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Binding Isotope Effects: Boon and Bane

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Summary of recent advances

Kinetic isotope effects are increasingly applied to investigate enzyme reactions and have been used to understand transition state structure, reaction mechanisms, quantum mechanical hydride ion tunneling and to design transition state analogue inhibitors. Binding isotope effects are an inherent part of most isotope effect measurements but are usually assumed to be negligible. More detailed studies have established surprisingly large binding isotope effects with lactate dehydrogenase, hexokinase, thymidine phosphorylase and purine nucleoside phosphorylase. Binding reactants into catalytic sites immobilizes conformationally flexible groups, polarizes bonds and distorts bond angle geometry, all of which generate binding isotope effects. Binding isotope effects are easily measured and provide high-resolution and detailed information on the atomic changes resulting from ligand-macromolecular interactions. Although binding isotope effects complicate kinetic isotope effect analysis, they also provide a powerful tool for finding atomic distortion in molecular interactions.

Introduction

Isotope effects for enzymatic reactions cause a change in rate or equilibrium as a result of an atomic substitution [1]. Enzymatic applications of isotope effects began in the early 1960's [2-4] and became a common tool of mechanistic enzymology following the Sixth Annual Harry Steenbock Symposium in 1976 [5]. Typically, ^2H or ^3H is substituted for ^1H , ^{13}C or ^{14}C is substituted for ^{12}C , ^{15}N is substituted for ^{14}N or ^{18}O is substituted for ^{16}O in otherwise normal substrates [6]. Reaction rates and binding equilibria are sensitive to isotopic substitution because increased atomic mass alters the bond vibrational environment of reactants [1]. Changes in bond vibrational status surrounding the isotopic substitution between free and bound states give rise to a binding preference for light or heavy isotope in the bound state and hence an experimentally accessible isotope effect. Likewise, differences between bond vibrational environments between free substrate and that at an enzymatic transition state report on bond vibrational environments at the transition state [7].

Experimental measurement of isotope effects typically uses a mixture of labeled and unlabeled reactants with individual isotopic substitutions at each atom of interest. The enzyme discriminates between the labeled and unlabeled reactants to generate an experimentally accessible isotope effect. For catalysis, competitive measurements of this type are called $k_{\text{cat}}/K_{\text{m}}$ kinetic isotope effects (KIEs, or V/K KIE) and values obtained by competitive methods include every step between substrate binding and the first irreversible step in the reaction (Figure 1) [8]. Although V/K KIEs are a combination of binding and catalytic isotope effects, they are useful since they can be used to distinguish between the bond vibrational environment

of the reactant free in solution compared to the enzyme-directed transition state [9]. By measuring binding isotope effects (BIEs) specifically [10], atomic distortion upon formation of the Michaelis complex can be understood. Binding isotope effects together with V/K KIEs can separate these distinct effects. As we shall see, BIEs can be insignificant, or can contribute in the same direction or in the opposite direction to the intrinsic KIEs that arise from transition state chemistry. A simple qualitative interpretation of BIEs is that binding of the molecule containing the heavier isotope will be diminished when bond(s) to the heavier isotopic atom are weaker in the bound complex (a normal isotope effect). Conversely, when bonds to the isotopic label are tighter in the bound complex, binding of the heavier isotopic ligand will be preferred (inverse isotope effect). Normal and inverse BIEs will be reported as positive and negative values expressed as percentages, that is, the percent preference for binding the labeled isotopomer. Recent reviews considering the atomic origins and kinetic effects of BIEs in catalysis are important contributions to this field [11•,12••,13••]

BIE in Lactate Dehydrogenase

Anderson's laboratory pioneered the field of BIE by reporting a normal BIE (preferred binding of the light isotope) of 8.5% for the binding of [4-³H]NAD⁺ to lactate dehydrogenase [14]. Thus, interactions of the conjugated nicotinamide ring with the enzyme caused the C4-H bond (the site of hydride chemistry) to weaken in the Michaelis complex. Partial loss of the C4-H bond makes C4 more chemically reactive to the addition of a hydride ion, thus the Michaelis complex is distorted toward the transition state, even before the second substrate (the hydride donor) adds to the catalytic site (Figure 2). Binding of oxamate, an analogue of the lactate/pyruvate substrates is also influenced by a binding isotope effect such that [¹⁸O]oxamate gives an inverse BIE of (negative) -1.6%, thus the bond environment of the ¹⁸O is more constrained on the enzyme than in water. Computational analysis of bond vibrational frequencies of oxamate in water and on the enzyme revealed that a bifurcated hydrogen bond between the oxamate carboxylate and the guanidinium group of an active site histidine caused the inverse BIE (Figure 2) [15]. BIEs that occur with atoms near the reaction center can be interpreted in terms of electron distribution and bond vibrational modes being influenced by close amino acid contacts in the Michaelis complex [16••]. Thus, ionic, steric or H-bond contacts that weaken a covalent bond containing the isotopic label will cause a normal BIE. Contacts between the protein and ligand that constrain or strengthen such an atomic center will cause an inverse BIE, as in the case of [¹⁸O]oxamate (described above), and in one of the BIEs for glucose-hexokinase (described below).

Nucleoside Hydrolase

A nucleoside hydrolase from *Crithidia fasciculata* (a protozoan) exhibited a 5.1% remote V/K KIE with [5'-³H]inosine reacting as the substrate (Figure 3). Although this was a V/K KIE experiment, the [5'-³H] bond is four bonds away from the reaction center, and the general rule for kinetic isotope effects is that effects are negligible more than two bonds away from the reaction center. This surprising isotope effect was interpreted as a BIE caused by distortion of sp³ geometry of the 5'-carbon [17]. An internal hydrogen bond was proposed to exist between the enzyme and the 5'-hydroxyl group such that the 5'-hydroxyl oxygen is placed near the 4'-ribose ring oxygen. In solution, the 5'-hydroxyl is free to rotate, but when fixed at the catalytic site, the fixed alignment of the hydroxyl weakens the C5'-H bonds to give the relatively large normal BIE. This interpretation was founded completely from KIE studies and subsequent x-ray crystallographic structures supported the proposal (Figure 3) [18]. The nucleoside hydrolase result suggested that remote BIEs might be a normal expectation for enzymes rather than isolated anomalies. This expectation was supported by computational and NMR studies on substituted alcohols [17,19].

Hexokinase

The extent and magnitude of BIEs was tested systematically using binary and ternary complexes of human brain hexokinase [20,21]. Tritium was incorporated at every carbon-H bond to give a family of [1-³H]-, [2-³H]-, [3-³H]-, [4-³H]-, [5-³H]- and [6-³H₂]glucoses [20]. Hexokinase-catalyzed phosphorylation of glucose occurs at the C6 primary hydroxyl group, making this the reaction center with all other BIEs being remote (more than two bonds away from the reacting atom, O6). Since distortion of sp³ centers and fixing rotational angles of primary and secondary hydroxyl groups are both known to contribute to bond vibrational changes that give rise to BIEs, isotope effects at each carbon-tritium bond indicate the local bond vibrational environment that is changed upon glucose binding. Tritium BIEs were observed from every carbon-tritium center of glucose in the binary complex of hexokinase•glucose [20]. The magnitude of the isotope effects varied from -7.3% for [2-³H]glucose to 6.5% for [6-³H₂]glucose (Figure 4). Thus the C2-H bond becomes stiffer when glucose is bound to the enzyme but the C6-H bond become weaker upon glucose binding. Position-specific BIEs demonstrated partial deprotonation of O6 by the catalytic site Asp657 and steric compression of C2-H by contact with the carbonyl of Ser603 [20]. BIEs with the ternary complex of hexokinase•glucose•β-γ-CH₂-ATP gave substantial changes at C6-H₂, C5-H and C1-H (Figure 4). Using the crystal structure of human hexokinase, the changed BIEs for the ternary complex were interpreted in terms of conformational changes of Glu742 positioned near C1 and Asn683 located under the glucosyl ring near C5 (Figure 5) [21]. Computational modeling with 2-propanol was used to interpret isotope effects at each position. Significant BIEs occur with subtle bond length, angle and sp³ geometric distortions that are too small to be observed by crystallography, but when combined with crystallography, interpretation of the BIE effect changes is augmented [21]. The isotope effects on the α-, β-anomeric equilibrium of glucose were also considered and were shown to have an insignificant effect on the BIE shown in Figure 4. The transition state structure for the anomeric equilibrium of glucose has also been established based on intrinsic KIEs [22•].

Thymidine Phosphorylase

Human thymidine phosphorylase (TP) catalyzes the reversible phosphorylation of thymidine to give thymine and α-D-2-deoxyribosyl 1-phosphate (Figure 6). The enzyme is an angiogenic factor and is involved in the metabolism of 5-F-uracil [23]. Unlike most N-ribosyl transferases, the arsenolysis reaction catalyzed by TP has a near-symmetrical nucleophilic displacement that characterizes its transition state [24]. Although this conclusion has been disputed by computational proposals [25-27], there is no other logical interpretation for the large (13.9%) [1'-¹⁴C]thymidine kinetic isotope effect [24,28]. A remote kinetic isotope effect for the [5'-³H₂]thymidine was 6.1%, similar to that reported earlier for nucleoside hydrolase and attributed to a BIE [17,28]. BIEs are readily measured for two-substrate reactions but not for hydrolases like nucleoside hydrolase. Thymidine phosphorylase provided an opportunity to distinguish BIEs from V/K KIEs. A BIE for [5'-³H₂]thymidine was measured to be 6.0%, within experimental error of the V/K KIE. Therefore, the remote [5'-³H₂]thymidine V/K KIE is a BIE developed fully on formation of the Michaelis complex and independent of transition state formation. As the reaction proceeds from the Michaelis complex to the transition state, no additional changes occur to the electronic environment of the C5'-H bonds. The C5'-H bonds are weaker in both the Michaelis complex and at the transition state than in thymidine free in solution [13,28]. Crystal structures of human TP are not sufficiently developed to provide geometric information on the disposition of the 5'-hydroxyl at the catalytic site [29,30], but distortion of the sp³ center at C5' to increase freedom in the out of plane bending modes for the H5' atoms or freezing of the hydroxyl geometry to increase the length of the C5'-H bonds can cause these remote effects (Figure 7) [17,31].

Purine Nucleoside Phosphorylase

Genetic deficiency of human purine nucleoside phosphorylase (PNP) causes apoptosis in dividing T-cells making PNP a target for T-cell leukemias and autoimmune diseases [32,33]. The transition state structures for mammalian PNPs have been used to design transition state analogues with pM affinity [34,35•]. Two of these PNP inhibitors are in clinical trials for T-cell diseases [33,36]. Analysis of the V/K KIEs for arsenolysis of [5'-³H₂]inosine indicated a 5.4% KIE [37]. As the C5'-H bonds are four bonds from the reaction center, it was assumed that these isotope effects arise primarily from binding effects as was found for the remote BIE for TP (see above). Crystallography of mammalian PNPs with substrate nucleosides or transition state analogues has shown the 5'-hydroxyl to be in a hydrogen bond with His257 to locate the hydroxyl oxygen within 2.8 Å of the O4' in the ribosyl ring in both the Michaelis complex and in complexes with transition state analogues [38] (Figure 8). Direct BIE experiments established that only 1.5% of the V/K KIE is generated in the Michaelis complex [39••]. An additional 4.6% isotope effect occurs as the Michaelis complex is converted to the transition state. Both BIE and V/K KIE are in the same direction, indicating partial weakening of C5'-H bonds in the Michaelis complex and additional bond vibrational freedom for these bonds at the transition state [39••].

Mutations His257Phe, His257Gly and His257Asp were generated to probe the contributions of the C5'-hydroxyl His257 to BIE and KIE in human PNP. These three mutations altered the BIE values to 2%, 2% and -2%, respectively, while the transition state contribution further altered the C5'-H bond vibrational environment to contribute -3%, -14% and 7% from the intrinsic KIE, respectively [39••]. These surprising results establish that forces in the Michaelis complex, established from the BIEs, can be reversed or enhanced by the formation of subsequent forces developed in the transition state. Crystal structures of these mutants with transition state analogues at their catalytic sites and computational analysis of the BIE values indicated that polarization of the O5'-H bond both in the Michaelis complex and at the transition state is a dominant factor contributing to these effects [39••].

Adenosine Deaminase

Adenosine deaminase (ADA) uses a catalytic site Zn²⁺ to ionize a bound water molecule to a reactive hydroxyl ion that attacks C6 of adenosine to form a tetrahedral Meisenheimer intermediate with the subsequent protonation and loss of the amino group (Figure 9). The transition state structures of human, bovine and *Plasmodium falciparum* (a malaria parasite) demonstrate that the transition states are close to the covalent intermediate, but that the state of N1 protonation differs between these three enzymes [40••]. In the measurement of the V/K KIE values for atoms involved in the reaction coordinate, [1'-³H]adenosine, with the isotope located five bonds away from the reactive center, was used as a remote label. Surprisingly, this center showed remote isotope effects of 1.2% for *P. falciparum* ADA, 0.7% for human ADA and 0% for the bovine ADA. Possible origins for this unexpected remote isotope effect include rotation around the N-ribosidic bond, altering the electron distribution to C1'-H or sp³ geometric distortion arising from asymmetrical binding interactions of the adenine and ribosyl portions of the substrate. Binding interactions can differ between Michaelis and transition states and the origin of these recently observed isotope effects have not yet been assigned. However, they provide a potentially interesting tool to use in analysis of conformational interactions for nucleosides or oligonucleotides at the catalytic sites of the large number of enzymes using these structurally complicated substrates.

Conclusions

Although BIEs for enzyme-catalyzed reactions have been known since 1989, it is only in the last few years that attempts have been made to quantitate both BIEs and KIEs in specific reactions. When this is done, atomic alterations occurring in the Michaelis complex can be clearly distinguished from those in the transition state. From the few examples now available, the magnitudes of binding isotope effects are large enough to require consideration in full analysis of isotope effect investigation of enzymatic transition states. Importantly, BIEs from the Michaelis complex do not always cause isotope effects that are in the same direction as those generated at the transition state. Thus, competitive V/K KIEs can arise completely from BIEs or BIE values can be negligible, can contribute to or can decrease the observed value of the experimentally determined V/K KIE values. Despite the added complication BIE values add to the analysis of isotope effect studies, BIEs measured alone have potential for understanding geometric and electronic distortions of ligands interacting with macromolecules. Catalysis is not involved in BIEs, so binding interactions can potentially be mapped for any ligand binding event, including interactions with receptors.

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Figure 1.

The relationship between binding isotope effects (BIE), kinetic isotope effects measured by competition between isotopically labeled and unlabeled reactants (V/K KIE) and intrinsic kinetic isotope effects (intrinsic KIE) using the arsenolysis reaction of purine nucleoside phosphorylase as an example [39••]. In this reaction inosine (Ino) is converted to hypoxanthine (Hx) and ribosyl 1-arsenate (R1As) as immediate products. R1As is unstable and hydrolyzed rapidly. V/K KIE report on bond vibrational differences between the labeled reactants free in solution and those at the transition state when its formation is irreversible, thus mixing BIE and the isotopes from chemistry from the intrinsic KIE. BIE and V/K KIE measurement permits solution of the intrinsic KIE. Isotope effects from substrate distortion on binding can then be compared to those generated in the chemical step.

Figure 2. Crystal structure contacts between horse liver lactate dehydrogenase (LDH), NAD⁺ and oxamate (PDB ID 1I0Z) and illustrated in [16••]. The binding isotope effects are from [14, 15].

V/K KIE for Nucleoside Hydrolase

Figure 3. Kinetic isotope effects near the site of bond loss (the C-N bond connecting ribose and hypoxanthine) compared to the remote 5'- ^3H V/K KIE for nucleoside hydrolase from *Crithidia fasciculata* [17].

Binary and **ternary** BIE for human brain hexokinase **Figure 4.**

Binding isotope effects for individually labeled ^3H -labeled glucose molecules binding to the catalytic domain of human brain hexokinase [20,21]. The binary complex consists of glucose and hexokinase. The ternary complex includes the β,γ -methylene-ATP in complex with magnesium ion.

Figure 5.

Amino acid contacts to glucose in the catalytic site of human brain hexokinase [21,41]. Serine 603 is not shown here, but is in steric contact with the hydrogen at the 2-H of glucose, resulting in an inverse isotope effect for this position (Figure 4). The interactions of Asn656 to the 4-hydroxyl group are weak compared Asp657 and Asn683, indicated by the wavy lines.

Figure 6.

The reaction catalyzed by human thymidine phosphorylase [24]. The transition state is unusual for an N-ribosyltransferase reaction by featuring a nearly symmetrical nucleophilic displacement reaction. Other enzymatic N-ribosyltransferases feature dissociative transition states, as does the chemical reaction [42•].

Binding and V/K KIE for human thymidine phosphorylase

Figure 7.

Comparison of BIE (in black) and V/K KIE (in blue) for human thymidine phosphorylase. The KIE reported here are for arsenolysis of thymidine. Use of arsenate as the nucleophile introduces an irreversible step following the transition state formation. Binding isotope effects were measured in the presence of sulfate as a non-reactive phosphate mimic [28].

Kinetic isotope effects for human PNP and His257 mutants. V/K KIE, BIE and intrinsic KIE are summarized for [5'-³H₂]inosine.

Enzyme	V/K KIE	BIE	Intrinsic KIE
Native PNP	5.4 ± 0.2	1.5 ± 0.3	4.6 ± 0.4
His257Phe	-0.8 ± 0.3	2.2 ± 0.4	-3.2 ± 0.5
His257Gly	-7.5 ± 0.5	2.4 ± 0.6	-14.1 ± 0.7
His257Asp	4.6 ± 0.4	-2.0 ± 0.5	6.9 ± 0.7

Figure 8.

Alignment of inosine, phosphate and His257 near the transition state in the catalytic site of human PNP (lower figure). Vibrational modes of the protein are coupled into His 257 and to the phosphate to create periodic compression motions of the three oxygen atoms shown in bold [39,43••]. The effects of these compression modes is to destabilize electrons from the central oxygen, causing them to depart with the leaving group, creating a ribooxacarbenium ion at the transition state. The upper table resolves the relative contributions of BIE and the intrinsic KIE to the experimentally observed V/K KIE.

Figure 9.

The reaction catalyzed by adenosine deaminase involves a zinc-activated water and a short-lived covalent intermediate, the Meisenheimer complex (A). Coformycin is a tight-binding transition state analogue of the reaction. Kinetic isotope effects (V/K KIE as shown in Fig. 1) using the indicated atomic labels. KIE were measured with adenosine deaminases from *Plasmodium falciparum* (Pf ADA), human and bovine sources [40••]. The $1'\text{-}^3\text{H}$ isotope effect is five bonds from the deamination chemistry and differs for the three enzymes. It is proposed to be a hyperconjugative binding isotope effect arising from rotation or distortion of the purine ring relative to the ribosyl ring when bound to the specific enzymes.