However, because we can reasonably explain the observed data in terms of the available information, both the active site structures and the present interpretations are probably representative of binary and ternary complexes.

Binding isotope effects are easily measured for both binary and ternary complexes of hexokinase. They provide unique and valuable insights into hydrogen-bond contacts in structures deduced by X-ray crystallography.

Acknowledgment. This work was supported by the National Institutes of Health. The authors would like also to express gratitude to Dr. Kelly Tanaka and to Drs. Philip Williams and Hiromi Morimoto of the National Tritium Labeling Facility (formerly Berkeley, CA) for their assistance in the synthesis of [4-^{3}H]glucose. The completion of this work would not have been possible without their help.

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JA029852K