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Correction of Pre-Steady-State KIEs for Isotopic Impurities and the Consequences of Kinetic Isotope Fractionation†

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We show, both experimentally and by kinetic modeling, that enzymatic single-turnover (pre-steady-state) H-transfer reactions can be significantly complicated by kinetic isotope fractionation. This fractionation results in the formation of more protiated than deuterated product and is a unique problem for pre-steady-state reactions. When observed rate constants are measured using rapid-mixing (e.g., stopped flow) methodologies, kinetic isotope fractionation can lead to a large underestimation of both the magnitude and temperature dependence of kinetic isotope effects (KIEs). This fractionation is related to the isotopic purity of the substrates used and highlights a major problem with experimental studies which measure KIEs with substrates that are not isotopically pure. As it is not always possible to prepare isotopically pure substrates, we describe two general methods for the correction, for known isotope impurities, of KIEs calculated from pre-steady-state measurements.

Introduction

Arguably, the most useful experimental probe of an enzyme reaction mechanism involves the measurement of perturbations—equilibrium, binding, steric, or kinetic (KIE)—arising from specific isotopic substitutions within the enzyme, substrate, or solvent.[1−4] Observed KIEs, such as the deuterium 1° KIE, k_H/k_D, reflect the influence of an isotopic substitution on a partially or fully rate-limiting rate constant. In enzymology, this observed rate constant, k_{obs}, is typically measured in one of two ways: by steady-state enzyme turnover yielding $k_{obs} = V/e$ or V/K, or by pre-steady-state methods such as rapid-mixing or flash photolysis methods.[5,6] These latter methods often allow the direct measurement of the evolution of a chemical species with time and, if the kinetic complexity is low, then k_{obs} approaches the actual rate constant of interest. The data are fit to exponential functions to directly extract k_{obs}:

$$A(t) = \Delta A \exp(-k_{obs}t) \tag{1}$$

If the observed rate constant is not fully rate-limiting, then k_{obs} or the KIE can be corrected for commitments to catalysis in the same manner as steady-state data.[7] It is less obvious, however, how to extract intrinsic rate constants and/or KIEs from pre-steady-state measurements when there is a mixture of isotopically labeled substrates present—essentially a special case of competitive inhibition. This is a particular problem during KIE measurements where it is not always possible to use isotopically pure substrates.

Correction of steady-state reaction rates for isotopically impure substrates is usually straightforward as, typically:

$$V_{obs} = V_H\chi + V_D(1 - \chi) \tag{2}$$

where V_D and V_H are the rate of the reaction (e.g., the Michaelis−Menten parameters k_{cat} or k_{cat}/K_m) with isotopically pure deuterated and protiated substrates, respectively. Note, we will consider H/D KIEs for the remainder of this study, but extension to other isotopes should be obvious. In eq 2, χ is the fraction of isotopic impurity to the total substrate concentration, e.g.:

$$\chi = \frac{[S_H]}{[S_H] + [S_D]} \tag{3}$$

where S_H and S_D are the initial concentrations of protiated and deuterated substrate, respectively. Equation 2 leads to a linear relationship between V_{obs} and χ, thus allowing a linear extrapolation of V_D from known values of V_H and χ. Values of χ can usually be determined quite accurately by ^{1}H NMR[8,9] or possibly by mass spectrometry.[10] When measuring the rate of a pre-steady-state reaction, the data are typically fit to a single-exponential function (eq 1), yet when the substrate is not isotopically pure, the data should really be fit to a double-exponential function:

$$A(t) = A_H \exp(-k_{obs(H)}t) + A_D \exp(-k_{obs(D)}t) \tag{4}$$

where A_H and A_D are the changes in signal amplitude due to the reaction with protiated and deuterated substrate, respectively. Experimentally, one strives to make the substrate as isotopically pure as possible so χ tends to be relatively low (less than 10% usually) and as: $\Delta A = A_H\chi + A_D(1 - \chi)$, it is often not possible to deconvolute the data into its two constitutive exponential functions, as A_D approaches zero when χ approaches zero. Further, when the values of k_H and k_D are not very different—i.e., the magnitude of the KIE is less than about 5−10—it is generally not possible to accurately resolve, by fitting to a double-exponential function, the two kinetic phases in eq 4 even if they have similar amplitudes (see below and ref [11]). Consequently, during KIE measurements, it is not uncommon to fit the data to a single-exponential function (eq 1) and ignore the (usually small) contamination from the fraction of nondeuterated substrate.[12] This approach is far from ideal and while we have previously made an attempt to correct pre-steady-state k_{obs} values for

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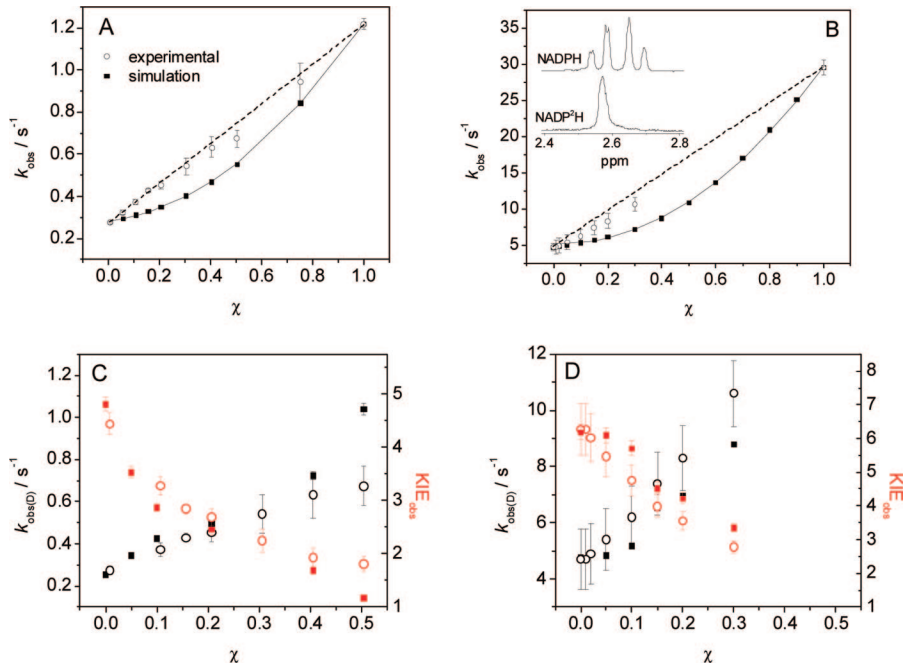

Figure 1. Effect of the substrate/coenzyme isotopic composition, χ, on the observed maximum rate (open circles) determined by fitting a single-exponential function to stopped-flow traces of (A) the reduction of AADH with benzylamine, and (B) the reduction of PETNR with NADPH, both at 25 °C. See the Experimental Section for conditions. The panel B insert shows ^{1}H NMR spectra of the C4 protons of the NADPH and (*R*)-[4,^{2}H]-NADPH used in this study. Simulated data (filled squares; see text for details) are shown for comparison and the dotted lines are linear (eq 9). Panels C and D show a comparison between fitting the above data (C, AADH/benzylamine; D, PETNR/NADPH) to a single-exponential (open circles) vs a double-exponential function (closed squares) where the faster rate constant (k_H) is fixed and the slower rate constant (k_D) plotted. Both the observed rate constants (black symbols) and corresponding observed KIEs (red symbols) are shown. Reaction traces and residuals are shown in Figures S1 and S2 of the Supporting Information.

isotopic impurities,[12] we are not aware of a general method to do so. Therefore, this current study attempts to derive a simple and general method of correcting pre-steady-state $k_{obs(D)}$ and/or KIE values for isotopic impurities.

Results and Discussion

We have previously made an attempt to correct values of k_{obs} determined from stopped-flow measurements of the reductive half-reaction (RHR) of morphinone reductase (MR) with deuterated NADH containing a known isotopic impurity of protiated NADH.[12] Briefly, we simulated stopped-flow traces using eq 4 with known values of $k_{obs(H)}$, A_H, and A_D. The value of $k_{obs(D)}$ was then varied until k_{sim}, the value of k_{obs} determined by fitting a single-exponential function to these traces, was in agreement with the experimentally determined value of k_{obs}. The corrected KIE was then calculated from the corrected value of $k_{obs(D)}$. In the current study, we have experimentally tested this approach. Initially, values of k_{obs} were experimentally measured as a function of χ using a stopped-flow spectrometer, i.e., by measuring pre-steady-state KIEs. We chose two different enzyme-catalyzed RHRs for these measurements—the reduction of aromatic amine dehydrogenase (AADH) with benzylamine, and the reduction of pentaerythritol tetranitrate reductase (PETNR) with NADPH.[12−15] These reactions were chosen because (i) they are well characterized, (ii) they allow the direct measurement of the H-transfer chemical step, (iii) they have reasonably small observed KIEs of 5−6, and because (iv) both perdeuterated benzylamine and (*R*)-[4-^{2}H]-NADPH (NADPD) can be obtained with very high isotopic purity (>99%; see the Experimental Section). Note that the PETNR/NADPH reaction is homologous to the MR/NADH reaction and that we chose the former reaction as we can currently prepare (*R*)-[4-^{2}H]-NADPH with a higher isotopic-purity than (*R*)-[4-^{2}H]-NADH.

The k_{obs} vs χ data for the two RHRs are shown in Figure 1A,B and are compared with k_{sim} values determined as described above. It is immediately evident that there is poor agreement between the k_{obs} and k_{sim} values for both reactions. In fact, to a first approximation, k_{obs}, like V_{obs} (eq 2) is linearly dependent on χ. In addition to fitting the stopped-flow traces to a single-exponential function (eq 2), we also fit the traces to a double-exponential function (eq 4) with $k_{obs(H)}$ fixed to the value of k_{obs} measured when $\chi = 1.0$. Stopped-flow traces, fits, and residuals are shown in the Supporting Information. In Figure 1C,D, the resulting $k_{obs(D)}$ and observed KIE values are compared with those determined from a single-exponential fit (shown in Figure 1A,B). When χ is less than 0.3−0.4, the k_{obs} values obtained by both methods are essentially identical. Further, the residuals from single and double exponential fits are also similar. Together, these findings suggest that fitting such data to a double-exponential function (eq 4) does not yield reliable values of $k_{obs(D)}$ and thus the KIE.

In Figure 1A,B, the rate constants were consistently underestimated for all values of k_{sim} so it is most likely that the disagreement between these k_{obs} and k_{sim} values is due to some form of isotopic discrimination in the reaction, this discrimination causing H to be transferred more often than D. We turned to kinetic modeling for an explanation and chose to further examine the PETNR/NADPH reaction in preference to the AADH/benzylamine reaction due to the simplicity of the PETNR RHR[12,15] over the AADH RHR.[13,14]

The RHR of PETNR with NADPH can be described by

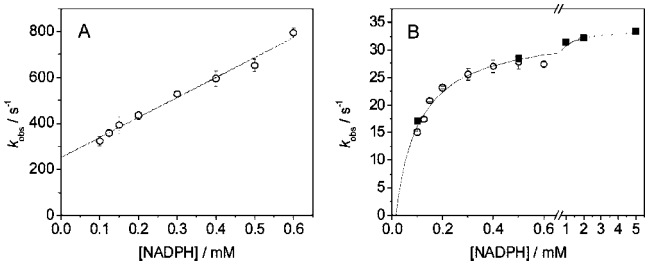

Figure 2. Effect of NADPH concentration on the rate of NADPH binding to PETNR (A) and the rate of the following forward and back H-transfer (B) measured by stopped flow at 25 °C. (A) The apparent rate of formation of the charge-transfer (CT) absorbance at 555 nm, corresponding to the E·NADPH binary complex. The data are fit to $k_{obs} \sim k_{off} + k_{on}$[NADPH] with $k_{on} = (8.7 \pm 0.4) \times 10^5$ M^{-1} s^{-1} and $k_{off} = 251 \pm 14$ s^{-1}. (B) The apparent rate of CT decay at 555 nm (open circles) and FMN reduction at 466 nm (closed squares). Both of these spectral changes reflect H-transfer and together the data are fit to $k_{obs} \sim k_{ox} + k_H$[NADPH]/{($k_{off}/k_{on}$) + [NADPH]} with $k_{ox} = -6 \pm 8$ s^{-1}, $k_H = 38 \pm 8$ s^{-1}, and $k_{off}/k_{on} = 0.08 \pm 0.03$ mM. The 466 nm data is taken from ref 12. Note, in order to keep the coenzyme in reasonable excess, it was not possible to use lower concentrations of NADPH.

$$E_{ox} + NADPH \underset{k_{off}}{\overset{k_{on}}{\rightleftharpoons}} E_{ox} \cdot NADPH \underset{k_{ox}}{\overset{k_H}{\rightleftharpoons}} E_{red}H \cdot NADP^+ \quad (5)$$

where E_{ox} is the oxidized enzyme, E·NADPH the experimentally observable enzyme/coenzyme binary complex, and $E_{red}H \cdot NADP^+$ the complex of reduced enzyme and $NADP^+$. The k_{obs} values in Figure 1B can be assigned to k_H[14,15] so in order to accurately model this reaction, it was necessary to determine the remaining three rate constants. For the PETNR RHR this is possible and together these measurements are shown in Figure 2. Of significance is that (i) k_{off} is significantly faster than k_H, and (ii) k_{ox} is, at most, 2 s^{-1} and thus much less than k_H; i.e., H transfer is nearly irreversible. In the homologous RHR of MR with NADH, we have recently shown the driving force of H-transfer is large (~600 kJ mol^{-1})[16] and thus the H-transfer back reaction in these homologous Old Yellow Enzymes should be very slow, rendering the H-transfer step irreversible on the time scale of the experiment.

It is possible to model the RHR of PETNR (eq 5) computationally by treating the reaction as a series of coupled differential equations (see Experimental Section for details). We assumed that there is no KIE on either k_{on} or k_{off} as previous measurements with both MR and PETNR have shown no significant KIE on the ratio k_{off}/k_{on}.[12] Representative reaction profiles are shown in Figure 3A−C. When there is a mixture of both NADPH and NADPD present (i.e., $0 < \chi < 1$), we found that a significant kinetic fractionation occurs in the reaction if $k_H/k_D > 1$ and k_{off} is at least as fast as ~$0.1 \times k_H$. Note that fractionation will lead to the formation of more $E_{red}H \cdot NADP^+$ than $E_{red}D \cdot NADP^+$ (species d vs e in Figure 3A−C). Kinetic isotope fractionation is not a new idea. Northrop discussed this effect nearly 30 nearly years ago,[17] and others have also addressed this issue (see, e.g., refs 18 and 19). In the context of steady-state analysis, this fractionation can be ignored because, after several turnovers, the observed isotope effect becomes dominated by the KIE on the isotopically sensitive step. When the H-transfer step is not irreversible, then the fractionation may still be observed when $k_{off} \gg k_H$ if a reaction intermediate cf. the product is measured, as is the case when we measure the RHR of PETNR and MR (i.e., the CT complex in Figure 2). This phenomenon is shown in Figure 3C where, if one was to follow the reaction by monitoring the loss of the binary complex (species b and c), then the experiment would

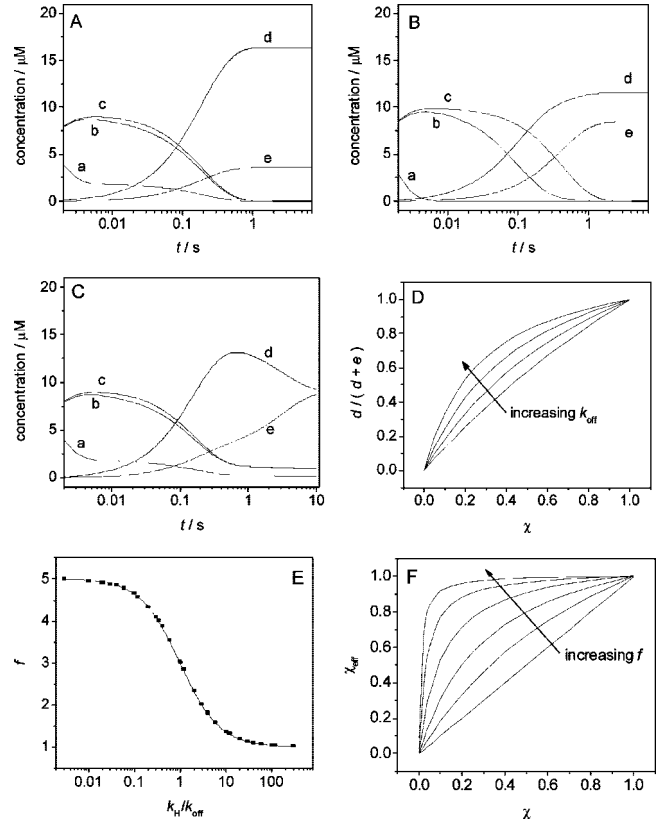

Figure 3. Kinetic modeling of the reaction of PETNR with mixtures of NADPH and NADPD (eq 10). (A−C) Simulated reaction profiles when [PETNR] = 20 μM, [NADPH] = [NADPD] = 0.5 mM (χ = 0.5), $k_H = 30$ s^{-1}, $k_{on} = 10^6$ M^{-1} s^{-1}, $k_H/k_D = 5$ and (A) $k_{off} = 10 \times k_H$ and $k_{ox} = 0$; (B), $k_{off} = 0.1 \times k_H$ and $k_{ox} = 0$; (C) $k_{off} = 10 \times k_H$ and $k_{ox} = 0.1 \times k_H$. The species are (a) E_{ox}, (b) E_{ox}·NADPH, (c) E_{ox}·NADPD, (d) $E_{red}H \cdot NADP^+$, and (e) $E_{red}D \cdot NADP^+$. Note that the majority of the reaction a → b/c occurs at faster time scales than shown. See text for more details. (D) Effect of χ on the apparent total fractionation (at $t = 10$ s) of the reduced product d when $k_H = 30$ s^{-1}, $k_{ox} = 0$, and $k_{off} = 3$, 10, 30, and 300 s^{-1}. (E) Influence of the ratio of k_H/k_{off} on f determined by kinetic modeling (filled squares) and calculated from eq 7 (when $k_H/k_D = 5$ and $k_{ox} = 0$). (F) Correlation between χ_{eff} and χ when $f = 1$, 2, 4, 10, 30, and 100 calculated from eq 8.

appear to have gone to completion after ~1 s, at which point the fractionation is maximal and approaches that value observed when $k_{off} = 0$ (compare the amount of species d in Figure 3A and 3C). The data in Figure 3A−C were modeled with χ = 0.5. Figure 3D shows the effect of χ on the fractionation for differing values of k_{off}. Clearly, the fractionation is both dependent on k_{off} and on χ. In order to normalize the fractionation with regard to χ, we can define the fractionation, f, as

$$f(t) = \frac{[P_H]/[P_D]}{[S_H]/[S_D]} \quad (6)$$

where P_i and S_i are the isotopically labeled product and substrate (or species on the "right" and "left" of the isotopically sensitive step), respectively, at a given time t. Note that $[S_H]/[S_D] = \chi/(1 - \chi)$. In the case of the modeling in Figure 3, P_H = species d and we will consider the fractionation at the apparent completion of the reaction, not the completion itself, i.e., if we were measuring the binary complex (b + c) in Figure 3C then the reaction would appear to have gone to completion after ~1 s, whereas the actual completion is not reached until after 10 s.

The kinetic fractionation shown in Figure 3 occurs when the rate of the isotopically sensitive step (k_H) is comparable or slower to the rate of the preceding back reaction (k_{off}) and it would be preferable to find an expression to relate f to k_H, k_{off}, and k_H/k_D. The fractionation is essentially an equilibrium isotope effect on product formation and arises due to an isotope effect on *productive* substrate capture, i.e., a KIE on only that fraction of the binary complex that proceeds to product formation. If we recall that the steady-state parameter V/K is essentially the second-order rate constant for productive substrate capture,[2,20] then f may be equivalent or proportional to the KIE on V/K. Northrop has shown[2] that the KIE on V/K is determined by k_H, k_{off}, and k_H/k_D so, if f has the same form as the KIE on V/K, then

$$f \propto \frac{(V/K)_H}{(V/K)_D} = \frac{k_H/k_D + k_H/k_{off}}{k_H/k_{off} + 1} \quad (7)$$

If eq 7 is valid, then the fractionation can be as large as the KIE and the magnitude of the fraction is determined by the ratio of k_H to k_{off} (or equivalent steps) and is maximal when $k_{off} \gg k_H$. We verified eq 7 by calculating f from modeled reaction profiles (as shown in Figure 3A,B) over a range of k_{off} values and comparing the values to eq 7. The values are equivalent and these data are shown in Figure 3E. We should stress that eq 7 will tend to overestimate the actual fractionation for a number of reasons. Unless the substrate is in a very large excess, the fractionation will not be fully realized at low values of χ. For single turnover experiments, saturating substrate concentrations are typically considered to be $10 \times K_S$, where K_S is the apparent saturation constant ($K_S = k_{off}/k_{on}$ in Figure 2B). However, when $\chi < 0.1$, then the actual concentration of protiated substrate will be less than K_S, resulting in an apparent inverse binding isotope effect that increases as χ approaches zero. More generally, eq 8 will breakdown if there are additional isotopically sensitive steps in the reaction, such as on substrate binding or preorganization. Further, the fractionation will be overestimated if the isotopically sensitive step is not fully irreversible. As it is often difficult to measure precisely the rates of k_{off} and k_{ox} (or equivalent) and/or the equilibrium constant for both the isotopically sensitive step and preceding step (see, e.g., Figure 2 and ref 16), one will typically not be able to measure precise values of f. However, if f is equivalent to the KIE on V/K (eq 7), it may be possible to estimate the magnitude of f from steady-state measurements. This will only be possible if the reaction of interest is at least partly rate-limiting during enzyme turnover. The *oxidative* half-reactions of PETNR and MR are fully rate-limiting so it is not possible to estimate f using this method.[15,21] However, because we can directly measure k_{off}, k_H, and k_D (Figure 2), we can estimate f to be ~5.7. Conversely, the RHR of AADH is complex and the steps preceding H transfer cannot be directly measured.[13,14] Nevertheless, the RHR with benzylamine is partly rate-limiting and the KIE on $V/K \sim 2$ ($k_{cat(H)}/k_{cat(D)} = 2.2 \pm 0.1$; $K_{m(H)} \sim K_{m(D)} = 14 \pm 1$ μM; from ref 14).

With estimates of f for the RHR of both AADH and PETNR, we attempted to correct the simulated data in Figure 1 by repeating the simulation process using the effective cf. absolute values of χ. By rearranging eqs 3 and 6 we can calculate the effective χ for given values of f and χ:

$$\chi_{eff} = \frac{[P_H]}{[P_H] + [P_D]} = \frac{f\chi/(1-\chi)}{1 + f\chi/(1-\chi)} \quad (8)$$

In Figure 3F, χ_{eff} is plotted vs χ for increasing values of f. It is apparent that the fractionation can become very large as χ approaches zero (if f is also large) and that even a relatively small isotopic contamination ($\chi < 10\%$) could result in a large uncertainly in k_D and thus the KIE. As the RHR of both AADH and PETNR appear to have values of f that are significantly greater than unity, it is then not surprising that we observe considerable deviation from the experimental and observed values of k in Figure 1. This also explains, in part, why it is not possible to accurately extract $k_{obs(D)}$ by fitting the stopped-flow traces to a double-exponential (Figure 1)—A_D (eq 4) will be much smaller than expected. Note that χ_{eff} in Figure 3D is equivalent to a normalized value for A_H.

In Figure 4, the experimental k_{obs} vs. χ data for the AADH and PETNR RHRs are compared with new k_{sim} values determined using χ_{eff} cf. χ. There is good agreement between the k_{obs} and new k_{sim} values for the AADH data, suggesting that the value of f estimated from the steady-state KIE on V/K is reasonable. The PETNR k_{sim} values significantly overestimate the experimental values when we use the value of $f = 5.7$ to determine the values of χ_{eff}. A good agreement is achieved when we reduce the value of f to 2.5 (Figure 4B), suggesting that, as discussed above, eq 7 will tend to overestimate the fractionation and thus the values of f.

While we can explain the experimentally observed dependence of k_{obs} on χ in Figure 4, the method described above of "correcting" the simulated values in order to estimate k_{obs} is cumbersome. Further, in the case of the PETNR reaction, the method is probably not sufficiently accurate to be useful. Inspection of Figure 4 shows that both the AADH and PETNR experimental data are described equally well by eq 8 (when f is adjusted) and by a simple linear function:

$$k_{obs}(\chi) = k_H\chi + k_D(1-\chi) \quad (9)$$

Therefore, a simple linear extrapolation of the experimental data will yield values of k_D that are comparable to those values determined by our more elaborate correction method. Consequently, eq 9 provides a simple method to calculate k_H/k_D from k_H and k_{obs} at a known value of χ. However, it is not obvious how generally applicable eq 9 will be. At both high and low values of f, eq 8 predicts that there should be a considerable deviation of the χ dependence of k_{obs} from linearity. As it is reasonably trivial to measure k_{obs} at several values of χ (analogous to a proton inventory experiment), it is possible to experimentally verify whether there is a linear dependence of χ on k_{obs}. Note that it is not necessary to measure k_{obs} at very small χ values, but rather show a reasonably linear trend over as wide a range of χ values as possible. Therefore, in principle,

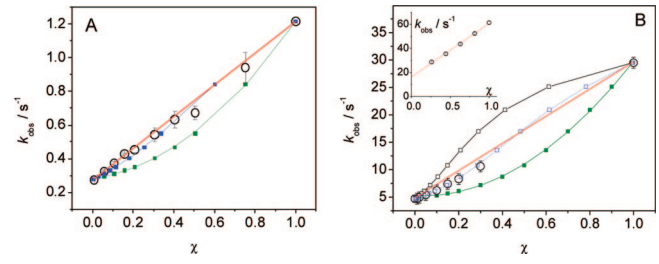

Figure 4. Correction of k_{sim} values for kinetic isotopic fraction. The k_{sim} values are compared to the experimental values (open circles) for (A) the reduction of AADH with benzylamine, (B) the reduction of PETNR with NADPH and (B, inset), the reduction of MR with NADH. The k_{sim} values were corrected using eq 7 and 8 with values of $f = 1$ (AADH and PETNR, green squares), $f = 2.0$ (AADH; blue squares), $f = 2.5$ (PETNR; blue open squares, dotted line) and $f = 5.7$ (PETNR; black open squares). The solid red lines are linear functions (eq 9). See text for more details. The MR data are fit to eq 9 only.

TABLE 1: Correction of Previously Determined k_{obs} and KIE Values for the RHR of MR

coenzyme	χ	$k_{obs(D)}$ (s^{-1})	k_D[a] (s^{-1})	KIE[b]	KIE[c]
(*S*)-[4-^{2}H]-NADH	0.99	47.8 ± 0.4	47.7 ± 1.0	1.18 ± 0.02	1.18 ± 0.04
(*R*)-[4-^{2}H]-NADH	0.94	12.7 ± 0.1	9.9 ± 0.2	4.6 ± 0.1	5.7 ± 0.2
(*R,S*)-[4,4-^{2}H$_2$]-NADH	0.91[d]	11.1 ± 0.4	6.6 ± 0.3	5.4 ± 0.2	8.5 ± 0.5

[a] k_D was determined using eq 9 with $k_H = 56.4 \pm 0.8$.[12] [b] The previous values of the corrected KIE from ref 12. [c] The KIE corrected from k_D values determined from a linear extrapolation (eq 9). [d] The (*R,S*)-[4,4-^{2}H$_2$]-NADH had 9% H at the C4 *R* position and 1% H at the C4 *S* position, and the 1% contamination was ignored for this correction.

eq 9 allows the trivial correction of the KIE for isotopic impurity.

As an example, we tested eq 9 for a third enzymatic RHR: the RHR of MR with NADH. We have previously measured various KIEs on the RHR of MR with NADH, and these values were measured at known values of χ which are significantly greater than zero. In the insert to Figure 4B we show the dependence of k_{obs} on χ for the RHR of MR with NADH; note that we varied χ from 0.2 to 1.0. As the trend is fairly linear, the data can justifiably be fit to eq 9 to extrapolate a value of k_D. This linear correction was then performed with our previously published values of $k_{obs(D)}$ for the reaction of MR with (*S*)-[4-^{2}H]-NADH, (*R*)-[4-^{2}H]-NADH, and (*R,S*)-[4,4-^{2}H$_2$]-NADH.[12] These values and the correspondingly corrected KIEs are listed in Table 1. Of interest is that, based on our previous correction of the KIE values, we showed that the rule of geometric mean was not *significantly* violated for the RHR of MR.[12] Using the corrected values in Table 1, it is now clear that we had underestimated the KIEs in our previous study and, as a consequence, the product of the 1° and 2° KIE values is significantly smaller than the measured KIE for the dideuterated species. Therefore, the rule of geometric mean is violated; the KIE for the dideuterated reaction is 8.5 ± 0.5 while the product of the 1° and 2° KIEs is only 6.7 ± 0.4. As eq 9 is not strictly correct, it is possible that the newly corrected 1° and 2° KIEs in Table 1 are overestimated. However, we have demonstrated that kinetic fractionation occurs during the RHR of MR with NADH (Figure 4 inset), so the previously reported KIEs are almost certainly underestimated. The isotopic contamination present during the dideuterated KIE measurements was significantly larger than that during the 1° and 2° KIE measurements (Table 1). Therefore, correction of the KIEs will lead to a greater increase in the magnitude of the dideuterated KIE relative to the others and thus a significant violation of the rule of geometric mean (as the product of the published 1° and 2° KIE values is identical to the published dideuterated KIE[12]). A violation of the rule of geometric mean is expected for reactions that involve a high degree of quantum mechanical tunneling.[22−25] We now have a body of evidence suggesting that H transfer during the RHR of MR with NADH does occur predominantly by tunneling,[26−28] so the violation of the rule of geometric mean is consistent with this interpretation.

If the magnitude of a KIE is underestimated due to isotopic contamination of the isotopically labeled substrate, then the temperature dependence of the KIE (i.e., ΔE_a or $\Delta\Delta H^{\ddagger}$) will also be underestimated. This effect is demonstrated in Figure 5, where the effect of χ on the apparent value of ΔE_a is modeled using eq 9 when the KIE = 5.0 at 298 K. Even modest values of χ can lead to significant error in ΔE_a and as the magnitude of both the KIE and of ΔE_a become larger, this effect becomes more pronounced. Temperature-dependent KIEs are often used as evidence for environmental coupling to the reaction,[29−31] and the magnitude of ΔE_a or $\Delta\Delta H^{\ddagger}$ (which are equivalent) is now routinely used to model such reactions.[26,32−34] Consequently, care must be taken to correct not only values of the KIE for isotopic contamination but also values of ΔE_a and the Arrhenius/Eyring prefactor ratios.

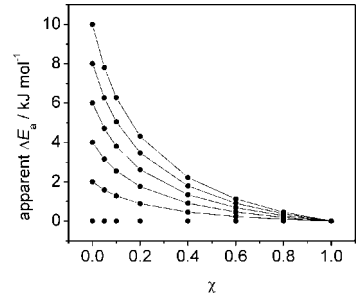

Figure 5. Effect of χ on the observed difference in activation energy (ΔE_a) of a modeled reaction when the KIE = 5.0 at 298 K when $\Delta E_a(\chi=0)$ is varied between 0 to 10 kJ mol^{-1}. See text for more details.

As the magnitude of the isotopic fractionation appears to be governed by both the k_H/k_D and k_H/k_{off} ratios (eq 7), it may be possible to use this fractionation as an additional mechanistic probe of enzyme mechanisms, i.e., to estimate values of the rate constant equivalent to k_{off}, which may otherwise be impossible to measure. As an example, if we take the magnitude of the fractionation during the RHR of AADH with benzylamine to have a value of $f = 2.0 \pm 0.5$ (Figure 4A), then, by rearranging eq 7, we can estimate a value of $k_{off} = 0.5 \pm 0.2$ s^{-1}. Note that while k_{off} appears to be slower than k_H (1.2 s^{-1}), these values are similar and thus only partial fractionation is observed. The step preceding H-transfer in AADH is spectrally silent and we have no estimate of the rate or equilibrium constant for this reaction.[13] However, as H-transfer is (at least partially) rate limiting during the RHR of AADH with other substrates such as tryptamine[13]—a reaction that proceeds at >2000 s^{-1} at room temperature with a KIE of >50—then the forward rate of the reaction preceding H-transfer must be faster than H-transfer. If we assume that this reaction step is similar with benzylamine, then we can estimate that the equilibrium constant for this step to be $>10^3$ as $k_{off} = 0.5 \pm 0.2$ s^{-1} (see above) and $k_{on} \gg 2000$ s^{-1}. Further, in order for fractionation to occur, the reverse rate of H-transfer must be slow on the time scale of the experiment (see above), so the kinetic equilibrium constant for the actual H-transfer step during the RHR of AADH with benzylamine can also be estimated to be significantly greater than unity and thus $k_{ox} \ll 1$ s^{-1}. Conversely, if similar values of k_{off} and k_{ox} are found during the RHR with tryptamine, then essentially no fractionation will occur during this reaction ($k_H \sim 2000$ $s^{-1} \gg k_{off} \sim 0.5$ s^{-1}) and H-transfer will effectively be irreversible as $k_H/k_{ox} \gg 10^3$.

Conclusions

We have shown that there can be a significant kinetic isotope fractionation during the first turnover of an enzyme-catalyzed reaction. This fractionation makes the correction of KIEs for isotopic contamination difficult but may allow the estimation of details of additional "silent" reaction steps. We describe two

general methods to correct KIEs derived from single-turnover experiments for isotopic contaminations. These methods are applicable both to enzyme and chemical systems where the measurements are made using stopped-flow, flash photolysis, or similar such instruments—any single-turnover measurement where observed rate constants are obtained by fitting the time evolution of the concentration of a chemical species to exponential functions. The only requirement is that the reaction must have at least one reversible chemical step preceding the H-transfer. As a word of caution to the experimental community, the correction of KIEs for isotopic contamination is crucial. Even quite modest protium contaminations can cause large underestimations of the magnitude of the KIE. This error in the KIE will also be manifest on the temperature dependence of the reaction (if the KIE is temperature dependent), a key diagnostic of the presence of environmental coupling to the reaction.[29−31] We suggest that, when mechanistic details are gleaned from the *magnitude* of KIEs and/or their temperature dependencies, care must be taken to ensure that substrates are isotopically pure. When it is not possible to prepare isotopically pure substrates, every attempt must be made to appropriately correct the magnitude of the KIE. If eq 9 proves to be generally applicable, then this correction is not difficult.

Experimental Section

All materials were obtained from Sigma-Aldrich (St. Louis, MO), except deuterated benzylamine HCl ($C_6D_5CD_2NH_2$ HCl, 99.6%; CDN Isotopes) NAD^+, $NADP^+$, NADH, and NADPH, (Melford Laboratories (Chelsworth, UK) and $^2H_6$-ethanol (Cambridge Isotope Laboratories, Andover, MA).

Aromatic Amine Dehydrogenase. AADH was purified as described previously.[13,14,35] Immediately prior to use in kinetic studies, AADH was oxidized with potassium ferricyanide and exchanged into 10 mM potassium phosphate (pH 7.0) by gel-exclusion chromatography and the enzyme concentration determined by $\varepsilon = 27.6\ mM^{-1}\ cm^{-1}$ at 433 nm. Benzylamine solutions were made fresh, and their concentrations were determined by $\varepsilon = 13.8\ mM^{-1}\ cm^{-1}$ at 250 nm.

Pentaerythritol Tetranitrate Reductase and Morphinone Reductase. PETNR was purified as described previously,[12,36] and the enzyme concentration was determined by $\varepsilon = 11.3\ mM^{-1}\ cm^{-1}$ at 462 nm. MR was purified as described previously,[12,21] and the enzyme concentration was determined by $\varepsilon = 11.3\ mM^{-1}\ cm^{-1}$ at 464 nm. (*R*)-[4-^{2}H]-NADPH was prepared essentially as described previously,[12,36] the difference being that the catalytic reduction of $NADP^+$ by equine liver alchohol dehydrogenase was performed in ammonium bicarbonate-buffered D_2O (pD 9). ^{1}H NMR showed at least 99% deuteration of the C4-*R* proton (Figure 1B insert). (*R*)-[4-^{2}H]-NADH was prepared in protiated buffer essentially as described previously.[12] Stopped-flow experiments with both PETNR and MR were performed in 50 mM potassium phosphate (pH 7.0). The buffer also contained 1 mM 2-mercaptoethanol when working with MR. Coenzyme solutions were made fresh, and their concentrations were determined by $\varepsilon = 6.22\ mM^{-1}\ cm^{-1}$ at 340 nm.

Pre-Steady-State Kinetics. Rapid reaction kinetic experiments were performed using an Applied Photophysics SX.18MV-R stopped-flow spectrophotometer. All experiments involving PETNR and MR were performed in a Belle Technology anaerobic glovebox. Spectral changes accompanying TTQ reduction in AADH were monitored at 456 nm, while those accompanying FMN reduction in PETNR and MR were monitored at 464 nm. Saturating final concentrations of benzylamine (200 μM), NADPH (1 mM), and NADH (5 mM) were used in order to confer pseudo-first-order kinetics. All experiments were performed at 25 °C.

Reaction Modeling. The RHR of PETNR with mixtures of NADPH and NADPD was modeled assuming the following reaction:

$$E_{ox}\cdot NH + ND \underset{k_{off}}{\overset{k_{on}}{\rightleftharpoons}} E_{ox}\cdot NH + E_{ox}\cdot ND$$

$$E_{ox}\cdot NH \underset{k_{ox(H)}}{\overset{k_H}{\rightleftharpoons}} E_{red}H\cdot N^+$$

$$E_{ox}\cdot ND \underset{k_{ox(D)}}{\overset{k_D}{\rightleftharpoons}} E_{red}D\cdot N^+ \qquad (10)$$

where NH and ND are NADPH and NADPD, respectively, and N^+ is $NADP^+$. The reaction was modeled by the (approximate) numerical solution of the following coupled partial differential equations as a function of time using NDSolve in Mathematica version 6.0 (Wolfram Research, Champaign, IL):

$$\partial[E_{ox}]/\partial t = -k_{on}([E_{ox}]_t[NH]_t + [E_{ox}]_t[ND]_t) + k_{off}([E_{ox}\cdot NH]_t + [E_{ox}\cdot ND]_t)$$

$$\partial[NH]/\partial t = -k_{on}[E_{ox}]_t[NH]_t + k_{off}[E_{ox}\cdot NH]_t$$

$$\partial[ND]/\partial t = -k_{on}[E_{ox}]_t[ND]_t + k_{off}[E_{ox}\cdot ND]_t$$

$$\partial[E_{ox}\cdot NH]/\partial t = -(k_{off} + k_H)[E_{ox}\cdot NH]_t + k_{on}[E_{ox}]_t[NH]_t + k_{ox(H)}[E_{red}H\cdot N^+]_t$$

$$\partial[E_{ox}\cdot ND]/\partial t = -(k_{off} + kD)[E_{ox}\cdot ND]_t + k_{on}[E_{ox}]_t[ND]_t + k_{ox(D)}[E_{red}H\cdot N^+]_t$$

$$\partial[E_{red}H\cdot N^+]/\partial t = -k_{ox(H)}[E_{red}H\cdot N^+]_t + k_H[E_{ox}\cdot NH]_t$$

$$\partial[E_{red}H\cdot N^+]/\partial t = -k_{ox(D)}[E_{red}H\cdot N^+]_t + k_D[E_{ox}\cdot ND]_t \qquad (11)$$

Initial concentrations were typically as follows: $[E_{ox}] = 20\ \mu M$, $[NH] + [ND] = 1$ mM ($\chi = 0-1$), and $[E_{ox}NH]$, $[E_{ox}ND]$, $[E_{red}H\cdot N^+]$, and $[E_{red}D\cdot N^+] = 0$. The following assumptions were made: (i) k_{on} and k_{off} are the *same* for NADPH and NADPD; (ii) the KIE on k_H/k_D is the same for the reverse (oxidative) reaction such that $k_H/k_D = k_{ox(H)}/k_{ox(D)}$.

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Nomenclature

AADH = aromatic amine dehydrogenase
MR = morphinone reductase
PETNR = pentaerythritol tetranitrate reductase
RHR = reductive half-reaction
KIE = kinetic isotope effect

Supporting Information Available: Reaction traces, fits, and residuals for reactions of AADH with benzylamine and PETNR with NADPH. This material is available free of charge via the Internet at http://pubs.acs.org.

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