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Triple Isotopic Labeling and Kinetic Isotope Effects: Exposing H-Transfer Steps in Enzymatic Systems

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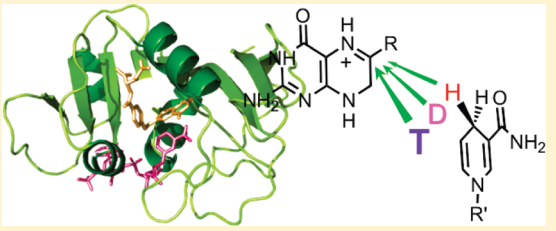
Supporting Information

ABSTRACT: Kinetic isotope effect (KIE) studies can provide insight into the mechanism and kinetics of specific chemical steps in complex catalytic cascades. Recent results from hydrogen KIE measurements have examined correlations between enzyme dynamics and catalytic function, leading to a surge of studies in this area. Unfortunately, most enzymatic H-transfer reactions are not rate limiting, and the observed KIEs do not reliably reflect the intrinsic KIEs on the chemical step of interest. Given their importance to understanding the chemical step under study, accurate determination of the intrinsic KIE from the observed data is essential. In 1975, Northrop developed an elegant method to assess intrinsic KIEs from their observed values [Northrop, D. B. (1975) Steady-state analysis of kinetic isotope effects in enzymic reactions. Biochemistry 14, 2644−2651]. The Northrop method involves KIE measurements using all three hydrogen isotopes, where one of them serves as the reference isotope. This method has been successfully used with different combinations of observed KIEs over the years, but criteria for a rational choice of reference isotope have never before been experimentally determined. Here we compare different reference isotopes (and hence distinct experimental designs) using the reduction of dihydrofolate and dihydrobiopterin by two dissimilar enzymes as model reactions. A number of isotopic labeling patterns have been applied to facilitate the comparative study of reference isotopes. The results demonstrate the versatility of the Northrop method and that such experiments are limited only by synthetic techniques, availability of starting materials, and the experimental error associated with the use of distinct combinations of isotopologues.



Kinetic isotope effect (KIE) experiments compare the reaction rates of reactants that differ only in their isotopic composition (isotopologues).[1−3] As such, these experiments are sensitive to the shape of the reaction barrier while involving no perturbation of the potential energy surface. Since a wide array of analytical techniques can be used to measure KIEs, KIE experiments can be tailored to the particulars of the system being investigated. KIEs are thus a rich source of information for mechanistic chemistry and biochemistry. There are two categories of KIE experiments: noncompetitive and competitive. Noncompetitive experiments measure rates for each isotopologue separately; then the KIE is calculated from the ratio of these rates. Competitive experiments directly measure the ratio of reaction rates by allowing different substrate isotopologues to compete for the enzyme active site in a one-pot reaction and following isotopic fractionation as a function of reaction progress to obtain the KIE.[2] Noncompetitive KIE measurements can yield KIEs on rates from pre-steady-state measurements or on k_{cat} and k_{cat}/K_M from steady-state measurements; however, noncompetitive methods cannot directly evaluate intrinsic KIEs and can suffer from errors due to the presence of different impurities in the isotopologue reaction mixtures. Competitive experiments, while limited to measuring the isotope effect on the second-order rate constant k_{cat}/K_M,[4] are not affected by different contaminants (since both isotopologues compete in the same reaction mixture) and can utilize the radioisotope tritium (^{3}H or T) in trace amounts, thus allowing examination of all three isotopes of hydrogen (H, D, and T or ^{1}H, ^{2}H, and ^{3}H, respectively). Heavy-atom KIEs can be accurately determined with the superior precision of competitive experiments (e.g., ^{15}N V/K effects of 1.0022 ± 0.0003 using an isotope ratio mass spectrometer[5] or 1.034 ± 002 using radioisotopes ^{14}C and ^{3}H).[6] The competitive method is thus the preferred choice for high-precision measurements of KIEs and is the only method to utilize radioisotopes such as T in trace amounts. For example, a competitive experiment measuring H/T KIEs can follow the conversion of the tritiated substrate by direct measurement of radioactivity and that of the H-labeled material using a remote ^{14}C label. The observed H/T KIE (KIE_{obs}) can then be calculated using eq 1:[1]

$$KIE_{obs} = \frac{ln(1-f)}{ln\left[\left(1-f\left(\frac{R_f}{R_\infty}\right)\right)\right]} \quad (1)$$

where the fractional conversion f is the fraction of ^{14}C in the product relative to the total amount of ^{14}C in the sample, and R_f and R_∞ refer to the ratio of ^{3}H to ^{14}C in the products at f and at complete (100%) conversion, respectively. Equation 1 can be adapted for the measurement of H/D and D/T KIEs as well as for the study of binding isotope effects (BIEs; *vide infra* for a

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discussion of the same), though in each case different isotopic labeling patterns must be employed.

Complex reaction cascades, such as those commonly seen in enzymatic reactions, contain many isotopically insensitive steps that can mask the intrinsic KIE (a phenomenon referred to as kinetic complexity). Equation 2 relates the KIE_{obs} to the intrinsic value (KIE_{int}) by accounting for the impact of kinetic complexity:[1]

$$KIE_{obs} = \frac{KIE_{int} + C_f + C_r \cdot EIE}{1 + C_f + C_r} \quad (2)$$

where C_f and C_r are the commitments to catalysis in the forward and reverse directions, respectively, and EIE is the equilibrium isotope effect (see Supporting Information for a more detailed description of C_f, C_r, and the EIE). In order to use KIEs to understand the mechanism of a specific step in a complex kinetic cascade, it is important to "unmask" the KIE_{int} from the KIE_{obs}.[7]

In 1975, Dexter Northrop proposed an elegant method to assess the value of intrinsic hydrogen KIEs from the observed KIEs on the second-order rate constants (k_{cat}/K_M, or V/K) of enzymatic reactions.[1,8,9] In the following discussion of the Northrop method, the observed H/D, H/T, and D/T KIEs are represented by ${}^{D}(V/K)_H$, ${}^{T}(V/K)_H$, and ${}^{T}(V/K)_D$, respectively, while the associated intrinsic KIEs are denoted by k_H/k_D, k_H/k_T, and k_D/k_T, respectively. The Northrop method requires measurement of KIEs for all three hydrogen isotopes and has been expanded to include a variety of experimental designs.[9] A simultaneous measurement of KIEs using all three isotopes is impractical; hence, a combination of two KIEs must be measured for each reaction. The hydrogen isotope that is common to both combinations of measured KIEs then becomes the "reference isotope" in Northrop's nomenclature. For example, if ${}^{T}(V/K)_H$ and ${}^{D}(V/K)_H$ are measured, then H is considered the reference isotope. In this case, the Northrop method uses eq 2 written for ${}^{T}(V/K)_H$ and ${}^{D}(V/K)_H$ and by simple algebraic manipulation of the two equations eliminates the commitments to catalysis and yields eq 3, where the only unknown left is the KIE_{int}:[a]

$$\frac{{}^{D}(V/K)_H - 1}{{}^{T}(V/K)_H - 1} = \frac{(k_H/k_T)^{0.7008} - 1}{(k_H/k_T) - 1} \quad (3)$$

A similar equation can be written to calculate intrinsic KIEs with D as reference

$$\frac{{}^{D}(V/K)_H{}^{-1} - 1}{{}^{T}(V/K)_D - 1} = \frac{(k_H/k_T)^{-0.7008} - 1}{(k_H/k_T)^{0.2994} - 1} \quad (4)$$

and with T as reference isotope:

$$\frac{{}^{T}(V/K)_D{}^{-1} - 1}{{}^{T}(V/K)_H{}^{-1} - 1} = \frac{(k_H/k_T)^{-0.2994} - 1}{(k_H/k_T)^{-1} - 1} \quad (5)$$

The Northrop method requires that the reaction must be irreversible within experimental error (so $C_r = 0$) or, alternatively, that the EIE for the reaction is close to unity; that the mechanism does not branch before the first irreversible step; and that only one step is isotopically sensitive. Another important underlying assumption of the Northrop method is that the Swain−Schaad relations hold for the intrinsic KIEs being considered.[9,10] The Swain−Schaad equations relate the rates of the three isotopes of hydrogen based on their mass differences, and most theoretical studies suggest that these are valid at physiological temperatures most relevant to enzymatic reactions, though could differ at temperatures below 250 K.[11] Note that the original Northrop equations used Swain−Schaad exponents of 1.44 (relating k_H/k_T and k_H/k_D) and 3.26 (relating k_H/k_T and k_D/k_T) calculated based on atomic masses of H, D, and T. However, eqs 3, 4, and 5 employ Swain−Schaad exponents of 1.427 and 3.34, based on reduced mass considerations as per the recommendations of Streitwieser et al.[12] This is not a substantial issue as it has a small effect on the value of KIEs but none on the more important trends (e.g., temperature or pressure dependence of KIEs) and on the relationship between KIEs.

A numerical solution of the three equations simultaneously for the average values of the KIE_{obs}, where KIE_{int} and the two Swain−Schaad exponents are unknowns, appears tempting (i.e., solving three equations with three unknowns). Such a solution would not require the use of the Swain−Schaad assumption. Unfortunately, these are nonlinear equations, and the three variables are interdependent in the numerical solution. Consequently, strict boundary conditions are needed, and the Swain−Schaad relations are the only logical condition at this time. Thus, solving eqs 3, 4, and 5 independently is more useful and also simplifies the error propagation process.

In the past, both H and T have been used as reference isotopes (see ref 7 for examples), but no comparative studies have been conducted to determine whether both reference isotopes result in the same intrinsic value, or if one of these combinations is more advantageous than the others, or if using D as reference actually results in a lower error for the intrinsic KIE, as predicted by Northrop. Northrop has suggested that the isotope used as a reference in this technique plays an important role in determining the sensitivity and accuracy of such experiments.[9] Assuming an intrinsic deuterium isotope effect of 5, and a random error of ±3% on the observed KIEs, Northrop calculated the magnitude of the propagated error on the intrinsic KIE if H, D, and T were each used as reference isotopes.[13] This simulation indicated that the use of H as the reference isotope (eq 3) would lead to the largest errors, while D as the reference isotope (eq 4) would have the smallest error. This is an intriguing prediction since intuitively one would expect the combination of *larger* observed KIEs, i.e. with H as reference, to give smaller relative errors than measuring a combination of smaller KIEs, as is the case if D were used as reference (assuming the same number of experiments performed for each combination). Here, the choice of reference isotope is examined empirically. A direct comparison of all combinations of observed KIEs for the same enzyme, under the same experimental conditions, would greatly aid researchers in choosing the reference isotope.

Here we present such an experimental comparison, using two enzymes that catalyze the same reaction and two different substrates. We have competitively measured primary (1°) KIEs using two nicotinamide-dependent enzymes from *E. coli*: chromosomal, *fol*A-encoded dihydrofolate reductase (cDHFR) and the R plasmid-encoded R67 DHFR.[14] We have assessed the intrinsic KIEs by using all combinations of observed KIEs; i.e., we have used H, D, and T as reference isotopes and extracted the intrinsic KIEs via the Northrop method using all three possible combinations of KIEs. This required the development and synthesis of a new stereospecific labeling pattern of the cofactor NADPH, enabling competitive measurements of ${}^{D}(V/K)_H$ KIEs that had not been attempted previously.

Both cDHFR and R67 DHFR catalyze the conversion of 7,8-dihydrofolate (H_2F) to *S*-5,6,7,8-tetrahydrofolate (H_4F) (Figure 2C). With both enzymes, the pro-*R* hydride from C4 of NADPH is

transferred to the *si*-face of the substrate H_2F.[14] The two enzymes are genetically and structurally unrelated (Figure 2A,B), and the differences in their dynamic flexibility and overall catalytic pathways have been commented upon previously.[15] In the context of this article, it is important to note that under physiological conditions the hydride transfer step is rate-limiting in the catalytic cascade of the R67 enzyme but not for cDHFR (where hydride transfer is only partially rate limiting at pH > 8).[16] In order to examine the effect of different commitments on the method, we have measured all three KIEs for cDHFR with an alternative substrate, dihydrobiopterin (H_2B, in which the PABG tail is truncated): for this substrate, the commitment masking the intrinsic KIEs is reduced relative to H_2F. Since R67 shows no commitments even with its natural substrate H_2F, H_2B was not used for R67 kinetics.

Our results suggest that the reference isotope for KIE experiments should be chosen on a case-by-case basis, and this choice is only limited by the ease with which various isotopologues are synthesized as well as the experimental error associated with a particular experimental design. In light of these findings, we discuss the pros and cons of the different combinations of observed KIEs and suggest some guidelines for the design of effective KIE experiments. To the best of our knowledge, the data presented below are the first direct comparison of all three observed hydrogen KIEs ($^{T}(V/K)_{H}$, $^{T}(V/K)_{D}$, and $^{D}(V/K)_{H}$) as well as the first comparative analysis of the resulting intrinsic KIEs using eqs 3, 4, and 5. Such an analysis allows us to assess the validity of the assumptions associated with the Northrop method and evaluate the benefits of measuring one combination or the other. An additional merit of the work described below is the competitive measurement of H/D KIEs. Tritium KIEs are not commonly measured in noncompetitive experiments due to the requirement of carrier-free tritiated substrate (i.e., >99% tritium at the position of interest, or specific radioactivity of 28 Ci/mmol). Consequently, competitive measurements of $^{T}(V/K)_{H}$ and $^{T}(V/K)_{D}$ cannot be directly compared to $^{D}(V/K)_{H}$ measured by the more commonly used noncompetitive experiments. The method for the competitive measurement of $^{D}(V/K)_{H}$ presented here thus allows direct comparison to noncompetitive experiments. Finally, since about third of all enzymes catalyze some form of hydrogen transfer,[17] and since intrinsic KIEs in such systems can provide direct insight into the chemical step, the current study would impact investigations of nicotinamide-dependent enzymes as well as many other enzymes and biological systems involving H-transfer reactions.

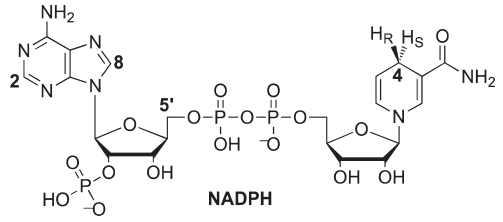

Figure 1. NADPH. The pro-*R* hydrogen (H_R) at C4 of the nicotinamide ring is the hydrogen substituted with D or T in the experiments presented here. In the case of [Ad-^{3}H]-labeled compounds, the ^{3}H is located at position 2 and 8 on the adenine ring, and position 5′ of the attached sugar. For [Ad-^{14}C]-labeled compounds, the adenine ring is universally labeled with ^{14}C. Table 2 summarizes the various labeling patterns available to determine competitive 1°KIEs in pro-*R* specific NADPH-dependent enzymes. The patterns for NADH-dependent enzymes would be the same, but the 2′-phosphate those cases would be replaced by a hydroxyl group.

MATERIALS AND METHODS

Materials. All materials were purchased from Sigma-Aldrich unless otherwise noted. 7,8-Dihydrofolate (H_2F) was synthesized from folic acid as described by Blakley.[18] [Ad-^{14}C]-NADPH, [Ad-^{3}H]-NADPH, 4*R*-[Ad-^{3}H, 4-^{2}H]-NADPH, 4*R*-[Ad-^{14}C, 4-^{2}H]-NADPH, and 4*R*-[4-^{3}H] NADPH were synthesized by previously published chemoenzymatic methods.[19−21] The synthesized cofactors were purified by semipreparative HPLC on a Supelco Discovery C-18 RP column as described elsewhere, lyophilized, and stored at −80 °C, at which temperature these are stable for two years.[22] cDHFR and R67 DHFR were expressed, purified, and stored according to well-established procedures.[15,23]

Methods. 1° $^{T}(V/K)_{H}$ and $^{T}(V/K)_{D}$ experiments with cDHFR and R67 DHFR were performed using experimental procedures described extensively elsewhere.[14,24] For 1° $^{D}(V/K)_{H}$ KIE

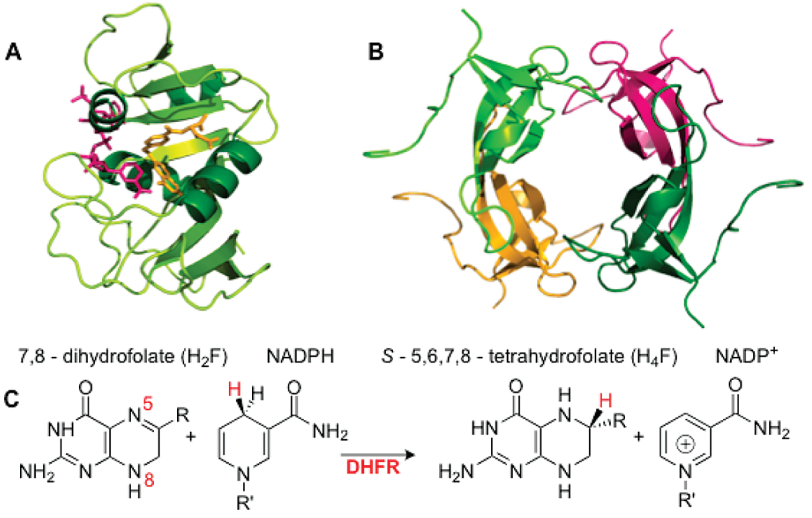

Figure 2. cDHFR, R67 DHFR, and the catalyzed reaction. (A) cDHFR (PDB ID 1RX2) shown bound to folate (orange) and $NADP^{+}$ (pink). (B) Homo-tetrameric R67 DHFR (PDB ID 1VIE). (C) Reaction catalyzed by both cDHFR and R67 DHFR. R = *p*-aminobenzoyl glutamate (PABG); R′ = 2′-phosphoadenosine diphosphate ribose.

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Table 1. Observed $^{i}(V/K)_{j}$ and Intrinsic (k_H/k_T) KIEs for cDHFR and R67 DHFR[a]

enzyme/substrate	$^{T}(V/K)_{H}$	$^{D}(V/K)_{H}$	$^{T}(V/K)_{D}$	k_H/k_T with H as reference	k_H/k_T with T as reference	k_H/k_T with D as reference
cDHFR/H_2F	4.84 ± 0.04	2.92 ± 0.03	1.66 ± 0.02	6.11 ± 0.54	6.16 ± 0.57	6.21 ± 0.50
cDHFR/H_2B	7.11 ± 0.09	3.55 ± 0.01	2.00 ± 0.02	13.28 ± 0.90	13.15 ± 0.89	13.17 ± 0.64
R67 DHFR/H_2F	6.04 ± 0.07	3.51 ± 0.03	1.74 ± 0.02	6.22 ± 0.47	6.66 ± 0.37	6.52 ± 0.33

[a] Standard deviations were calculated as described in the Supporting Information and are reported as ±.

measurements, these procedures were used with some modifications. Briefly, [Ad-^{14}C]-NADPH and 4R-[Ad-^{3}H, 4-^{2}H]-NADPH were combined in a 1:6 radioactivity ratio (to compensate for lower tritium efficiency during LSC counting; we have determined that varying this ratio to 1:4 or 1:9 does not affect the observed KIEs), copurified by RP-HPLC, and divided into 300 000 DPM ^{14}C aliquots for short-term storage at −80 °C. These aliquots were then used in competitive $^{D}(V/K)_{H}$ KIE experiments at 25 °C and pH 9 for cDHFR and pH 8 for R67 DHFR. For cDHFR, the procedure described by Wang et al.[24] was followed with one significant change: since the radioactive labels are remote from the site of hydride transfer, the tetrahydrofolate product need not be oxidized to eliminate problematic variable peaks in the ^{3}H radiogram. We experimentally verified that removal of the oxygen bubbling step did not impact the end result, and all $^{D}(V/K)_{H}$ experiments were subsequently performed without this time-consuming step. Similarly, competitive $^{D}(V/K)_{H}$ KIE experiments for R67 DHFR followed the procedure described in ref 14, but without the oxidation step. For both enzymes, at least two independent experiments were performed for each KIE, with three or more data points obtained per experiment over a broad range of fraction conversions (i.e., at f = 0.2−0.8). Intrinsic k_H/k_T KIEs were calculated using the Northrop method and the appropriate form of the Northrop equation: i.e., $^{T}(V/K)_{H}$ and $^{D}(V/K)_{H}$ KIEs were used in eq 3, $^{D}(V/K)_{H}$ and $^{T}(V/K)_{D}$ KIEs were used in eq 4, and $^{T}(V/K)_{H}$ and $^{T}(V/K)_{D}$ KIEs were input into eq 5. Data analysis was performed in the same manner as before.[24] Lastly, we used [Ad-^{14}C]-NADPH and [Ad-^{3}H]-NADPH as substrates in a binding isotope effect (BIE) experiment with cDHFR, using the same experimental and analytical methodology described above.

RESULTS

Table S1 lists the observed KIEs measured for all reactions, while Table 1 shows the averaged observed KIEs [$^{i}(V/K)_{j}$], along with the intrinsic k_H/k_T values calculated using the Northrop method, viz. numerical solutions to eq 3, 4, or 5. Since it is assumed that intrinsic KIEs closely follow the Swain−Schaad relationships,[10] intrinsic k_H/k_D and k_D/k_T KIEs may be easily calculated from the intrinsic k_H/k_T values. The validity of this assumption is confirmed by the fact that all intrinsic KIEs calculated using different reference isotopes resulted in similar average values (though different standard deviations; see Supporting Information for detailed description of intrinsic KIE calculation and the associated error propagation procedure). For cDHFR with dihydrofolate (H_2F), where H transfer is far from being rate limiting, intrinsic KIEs calculated using H, D, or T as reference isotope are in complete agreement with each other within experimental error. The smallest standard deviation (SD) is found for the intrinsic KIE calculated from D as reference and the largest with T as reference. This result shows that, for the same number of data points, the lowest error is obtained with D as a reference, which agrees with Northrop's simulation.[9] But in contrast to the outcome of that simulation, the largest error is seen when T is used as a reference.

When an alternative substrate (dihydrobiopterin or H_2B) is used with cDHFR, the SDs obtained using either H, D, or T as reference are similar, but the smallest SD is for D as a reference and the largest for H as a reference. From the fact that the intrinsic KIEs for both H_2F and H_2B are larger than the observed KIEs (Table 1), it is clear that the H transfer for the cDHFR reaction is far from being rate limiting. For H_2F, this is in accordance with the conclusions of ref 16 which measured and assessed the rate constants using single turnover experiments. As a control, we conducted the same measurements for the R67 DHFR-catalyzed reduction of H_2F, where the H-transfer step is rate-limiting[14] and where the observed and intrinsic KIEs are identical. Here, too, the Northrop method was used to calculate intrinsic KIEs in order to evaluate the impact of reference isotope on the calculated error. Again, we find excellent agreement between KIEs calculated from all three combinations for R67 DHFR, with the smallest error for D as reference and the largest for H as reference isotope. Thus, for cDHFR with H_2B and for R67 DHFR, the trend in calculated errors for the reference isotopes completely matches Northrop's predictions.[9]

One potential artifact when measuring $^{D}(V/K)_{H,obs}$ is the possibility of binding isotope effects (BIEs) arising from the remote T-labeling of the adenine ring.[25] To address this concern, we measured a BIE of 1.002 ± 0.001 for cDHFR using [Ad-^{14}C]-NADPH and [Ad-^{3}H]-NADPH as substrates and a similar analytical methodology as in the KIE measurements. This confirms that the remote-labeling scheme used in the $^{D}(V/K)_{H}$ measurements does not contribute significantly to the magnitude of isotope effects measured by competitive experiments with cDHFR.

DISCUSSION

The Northrop method has been used for a wide variety of enzymatic systems.[3,17] The validity of this method has, however, been questioned due to the assumptions implicit in its use as noted in the Introduction. While it is not difficult to show that a reaction is practically irreversible or that the EIE is close to unity, the use of the Swain−Schaad relations for the intrinsic KIEs is not straightforward.[11] To assess the robustness of that last assumption and the practicality of determining intrinsic KIEs using the Northrop method, we measured all combinations of competitive KIEs for three different systems involving similar H-transfer reactions but different degrees of kinetic complexity. The intrinsic KIEs calculated with different reference isotopes all result in the same intrinsic KIE (within experimental error). This result indicates that for primary (1°) KIEs, within experimental error (see Table 1), the intrinsic relations between the three isotopes of hydrogen indeed follow the semiclassical Swain−Schaad relations. This finding is of great general interest given the significant degree of tunneling associated with both the cDHFR and R67 DHFR catalyzed reactions.[14,26,27] This outcome has been proposed before but never directly demonstrated and

Table 2. Different Labeling Patterns for Nicotinamide Cofactors in KIE and BIE Experiments[a]

no.	adenine ring label	4*R* label	measured 1° KIE
1	^{14}C	^{1}H	H/T
	none	^{3}H	
2	^{14}C	^{2}H	D/T
	none	^{3}H	
3	^{14}C	^{1}H	H/D
	^{3}H	^{2}H	
4	^{14}C	^{1}H	BIE
	^{3}H	^{1}H	

[a] This table summarizes labeling patterns that may be used to study enzymes utilizing the 4*R*-hydrogen of a nicotinamide cofactor as a reducing agent. The second and third columns indicate the labeling of the nicotinamide cofactor, as per Figure 1. The remote radioactive label on the adenine is a tracer for nonradioactive C4-hydrogen isotope (i.e., H and D). All 1° KIEs reported herein were measured with ^{1}H in the 4*S* position, to eliminate possible effects due to 2° KIEs. Row 4 shows the labeling pattern utilized to measure H/T binding isotope effects (BIEs).

probably results from the similar mass dependence of semiclassical KIEs (mostly affected by different zero-point energies of the three isotopes)[28] and the mass dependence of quantum tunneling in full tunneling models.[17,29]

Note that here we only examined 1° KIEs and 1° Swain−Schaad relationship; this caveat is necessary given the large body of literature where inflated secondary Swain−Schaad exponents from mixed labeling experiments (2° H/T KIE with H transfer but 2° D/T KIE with D transfer) were used to study H-tunneling and coupled motion.[30−32] A new interpretation of that phenomenon suggests that this inflation results from deflation in 2° KIEs due to D transfer from shorter distance than H transfer, while both 1° and 2° KIEs themselves do follow the Swain−Schaad relationship,[32,33] but this issue is not examined here.

The above experiments may also assist in the design of new experiments incorporating the Northrop method. In order to rationally choose the appropriate reference isotope to assess the intrinsic KIEs using the Northrop method, the experimental design should consider all three possible reference isotopes. To establish a set of parameters that need to be considered in this design, we used all three combinations (i.e., H, D, and T as reference isotopes) for three systems with different levels of kinetic complexity and compared the intrinsic KIEs calculated by each method. These measurements included $^{T}(V/K)_{H}$, $^{D}(V/K)_{H}$, and $^{T}(V/K)_{D}$ KIEs with two enzymes that catalyze the same reaction (cDHFR and R67 DHFR), using two different substrates with different kinetic complexity (H_2F and H_2B) for cDHFR. In addition, we also measured the BIE for cDHFR using [Ad-^{14}C]-NADPH and [Ad-^{3}H]-NADPH as substrates.

Northrop's simulations[9,13] suggested that when H is used as the reference isotope, the intrinsic KIE will have the largest error, while the use of D as a reference will yield the smallest error. Our results indicate that the most precise intrinsic KIEs are indeed calculated when D is used as a reference isotope, but the different relative standard deviations are similar in all cases. Furthermore, no general trend was observed when H or T was used as reference isotopes. When D is the reference isotope, however, measurements of of $^{D}(V/K)_{H}$ and $^{T}(V/K)_{D}$ are needed. These require the synthesis and use of four different isotopologues (see Table 2). When H or T is the reference isotope, on the other hand, $^{T}(V/K)_{H}$ and $^{D}(V/K)_{H}$ are measured, which only requires the synthesis of three isotopologues. Consequently, when the small differences in accuracy between the three methods do not affect the qualitative conclusion drawn from the intrinsic KIEs, the ease of preparation of labeled substrates may weigh more heavily in the choice of reference isotope. Also, in some cases only one labeling pattern is feasible. For example, for formate dehydrogenase it is not possible to remotely label the substrate formic acid because it is a very small molecule, and ^{14}C labeling will result in an additional 1° KIE instead of functioning as a remote label.[34] In this case, measuring competitive $^{D}(V/K)_{H}$ KIEs is not possible, and the use of T as reference isotope is the only viable choice. This is also the preferred choice when remote labeling of the substrate that donates the H or D is laborious, but the remotely labeled acceptor is commercially available. One such case is that of the enzyme thymidylate synthase, where the H-acceptor labeled with ^{14}C at a position remote from the reacting atoms is commercially available, but remote labeling of the H-donor is effortful.[35] In short, our studies indicate that there is no general answer to the question posed in the Introduction ("*which isotope should be used as the reference isotope in multiple-isotope experiments?*), and the items presented above have to be considered on a per system basis.

In addition to the choice of reference isotope, other aspects of the experimental setup may also be optimized to lower errors on the observed KIEs and consequently on the calculated intrinsic KIEs. For example, when KIE experiments employ liquid scintillation counting (LSC) as an analytical technique, higher levels of radioactivity and longer counting times per sample increase the total count and lead to more accurate results. LSC measurements are more accurate at low concentrations of analyte than standard UV−vis absorbance or other spectroscopic methods aimed at measuring the fraction of light isotope converted to product (f in eq 1). As discussed extensively by Parkin[2] and many works cited therein, LSC measurements can contain errors from numerous sources, including inaccuracies due to incident ambient light. To minimize error from this latter source, samples containing LSC cocktail should be mixed and incubated for a minimum of 24 h before counting, or consecutive measurements need to be conducted until the value of counts per minutes for T stabilizes. Also, since T counting efficiency is most affected by incident light contributions, an increased ratio of ^{3}H/^{14}C in the reaction mixtures can further decrease errors (the experiments described above used a ^{3}H/^{14}C ratio of ∼6).

In summary, the results presented herein contribute to three critical aspects of the examination of C−H bond activations in enzymes and other kinetically complex systems. First, we have shown that 1° H/D KIEs may be viably measured by competitive experiments. Noncompetitive experiments commonly measure 1° H/D KIEs, and the results of such noncompetitive KIE experiments can now be compared to data from competitive KIE studies. This is important because the noncompetitive experiments commonly use synthesized D-labeled and commercial substrates (H-labeled). Consequently, different contaminants in the H- and D- labeled substrates can lead to artifacts such as inflated KIEs due to inhibitory effects of synthetic byproduct or reagents. Second, the comparison of intrinsic KIEs calculated using three different reference isotopes demonstrates and validates the use of the Northrop methods and its associated assumptions. Finally, these findings will assist in the design of experiments with new enzymatic systems and will enable a rational choice of reference isotope.

While only three systems have been examined here, the isotopic labeling described is on the ubiquitous nicotinamide

cofactor; hence, the methods discussed here are of rather broad use and interest. Furthermore, our conclusions are not limited to this cofactor, and it is likely that studies with other cofactors and substrates will benefit from the findings of the current study, as the essence of these experiments is very general. In the case of isotopic labeling remote from the reaction center, BIEs are a factor that must be taken into account for each new enzymatic system. Since BIEs are typically dependent on the environment of the binding site,[25] it is important that a precautionary BIE measurement be performed each time a new remote-labeling pattern is developed or whenever such a pattern is used to measure KIEs in a new enzyme.

■ ASSOCIATED CONTENT

Supporting Information. A definition of terms in eq 2 for a simplified enzyme reaction; detailed description of intrinsic KIE calculation and propagation of the corresponding errors. This material is available free of charge via the Internet at http://pubs.acs.org.

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■ ADDITIONAL NOTE

[a] Equations 3−5 and Table 1 refer to the intrinsic H/T KIE only because of its larger size relative to H/D and D/T KIEs. Since all intrinsic KIEs are assumed to be related by the Swain−Schaad relationships, one can be calculated from the other. Additionally, it is important to note that these transcendental equations cannot be solved analytically, despite having only one unknown, and are solved numerically (using common packages like MathLab or Mathematica, or the Java-based program we post free of charge on http://cricket.chem.uiowa.edu/~wang11/temp/intrin.html).

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