ELSEVIER

Binding isotope effects: boon and bane

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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.

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Introduction

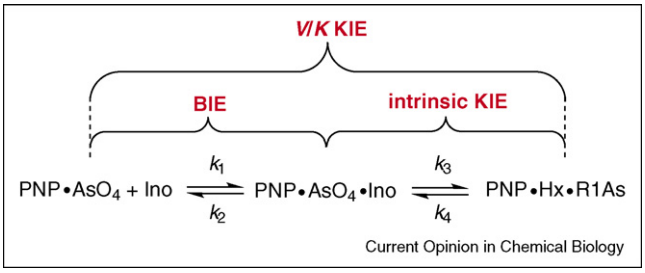
Isotope effects for enzymatic reactions cause a change in rate or equilibrium as a result of an atomic substitution [1]. Enzymatic applications of isotope effects began in the early 1960s [2–4] and became a common tool of mechanistic enzymology following the Sixth Annual Harry Steenbock Symposium in 1976 [5]. Typically, ^{2}H or ^{3}H is substituted for ^{1}H, ^{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_{cat}/K_{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 environments of the reactant free in solution compared with 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 bonds 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-^{3}H]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

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 is 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 isotope effects from chemistry. BIE and V/K KIE measurement permits solution of the intrinsic KIE. Isotope effects from substrate distortion on binding can then be compared with those generated in the chemical step.

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 [^{18}O]oxamate gives an inverse BIE of (negative) −1.6%, thus the bond environment of the ^{18}O 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 [^{18}O]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′-^{3}H]inosine reacting as the substrate (Figure 3). Although this was a V/K KIE experiment, the [5′-^{3}H] 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 sp^3 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′-ribosyl 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-^{3}H]glucose, [2-^{3}H]glucose, [3-^{3}H]glucose, [4-^{3}H]glucose, [5-^{3}H]glucose, and [6-^{3}H$_2$]glucose [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 sp^3 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-^{3}H]glucose to 6.5% for [6-^{3}H$_2$]glucose (Figure 4). Thus, the C2–H bond becomes stiffer when glucose is bound to the enzyme, but the C6–H bond becomes 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-CH$_2$-ATP gave substantial changes at C6–H$_2$, C5–H, and C1–H of glucose (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 sp^3 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