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α-Secondary Isotope Effects as Probes of "Tunneling-Ready" Configurations in Enzymatic H-Tunneling: Insight from Environmentally Coupled Tunneling Models

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Abstract: Using α-secondary kinetic isotope effects (2° KIEs) in conjunction with primary (1°) KIEs, we have investigated the mechanism of environmentally coupled hydrogen tunneling in the reductive half-reactions of two homologous flavoenzymes, morphinone reductase (MR) and pentaerythritol tetranitrate reductase (PETNR). We find exalted 2° KIEs (1.17−1.18) for both enzymes, consistent with hydrogen tunneling. These 2° KIEs, unlike 1° KIEs, are independent of promoting motions—a nonequilibrium pre-organization of cofactor and active site residues that is required to bring the reactants into a "tunneling-ready" configuration. That these 2° KIEs are identical suggests the geometries of the "tunneling-ready" configurations in both enzymes are indistinguishable, despite the fact that MR, but not PETNR, has a clearly temperature-dependent 1° KIE. The work emphasizes the benefit of combining studies of 1° and 2° KIEs to report on pre-organization and local geometries within the context of contemporary environmentally coupled frameworks for H-tunneling.

Introduction

Hydrogen tunneling is increasingly being implicated in enzyme reactions.[1,2] Experimental studies have emphasized the inadequacies of the Bell tunnel correction model of semiclassical transition state theory (TST)[3] and the importance of environmental reorganization to the tunneling reaction.[1] Physical models[2] that account for experimental criteria for H-tunneling[4,5] have emerged, and computational analyses have furnished important new insight at the atomic level.[6] However, a requirement for fast (subpicosecond) "promoting motions" that enhance tunneling by barrier compression in some enzyme systems[4,7] is controversial.[8,9] This fast compressive motion is accommodated in physical models of enzymatic H-tunneling[2] and inferred from some experimental[4,7] and computational[6b,c,f] analyses. However, computational[8] and solution kinetic studies[9] have also argued against the need for fast compressive motion along the H-transfer coordinate.

The analysis of kinetic isotope effects (KIEs) has been at the heart of experimental descriptions of H-tunneling in enzyme systems. In particular, the temperature (in)dependence of primary (1°) KIEs has contributed to the understanding and formulation of modern, environmentally coupled models of H-tunneling in enzyme systems.[4,5] The reductive half-reactions of two homologous enzymes, morphinone reductase (MR) from *Pseudomonas putida* M10[10,11] and pentaerythritol tetranitrate reductase (PETNR) from *Enterobacter cloacae*, have been shown to exhibit temperature-dependent and temperature-independent (at least within the limit of experimental detection) 1° KIEs, respectively.[10] In the context of the environmentally coupled model of H-tunneling, the temperature dependence of

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reaction rates and temperature-independent KIEs arise from collective thermally equilibrated motions that lead to degenerate reactant and product states, i.e., the "tunneling-ready" configuration. Temperature-dependent KIEs are treated as arising from fast (subpicosecond) "gating motions" (motion along the reaction coordinate) that enhance the probability of tunneling by bringing the reactant and product wells closer together. The precise meaning of the term "temperature-independent KIE" needs to be clarified: within the formalism of this model, the KIE is only truly independent of temperature when tunneling is from the vibrational ground state and in the absence of gated motion. Also, it can be difficult to distinguish experimentally—as is the case with PETNR—between strictly temperature-independent and weakly temperature-dependent 1° KIEs. In a recent study, we have shown in the enzyme aromatic amine dehydrogenase that there is a need for a fast promoting motion during a H-tunneling reaction even though the 1° KIE is not demonstrably temperature-dependent.[6f]

α-Secondary (2°) KIEs arise when a hydrogen neighboring the transferred (1°) H is deuterated. The observed 2° KIEs arise as the consequence of a change in the C−H bond force constant of the 1° H caused by 2° deuteration.[12] Several arguments have been invoked to explain this change in force constant. The most widely accepted is that the force constant is altered due to a change in hybridization state,[13] that is, the rehybridization from sp^3 to sp^2 of a carbon atom adjacent to a 2° deuterium. The magnitude of the 2° KIE is considered to indicate the progress of reactants to products at the transition state.[12] In a classical description, 2° KIEs have been used to report on the nature of the transition state,[14] with values falling between unity and the equilibrium isotope effect (EIE), but computational studies have warned about such uses.[6d] The EIE for NAD(P)H to $NAD(P)^+$ has been determined as 1.13.[15] Values observed outside of this range,[16] so-called exalted 2° KIEs, were rationalized by Huskey and Schowen[17a] as being indicative of coupling of the motion between the 2° hydrogen and that of the 1° hydrogen being transferred, with a concomitant tunneling contribution to the reaction assuming a truncated Bell tunnel correction to the semiclassical isotope effect. As such, in the absence of kinetic complexity, studies of α-secondary KIEs are powerful independent probes of tunneling in enzymes. A number of excellent experimental and theoretical treatments on the utility of 2° KIEs have been published in recent years,[6d,18] with 2° KIEs often being evaluated in terms of their Swain−Schaad exponent[18] in the context of a Bell tunneling correction[18b,c] or variational transition state theory.[6d] We examine herein the nature of 2° KIEs exalted above the EIE in the context of the environmentally coupled model of H-tunneling.

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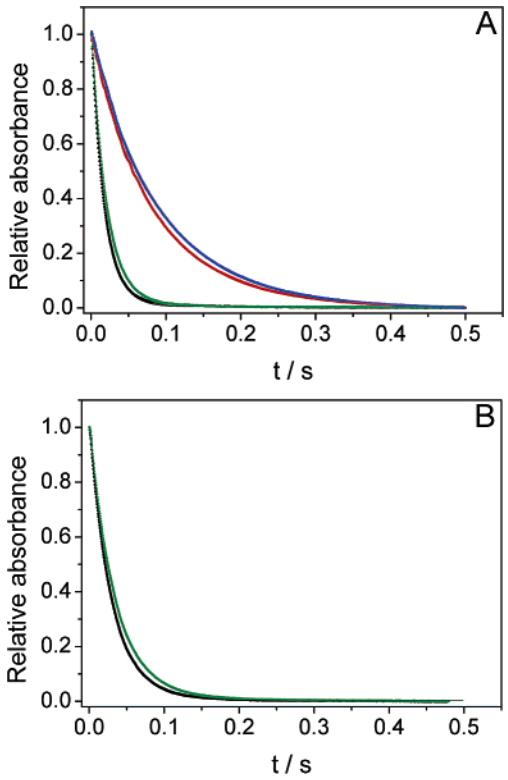

Figure 1. (A) Stopped-flow traces for the reaction of 20 μM MR with 5 mM NADH (black), 5 mM (*S*)-[4-^{2}H]-NADH (green), (*R*)-[4-^{2}H]-NADH (red), or 5 mM (*R*,*S*)-[4,4-$^2H_2$]-NADH (blue) at 25 °C in 50 mM potassium phosphate, 2 mM 2-mercaptoethanol, pH 7.0. (B) Stopped-flow traces for the reaction of 20 μM PETNR with 5 mM NADPH (black) or 5 mM (*S*)-[4-^{2}H]-NADPH (green) at 25 °C in 50 mM potassium phosphate, pH 7.0. The solid lines show the fits to a single-exponential expression (eq 3).

The reductive half-reactions of the homologous enzymes MR and PETNR involve hydride transfer from the C4 *R*-hydrogen (primary hydrogen, H_P) of β-NADH (MR) or β-NADPH (PETNR) to the N5 atom of flavin mononucleotide (FMN). This reaction is directly observed in a rapid mixing stopped-flow instrument and is kinetically resolved from steps involving coenzyme binding and formation of an enzyme−NAD(P)H charge-transfer complex, and the observed KIE is essentially the intrinsic KIE.[10,11] The reductive half-reactions of these two enzymes have been extensively characterized previously.[10,11] One of the crucial findings from these studies is that the 1° KIE of MR is highly temperature-dependent, whereas the 1° KIE of PETNR is, within the limit of experimental detection, independent of temperature. We have previously interpreted these data within the context of modern environmentally coupled models of H-tunneling.[2] Thus, MR requires a promoting motion to move the nicotinamide C4−H sufficiently close to the FMN N5 atom to facilitate tunneling, while PETNR requires no such motion. Herein, we further this study by measuring the temperature dependence of the α-2° KIEs in these enzymes using (*S*)-[4-^{2}H]-NAD(P)H. Here, H_P is hydrogen while the neighboring (secondary) isotope (H_S) at the C4 position is deuterium.[12] We have also studied the reaction in MR using dideuterated (*R*,*S*)-[4,4-$^2H_2$]-NADH to investigate the coupling between H_P and H_S.

Results and Discussion

The reductive half-reactions of MR and PETNR are essentially identical[10] and proceed by two kinetically resolved

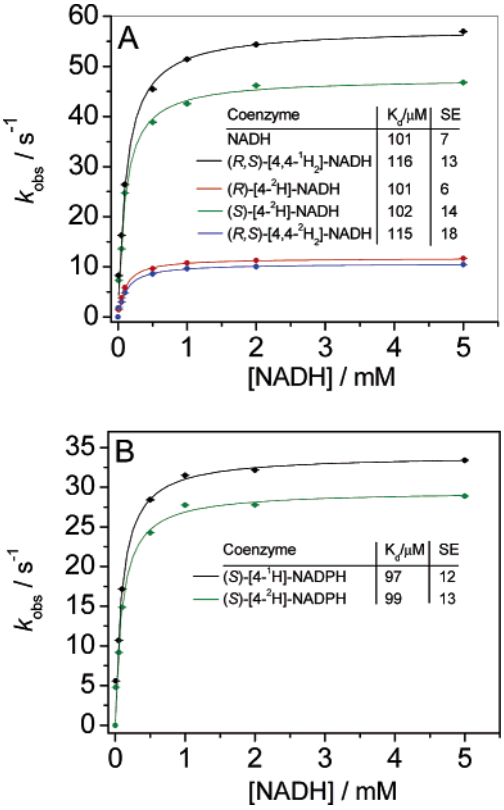

Figure 2. Dependence of the observed rate constant for hydride transfer on the concentration of synthesized coenzymes during the reductive half-reactions of morphinone reductase (A) and pentaerythritol tetranitrate reductase (B) at 25 °C. The solid lines show the fits to $k_{obs} = k_{max}$[NAD(P)H]/(K_d + [NAD(P)H]).

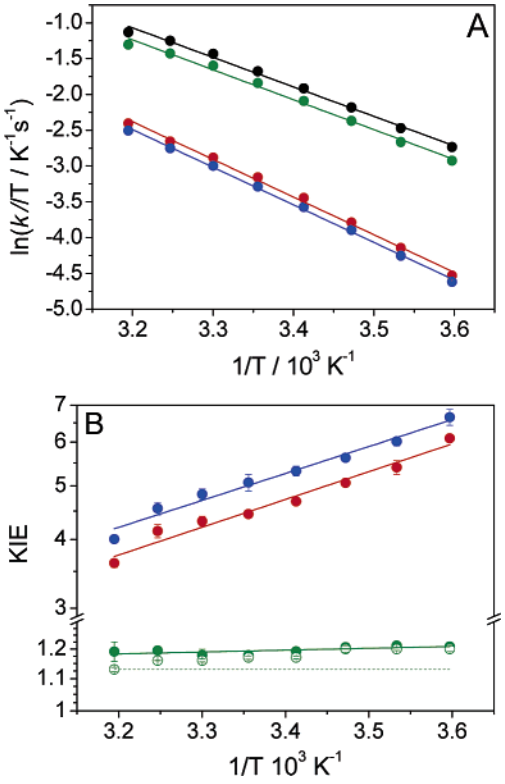

Figure 3. (A) Eyring plot of the reductive half-reaction of MR as measured using stopped-flow. The substrates are NADH (black), (*R*)-[4-^{2}H]-NADH (red), (*S*)-[4-^{2}H]-NADH (green), and (*R*,*S*)-[4,4-$^2H_2$]-NADH (blue). The data are fit to ln(k/T) = ln A' − $\Delta H^{\ddagger}$/RT, with the values for ln A' and $\Delta H^{\ddagger}$ given in Table 1. (B) Temperature dependence of the resulting KIEs. The same colors apply. Also shown are the 2° KIEs for PETNR (open green circles), while the dashed line represents the semiclassical limit of 1.13.[15]

steps. Initially, NADH binds with a second-order rate constant of 4.8×10^5 M^{-1} s^{-1} (5 °C, MR[11]) and NADPH with a constant of 9.5×10^5 M^{-1} s^{-1} (5 °C, PETNR[19]), forming a charge-transfer complex ($\lambda_{max} \approx 540-600$ nm). In the second step, the enzyme-bound flavin (FMN) is reduced in a pseudo-first-order hydride-transfer reaction from NAD(P)H. The latter step is observed as a monophasic (single-exponential) decrease in flavin absorbance at ~462 nm. The hydride transfer in both MR and PETNR has previously been shown to occur by a tunneling mechanism[10] and is further investigated in this study.

Figure 1 shows representative stopped-flow traces for the hydride-transfer step during the reductive half-reactions of MR and PETNR with the various coenzymes used in this study. The concentration dependence of the hydride-transfer step was investigated for each coenzyme, and apparent K_d values were found to be identical within error (Figure 2). Each coenzyme was used at a working concentration of 5 mM, thus ensuring that the coenzyme concentration was always at least 10-fold greater than the apparent K_d and that the observed rate constants were pseudo-first-order.

The temperature dependence of the rate of hydride transfer (flavin reduction) was determined by conducting stopped-flow experiments at 5 °C intervals over the temperature range 5−40 °C. The temperature dependence of the reduction of MR by the deuterated coenzymes is shown in Figure 3A, and the measured reaction enthalpies ($\Delta H^{\ddagger}$) and Eyring prefactors (ln A') for both MR and PETNR are given in Table 1. The observed rates at each temperature are also given in the Supporting Information. With MR, the enthalpies for NADH and the 1° deuterated coenzymes ((*R*)-[4-^{2}H]-NADH and (*R*,*S*)-[4,4-$^2H_2$]-NADH) are identical within error, with $\Delta\Delta H^{\ddagger} = 8.9 \pm 1.7$ and 8.6 ± 1.3 kJ mol^{-1}, respectively. The A'_H:A'_D ratios for (*R*)-[4-^{2}H]-NADH and (*R*,*S*)-[4,4-$^2H_2$]-NADH are also both significantly less than 1 (Table 1), the semiclassical limit.[20] We have previously shown for PETNR that there is little or no experimentally detectable difference in enthalpy between NADPH and (*R*)-[4-^{2}H]-NADPH ($\Delta\Delta H^{\ddagger} = 0.2 \pm 1.8$ kJ mol^{-1}) and A'_H:$A'_D = 4.1 \pm 0.3$.[10]

The 2° KIEs of both MR and PETNR are independent of temperature (within experimental error and the accessible temperature range), with $\Delta\Delta H^{\ddagger} = 0.6 \pm 1.3$ and 1.0 ± 2.0 kJ mol^{-1}, respectively (Table 1). At 25 °C, the 2° KIE for the reaction between MR and (*S*)-[4-^{2}H]-NADH is 1.18 ± 0.01, and at all temperatures measured the KIE is greater than 1.13, the EIE (Figure 3B). The 2° KIE for the reaction between PETNR and (*S*)-[4-^{2}H]-NADPH is 1.17 ± 0.01, identical to the value measured for MR (Figure 3B, Table 1).

Modern formalisms of enzymatic hydrogen tunneling invoke full quantum mechanical rate expressions that are based on the treatment by Marcus of Fermi's golden rule for non-adiabatic electron-transfer reactions.[21] We favor the treatment by Kuznetsov and Ulstrup[2b] with the nomenclature of Knapp et al.[2c]

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Table 1. Isotope Effects Obtained for the Reductive Half-Reaction of Morphinone Reductase and Pentaerythritol Tetranitrate Reductase

	MR				PETNR	
	NADH	(*S*)-[4-^{2}H]-'NADH	(*R*)-[4-^{2}H]-NADH	(*R,S*)-[4,4-$^2H_2$]-NADH	NADPH	(S)-[4-^{2}H]-NADPH
H_P, H_S [a]	H, H	H, D	D, H	D, D	H, H	H, D
ln A' [b]	12.8 ± 0.2	12.8 ± 0.3	14.9 ± 0.5	14.6 ± 0.3	10.8 ± 0.4	11.1 ± 0.4
A'_H:A'_D [b]		0.94 ± 0.51	0.12 ± 0.09	0.16 ± 0.09		0.79 ± 0.67
KIE_{obs} [c] (25 °C)		1.18 ± 0.02	4.44 ± 0.07	5.07 ± 0.18		1.17 ± 0.01
$KIE_{corrected}$ [d]		1.18 ± 0.02	4.6 ± 0.1	5.4 ± 0.2		1.17 ± 0.01
$\Delta H^{\ddagger}$ (kJ mol^{-1})	35.8 ± 0.6	36.4 ± 0.7	44.7 ± 1.2	44.4 ± 0.8	32.3 ± 1.0	33.3 ± 1.0
$\Delta\Delta H^{\ddagger}$ [e] (kJ mol^{-1})		0.6 ± 1.3	8.9 ± 1.7	8.6 ± 1.3		1.0 ± 2.1

[a] H_P and H_S are the primary and secondary hydrogen atoms at the nicotinamide C4 position, respectively. [b] The definitions of ln A' and A'_H:A'_D are given in Figure 3. [c] The lighter isotopomer in each case is H, H. [d] The KIEs were corrected for isotopic impurities. [e] $\Delta\Delta H^{\ddagger} = \Delta H^{\ddagger}_D - \Delta H^{\ddagger}_H$. See Experimental Section for details. Kinetic isotope effects are all relative to NAD(P)H, (H,H). Rate constants for each temperature studied are given in the Supporting Information.

where, in the simplest case,

$$k_{tun} = \frac{1}{2\pi}|V_{el}|^2\sqrt{\frac{4\pi^3}{\lambda RT\hbar^2}}\exp\left(\frac{-(\Delta G^\circ + \lambda)^2}{4\lambda RT}\right)\exp\left[\frac{-\mu_i\omega_i\Delta r^2}{2\hbar}\right] \quad (1)$$

The rate is determined by the electronic coupling between the product and reactant states, V_{el}, the driving force, ΔG°, the Marcus reorganization energy, λ, of the reaction, and the isotope-specific components of the Franck−Condon term shown in square brackets: the mass, μ_i, frequency, ω_i, and tunneling distance, Δr, of the transferred isotope. The other terms have their usual meaning. The temperature dependence (apparent enthalpy) of the observed rate of hydrogen transfer arises primarily from the Marcus term (in parentheses) and becomes more pronounced when $|\Delta G^\circ| \ll \lambda$. The magnitude of the KIE is largely determined by the tunneling distance,[2b,c] while, within the Kuznetsov and Ulstrup model,[2b] the temperature dependence of the KIE arises from an additional temperature-dependent gating term that decreases Δr and, to a lesser extent, by invoking transfer to, or from, vibrationally excited states, which adds an additional isotope-dependent vibrational energy component to the Marcus term. Both ΔG° and λ will vary slightly with isotopic substitution, which will lead to a temperature-independent contribution to the magnitude of the observed KIEs. This might contribute to the exaltation of the 2° KIE values above the EIE observed. Understanding the impact of these factors awaits development of a full tunneling formalism that accounts for 2° KIEs.

The 1° KIEs of the hydride transfer during the reductive half-reactions of MR and PETNR are quite different[1]—in MR the KIE is dependent on temperature, whereas in PETNR the KIE is independent of, or more weakly dependent on, temperature—yet their 2° KIEs are indistinguishable. This suggests that the origin of the measured 2° KIEs is not sensitive to the environmentally coupled gating motions in MR, which results in the temperature dependence of the 1° KIE. We suggest the identical 2° KIE values reflect similar local cofactor/coenzyme geometries in these enzymes in the "tunneling-ready" configuration.

Both MR and PETNR are homologues[10] of old yellow enzyme (OYE). A crystal structure of OYE with a bound NADPH analogue[22] indicates that the nicotinamide and FMN are coplanar, with the NADH C4 and FMN N5 atoms in close proximity. Stretching[23] of the nicotinamide C4−H_P bond (ω_i in eq 1) will position H_P closer to the FMN N5, facilitating transfer by decreasing the tunneling distance (Δr in eq 1). Deuteration of H_S will change the length and frequency of the C4−H_P bond stretch,[24] thus changing the tunneling distance. We do not expect the differently deuterated coenzymes to have significantly different ΔG° or λ values. We thus posit that this change in bond length, principally through a change in C−H bond frequency, is the origin of the identical 2° KIEs in MR and PETNR. If a change in the stretching frequency of H_P, caused by deuteration of H_S, significantly alters the coupling between the hydrogen tunneling coordinate and the promoting motion in the enzyme (Figure 4), then the measured $\Delta\Delta H^{\ddagger}$ values for the reaction of MR with (*R*)-[4-^{2}H]-NADH and (*R,S*)-[4,4-$^2H_2$]-NADH should be different. However, no difference is observed experimentally (Table l). As the promoting motion must be less than ~200 cm^{-1} (~k_BT)[1b] to be thermally accessible, it will be much slower than the stretching of C4−H_P (~3000 cm^{-1}).[1b] Small changes in the frequency of H_P, such as those caused by deuteration of H_S, will then *not* affect the promoting motion and hence the temperature dependence of the 1° KIE. Figure 4 illustrates the putative reaction geometry and how we envisage the promoting motion and C4−H_P bond stretch.

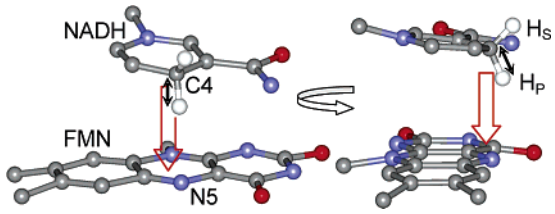

Figure 4. Orthogonal views of the putative reaction geometry in MR based on the coordinates of a complex of old yellow enzyme with a nicotinamide coenzyme analogue.[22] The black arrow shows the C4−H_P bond stretch, and the red arrow indicates the likely promoting (gating) motion of MR along the reaction coordinate.

Dideuteration of NADH also allows the coupling between H_P and H_S to be examined further in the context of the rule of the geometric mean (RGM).[17] A consequence of the RGM is that

$$k_H/k_{dideuterated} = 1^\circ\ KIE \times 2^\circ\ KIE \quad (2)$$

At 25 °C, 1° KIE × 2° KIE (4.6 ± 0.1 × 1.18 ± 0.02) = 5.4

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$\pm$ 0.2. This is not significantly different from the observed dideuterated KIE ($k_H/k_{dideuterated}$) = 5.4 $\pm$ 0.2, and therefore we do not observe a measurable violation of the RGM. Violations of the RGM[25] have been attributed by Huskey and Schowen,[17a] Ostović and co-workers,[17b] and Saunders[17c] to the influence of extra isotopic substitutions on the reduced mass of the hydrogen coordinate in reactions where tunneling is important. This occurs when there is strong vibrational coupling between the primary and secondary centers. This suggests a lack of *strong* coupling between H_P and H_S in MR and PETNR.

We have described herein two homologous enzymes that catalyze H-transfer by quantum mechanical tunneling. The mechanisms of H-transfer are different—MR, but not PETNR, appears to have a requirement for a promoting motion—yet both exhibit identical and exalted 2° KIEs. These exalted 2° KIEs provide further evidence for hydrogen tunneling in both enzymes. Importantly, we have demonstrated that the 2° KIEs—unlike 1° KIEs—are independent of promoting motions. Such a promoting motion may be considered to reflect the (nonequilibrium) pre-organization of cofactor and active-site residues into a "tunneling-ready" configuration. Thus, the 2° KIE, in conjunction with our other analyses, informs specifically on the "tunneling-ready" configuration but not on any preceding pre-organization steps. We infer that the local geometries of the "tunneling-ready" configuration are indistinguishable in MR and PETNR, despite differences in pre-organization (i.e., the inferred dominance of gated motions). When coupled with analysis of 1° KIEs, this study emphasizes the utility of 2° KIEs as additional probes of H-transfer within the context of contemporary environmentally coupled frameworks for H-tunneling.

Experimental Section

All materials were obtained from Sigma-Aldrich except NAD^+, $NADP^+$, NADH, and NADPH, which were from Melford Laboratories, and 1-[^{2}H]-glucose and 1-[$^2H_6$]-ethanol, which were from Cambridge Isotope Laboratories. Morphinone reductase (MR) and pentaerythritol tetranitrate reductase (PETNR) were purified as described.[10,11]

Synthesis of (*R*)-[4-^{2}H]-NADH. (*R*)-[4-^{2}H]-NAD(P)H was prepared through stereospecific reduction of NAD^+ with 1-[$^2H_6$]-ethanol using equine liver alcohol dehydrogenase and aldehyde dehydrogenase as described.[26] The deuterated NADH was then purified by anion-exchange chromatography and freeze-dried as described.[10]

Synthesis of (*S*)-[4-^{2}H]-NADH and (*S*)-[4-^{2}H]-NADPH. (*S*)-[4-^{2}H]-NADH and (*S*)-[4-^{2}H]-NADPH were prepared through stereospecific reduction of NAD^+ and $NADP^+$, respectively, with 1-[^{2}H]-glucose using glucose dehydrogenase as described.[27] The deuterated NADH and NADPH were then purified by anion-exchange chromatography and freeze-dried as described.[10]

Synthesis of (*R*,*S*)-[4,4-$^2H_2$]-NADH. [4-^{2}H]-NAD^+ was prepared by stereospecific oxidation of (*S*)-[4-^{2}H]-NADH with 100 mM cyclohexan-1-one using 10 μM morphinone reductase. The deuterated NAD^+ was purified in the same manner as for NAD^2H. (*R*,*S*)-[4,4-$^2H_2$]-NADH was then prepared through a further stereospecific reduction of [4-^{2}H]-NAD^+ with 1-[^{2}H]-glucose and glucose dehydrogenase and purified as per the previous steps.

Synthesis of Controls, (*S*)-[4-^{1}H]-NADH, (*R*,*S*)-[4,4-$^1H_2$]-NADH, and (*S*)-[4-^{1}H]-NADPH. (*R*,*S*)-[4,4-$^1H_2$]-NADH, (*S*)-[4-^{1}H]-NADH, and (*S*)-[4-^{1}H]-NADPH were prepared in exactly the same manner as their deuterated counterparts using unlabeled glucose or unlabeled ethanol.

(*S*)-[4-^{1}H]-NADH was used in kinetic measurements (Table S1 in the Supporting Information) of the (*S*)-[4-^{2}H]-NADH KIE. (*R*,*S*)-[4,4-$^1H_2$]-NADH and (*S*)-[4-^{1}H]-NADPH were used to establish the concentration dependence of the cofactors (Figure 2). The control compounds behaved identically to a commercial source of the coenzymes purchased from Melford Laboratories.

Table 2. Characterization of the Coenzymes Used in This Work

coenzyme	*m/z*[a]	isotopic purity (%)[b]
NADH	666.12	
(*R*)-[4-^{2}H]-NADH	667.14	94
(*S*)-[4-^{2}H]-NADH	667.13	98
(*R*,*S*)-[4,4-$^2H_2$]-NADH	668.15	90 (91% *R*, 99% S)
NADPH	nd[c]	
(*S*)-[4-^{2}H]-NADPH	nd	97

[a] Mass determined by mass spectrometry. [b] Extent of deuteration determined[28] by NMR. The NMR spectra are shown in Figure S1 in the Supporting Information. The error is estimated to be $\pm$1% impurity. [c] nd, not determined.

The identity and isotopic purity of the coenzymes were confirmed by mass spectrometry using a Waters LCT mass spectrometer (Table 2) and by 1-D NMR at 10 °C on a Bruker DRX600 spectrometer (Table 2, Figure S1 in the Supporting Information), prior to being stored dry (freeze-dried) at −80 °C. Working solutions of the coenzymes were made fresh in anaerobic 50 mM potassium phosphate pH 7.0 buffer, which was supplemented with 2 mM 2-mercaptoethanol when working with MR, and used immediately. The coenzyme concentration was determined at 340 nm (ϵ = 6.22 mM^{-1} cm^{-1}).

Kinetic Measurements and Analysis. To prevent the oxidase activities of MR and PETNR, all kinetic studies were performed under strict anaerobic conditions (<5 ppm O_2) within a glovebox environment (Belle Technology). Buffers were made anaerobic by bubbling nitrogen gas through solutions for ~1 h. Solutions were then placed in an anaerobic glovebox (Belle Technology) overnight to remove any residual traces of oxygen. Protein samples were made anaerobic by passing them through a small gel filtration (Bio-Rad 10 DG) column housed in the glovebox, which had been pre-equilibrated with anaerobic buffer.

Rapid reaction kinetic experiments were performed using an Applied Photophysics SX.18MV-R stopped-flow spectrophotometer contained within the glovebox. Spectral changes accompanying flavin reduction were monitored at 464 nm and analyzed as described below. A saturating coenzyme concentration of 5 mM was used, as this confers pseudo-first-order reaction kinetics.[10]

Individual reaction absorption traces were fit to a single-exponential expression (eq 3) using Origin 7 software (MicroCal):

$$\Delta A_{obs} = A \exp(-k_{obs} t) \quad (3)$$

The reported rate constant, *k*, is then the average rate constant, and the error is calculated as the standard deviation (SD) of the rate constants at each temperature. SD and standard error (SE) values are given in Table 1.

Arrhenius plots are curved and as such asymptotically approach infinity at high temperatures. It is therefore strictly correct to use the Eyring equation (eq 4) to analyze the temperature dependence of rate constants:

$$\ln(k/T) = \ln A' - \Delta H^{\ddagger}/RT, \quad \ln A' = \ln(k_B/h) + \Delta S^{\ddagger}/R \quad (4)$$

The parameters correspond to the semiclassical criteria: E_a (the slope), $\Delta H^{\ddagger}$, and ln A', which is analogous to the Arrhenius ln A term. Data were not fitted to the 40 °C data point due to the apparent partial denaturation of the enzymes at this temperature.

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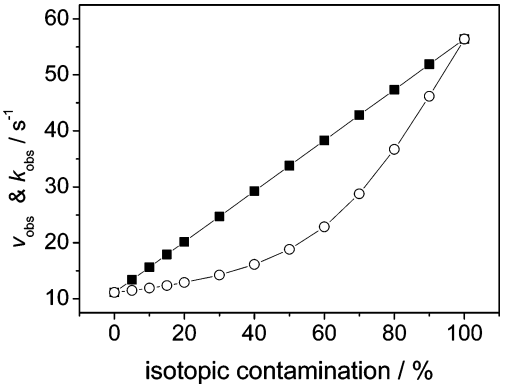

Figure 5. Effect of isotopic impurities on the observed rate constant extracted from a steady-state experiment (■, eq 5) and a stopped-flow experiment as performed in this work (○, eq 6). In both cases, $k_D = 11.13$ s^{-1} and $k_H = 56.41$ s^{-1}; see text for additional details.

Correction of Rate Constants and KIEs for Isotopic Contamination of the Coenzymes. Correction of steady-state reaction rates for isotopically impure substrates is typically performed by

$$v_{obs} = (1 - f_{impurity})k_D + f_{impurity}k_H \quad (5)$$

where $f_{impurity}$ is the fraction of isotopic impurity and k_D and k_H are the rates of the reaction with isotopically pure deuterated and protiated substrates, respectively. This leads to a linear relationship between the fraction of isotopic contamination and the observed reaction rate, which is shown in Figure 5. Unfortunately, this correction is *not* valid for stopped-flow data.

In this work, all stopped-flow traces were fit to a single-exponential function to extract a pseudo-first-order rate constant (eq 3), but when there are isotopic impurities,

$$\Delta A_{obs} = (1 - f_{impurity})A \exp(-k_D t) + f_{impurity}A \exp(-k_H t) \quad (6)$$

As the isotopic impurities in this work were all <10% (Table 2), fitting the stopped-flow traces to eq 6 was not successful, as the amplitude of the exponential describing the contamination is too small (<0.02 OD)—there is no significant improvement in the goodness of the fit when fitting from one versus two exponentials. As an alternative, the effect of isotopic impurities was estimated by simulating stopped-flow traces using eq 5 as a function of $f_{impurity}$, with $k_D = 11.126$ s^{-1} and $k_H = 56.41$ s^{-1} (i.e., the measured values for (*R*,*S*)-[4,4-$^2H_2$]-NADH and NADH at 25 °C, respectively, listed in Table S1). A single rate constant (i.e., k_{obs}) was then extracted by fitting these traces with eq 3, using exactly the same procedure as was used to analyze the experimental stopped-flow data. In Figure 5, the extracted rate constants are compared to those obtained from eq 5. Clearly, unlike in the case for steady-state data, when $f_{impurity}$ is small (<10%), then $k_{obs} \approx k_D$.

The rate constants at 25 °C for the reactions of MR with (*R*)-[4-^{2}H]-NADH and (*R*,*S*)-[4,4-$^2H_2$]-NADH were estimated by simulating stopped-flow traces (eq 6) with varying k_D values and fixed values for k_H (56.41 s^{-1}, Table S1) and $f_{impurity}$ [0.06 ± 0.01 and 0.09 ± 0.01 for NAD^{2}H and NAD$^2H_2$, respectively (Table 2)]. The small ~1% *S*-^{1}H contamination in (*R*,*S*)-[4,4-$^2H_2$]-NADH was *not* considered (see the arguments for the 2° KIEs below). The k_D values obtained are 12.21 ± 0.08 s^{-1} (cf. 12.69 s^{-1} observed) and 10.46 ± 0.08 s^{-1} (cf. 11.13s^{-1}) for (*R*)-[4-^{2}H]-NADH and (*R*,*S*)-[4,4-$^2H_2$]-NADH, respectively. The stated error is the difference in k_{obs} obtained when, for example, (*R*)-[4-^{2}H]-NADH $f_{impurity} = 0.06$ and $f_{impurity} = 0.07$ (the estimated error in the isotopic impurity). This error was then combined with the error in k_{obs} (Table S1) such that $k_D = 12.21 \pm 0.19$ s^{-1} and 10.46 ± 0.44 s^{-1} for (*R*)-[4-^{2}H]-NADH and (*R*,*S*)-[4,4-$^2H_2$]-NADH, respectively. This allows the correction of the observed 1° and dideuterated KIEs for isotopic impurity, and the values for the corrected KIEs are given in Table 1. The 2° KIEs were not corrected for isotopic impurities, as there was only ~1% protium contamination of the coenzymes (Table 2). Further, the effect of the impurity will be more pronounced when $k_D \ll k_H$, which is certainly not the case for these smaller KIEs.

The Eyring enthalpy and pre-exponential factors reported in the manuscript were calculated with the *observed* rates, *not* the corrected rates. We feel this is justified as the effect of the impurity will have a systematic and only very minor effect on the enthalpy and pre-exponential factor ratios. Again, there will be no measurable effect on the values for the reactions with (*S*)-[4-^{2}H]-NAD(P)H.

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Supporting Information Available: NMR determination of coenzyme isotopic purity and tabulated kinetic data. This material is available free of charge via the Internet at http://pubs.acs.org.

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