Unmasking of Hydrogen Tunneling in the Horse Liver Alcohol Dehydrogenase Reaction by Site-Directed Mutagenesis†

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ABSTRACT: Primary and secondary k_D/k_T and k_H/k_T kinetic isotope effects have been studied as a probe of hydrogen tunneling in the oxidation of benzyl alcohol catalyzed by horse liver alcohol dehydrogenase (LADH). In the case of the wild-type enzyme, isotope effects at 25 °C do not clearly support hydrogen tunneling; this result is consistent with a reaction rate that is partially limited by the release of product benzaldehyde. The three-dimensional structure for LADH was used to design site-directed mutations in an effort to enhance the rate of the product release step and to "unmask" tunneling. Substitutions that increased the size of the alcohol binding pocket resulted in minor changes in isotope effects. By contrast, reduction in the size of the alcohol binding pocket through substitution at residues 57 and 93, which are in van der Waals contact with bound alcohol substrate, produced a clear demonstration of protium tunneling from the breakdown of the semiclassical relationship between k_D/k_T and k_H/k_T isotope effects. The temperature dependence of k_D/k_T isotope effects has also been pursued, leading to the conclusion that tunneling does, in fact, occur in the reaction catalyzed by wild-type LADH. Despite the unmasking of protium tunneling in site-directed mutants, substitutions that decrease the size of the alcohol pocket appear to result in less extensive tunneling in the hydride transfer. It is noteworthy that the mutant enzyme (Leu^{57}→Phe), which shows the greatest evidence of tunneling, has the same catalytic efficiency (V_{max}/K_m) as the wild-type enzyme. The results show that isotope effects can reflect subtle changes in structure–function studies of enzymes that may not be apparent from initial rate parameters.

Protium, deuterium, and tritium have de Broglie wavelengths[1] of 0.6, 0.5, and 0.4 Å, respectively, at a total energy of 20 kJ/mol, which are similar to the typical distance they must travel during a hydrogen-transfer reaction. This property has led to the recognition that the behavior of hydrogen is poised between classical and quantum mechanical realms (Bell, 1980). Comparison of the reaction rates for H-, D-, and T-labeled substrates can effectively probe quantum effects under conditions of moderate levels of protium tunneling, in that the semiclassical interrelationship among k_H, k_D, and k_T [Swain–Schaad relationship (Swain et al., 1958)] is predicted to break down in a defined manner (Saunders, 1985). This has been confirmed in the hydride-transfer reaction between benzyl alcohol and NAD^+, catalyzed by yeast alcohol dehydrogenase (YADH)[2] (Cha et al., 1989). Using the temperature dependence of isotope effects as an alternative probe of tunneling, the proton-transfer reaction catalyzed by bovine serum amine oxidase has also been shown to occur with significant tunneling of both protium and deuterium (Grant & Klinman, 1989). Recent calculations indicate that the Swain–Schaad relationship does not necessarily break down when both protium and deuterium tunnel appreciably (Grant & Klinman, 1992).

Some features of a chemical reaction that affect the tunneling probability, and which are expected to be altered in an enzyme reaction, are the degree of participation of solvent reorganization, the thermodynamic relationship between reactants and products, and the shape (height and width) of the reaction barrier (Bell, 1980). The importance of the equalization of the energies of enzyme-bound reactants and products on the achievement of evolutionary perfection has received considerable attention (Albery & Knowles, 1976; Nambiar et al., 1983). Recently, the extent of hydrogen tunneling has been correlated with the $\Delta H°$ between reactant and product ternary complexes of YADH, with tunneling increasing as $\Delta H°$ decreases toward zero (Rucker et al., 1992). Another less commonly discussed feature of enzymes is the potential for catalysis resulting from the compression of the donor and acceptor atoms along the reaction coordinate (Rodgers et al., 1982; Bruno & Bialeck, 1992), leading to a concomitant reduction in barrier width and height. We have begun to use site-directed mutagenesis to explore the relationship between structure and function with respect to alterations in the potential energy surface of a reaction and the manifestation of tunneling. Although the wild-type form of alcohol dehydrogenase from horse liver (LADH) is too kinetically complex to reveal protium tunneling at 25 °C, we now show that rational mutagenesis at a single site can "unmask" this phenomenon. These findings support our view that tunneling is a general property of H-transfer reactions within enzyme active sites, thus opening up the possibility of further exploration of structure–function relationships.

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[1] The de Broglie equation $\lambda = h/(2mE)^{1/2}$ shows the $(1/m)^{1/2}$ mass dependence of the de Broglie wavelength. The probability of a particle tunneling is proportional to its de Broglie wavelength, leading to tunneling in the order H > D > T.

[2] Abbreviations: YADH, yeast alcohol dehydrogenase; LADH, horse liver alcohol dehydrogenase; HPLC, high-performance liquid chromatography; ESE, a chimeric combination of the E- and S-isozymes of LADH which differ from the E-isozyme by Thr^{94}→Ile, Arg^{101}→Ser, Phe^{110}→Leu, and the deletion of Asp^{115}.

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EXPERIMENTAL PROCEDURES

Preparation of LADH Mutants. The phagemid pBPP/LADH, which contains the LADH cDNA under the control of the *tac* promoter, also carries the replication origin for f1 bacteriophage (Park & Plapp, 1991). The phagemid was rescued from *Escherichia coli* as a single-stranded DNA (antisense) by infection with helper phage VCSM13 from Stratagene. The single-stranded template was used for site-directed mutagenesis by the Amersham mutagenesis kit, version 2, and also for dideoxy DNA sequencing by Sequenase 2.0 (U.S. Biochemical Corp.) to confirm mutations. The preparation of a chimeric combination of the E- and S-isozymes of LADH (see below) was described by Park and Plapp (1992). All results reported in this study employed enzymes which were expressed in *E. coli* XL1-blue using the phagemid pBPP/LADH; the appropriate form of the enzyme was purified according to procedures described by Park and Plapp (1991).

Steady-State Kinetics. Initial velocity studies were carried out with a SLM-4800C fluorometer with excitation at 340 nm and emission at 460 nm. The fluorescence intensity at 460 nm was collected using an IBM PS/2 computer interfaced to the fluorometer. Kinetics were measured at pH 7.0, 25 °C, with 10 mM NAD^+, 300 mM potassium phosphate, and 300 mM semicarbazide hydrochloride. The concentration of active sites was determined by spectrophotometric titration of about 20 μM enzyme with NAD^+ in the presence of 10 mM pyrazole using a double-difference technique (Theorell & Yonetani, 1963). Values of V_{max} and K_m and their standard errors were obtained by a nonlinear fit of the expression velocity = $V_{max}[S]/(K_m + [S])$.

Primary and Secondary Tritium Isotope Effects. The synthesis of the radiolabeled substrates used to measure competitive isotope effects was previously reported (Cha et al., 1989). The k_H/k_T isotope effects were measured with [*ring*-^{14}C(U)]benzyl alcohol (6.8 mCi/mmol) and [1-^{3}H]benzyl alcohol (16 Ci/mmol), which is randomly tritiated. The k_D/k_T isotope effects were measured with [1,1-$^2H_2$,*ring*-^{14}C(U)]benzyl alcohol (10.9 mCi/mmol) and [1,1-$^2H_2$, 1-^{3}H]benzyl alcohol (35 Ci/mmol), which is randomly tritiated.[3] The deuterium content of the [1,1-$^2H_2$,*ring*-^{14}C(U)]benzyl alcohol was determined by mass spectrometry of the urethane derivative to be 98.7 ± 0.3% at C-1. The [1,1-$^2H_2$,1-^{3}H]benzyl alcohol was synthesized from [1-^{2}H]benzaldehyde which was deuterated to a level >99.8%, as determined by mass spectral analysis.

The LADH-catalyzed oxidation of benzyl alcohol by NAD^+ produces benzaldehyde and NADH. For isotope effect measurements, the enzyme-catalyzed production of benzaldehyde was coupled with semicarbazide, which forms benzaldehyde semicarbazone as a final product. The coupled formation of the semicarbazone makes the enzyme reaction irreversible at the benzaldehyde product release step. Under the conditions of the experiments, irreversibility was tested by measuring isotope effects at varying enzyme activity, while keeping the concentration of semicarbazide hydrochloride constant.

[3] The primary and secondary k_D/k_T isotope effects are measured in a single experiment with 1,1-$^2H_2$ double-labeled substrates. As a result the primary k_D/k_T is determined with deuterium in the secondary position, and the secondary k_D/k_T is determined with deuterium in the primary position. By contrast, the primary and secondary k_H/k_T isotope effects are determined with hydrogen in the secondary and primary positions, respectively. Measuring isotope effects in this manner has been shown experimentally (Cha et al., 1989) and theoretically (Huskey, 1991) to be a sensitive means of demonstrating H-tunneling.

All kinetic isotope effects reported were measured at pH 7.0 in 300 mM potassium phosphate, 300 mM semicarbazide hydrochloride, and 10 mM NAD^+, and the temperature was thermostated to ±0.1 °C in a Neslab water bath. Typically, a reaction mixture of 6 mL was made, from which three aliquots (0.6 mL) were removed prior to the addition of LADH, for the measurement of the ^{3}H to ^{14}C ratio in the starting benzyl alcohol $(^3H/^{14}C)_0$. Radiolabeled benzyl alcohol was added so that a 0.5-mL high-performance liquid chromatography (HPLC) injection had approximately 5×10^5 dpm of ^{3}H and 5×10^4 dpm of ^{14}C. The amount of enzyme added was adjusted so that the reaction rate did not exceed 0.2% conversion/min to ensure proper semicarbazide coupling. Reaction aliquots of 0.6 mL were removed at fractional conversions of 5–30% and quenched by the addition of 20 μL of 15 mM $HgCl_2$, and the samples were frozen overnight (–20 °C). The aliquots of unreacted and reacted substrates were separated by HPLC with a C-18 reverse-phase column (20.0 × 0.46 cm) eluted isocratically at 1 mL/min with methanol/acetonitrile/water (12/12/76 v/v/v). The product NADH, substrate benzyl alcohol, and product benzaldehyde semicarbazone eluted at 2–6, 12–15, and 24–28 min, respectively. Fractions (1.5 mL) were collected and mixed with 12 mL of EcoLite cocktail (ICN), and the $^3H/^{14}C$ ratio was counted for 2 min with a dual $^3H/^{14}C$ program by a liquid scintillation counter (LKB Model 1209 Rackbeta).

Primary isotope effects were determined from the comparison of the ratio of ^{3}H in product NADH to ^{14}C in product benzaldehyde semicarbazone $(^3H/^{14}C)_t$ versus the $(^3H/^{14}C)_0$ of the initial benzyl alcohol substrate. Secondary isotope effects were determined by comparison of the $(^3H/^{14}C)_t$ ratio of product benzaldehyde semicarbazone versus the $(^3H/^{14}C)_0$. The k_H/k_T isotope effects were calculated by eq 1 (Melander & Saunders, 1987):

$$k_H/k_T = \ln(1-f)/\ln\{1-f[(^3H/^{14}C)_t/0.5(^3H/^{14}C)_0]\} \quad (1)$$

where f is the fractional conversion as determined by the ^{14}C in product versus substrate. The ratios of ^{3}H in the primary or secondary position at C1 to ^{14}C (ring label) are each equal to one-half of the $(^3H/^{14}C)_0$, since the benzyl alcohol is randomly tritiated at C1. Controls were routinely run which compared the $(^3H/^{14}C)_0$ of benzyl alcohol to the $(^3H/^{14}C)_\infty$ of product which was completely oxidized to benzoic acid and NAD[^{3}H] with LADH and aldehyde dehydrogenase (Sigma). The k_D/k_T isotope effects were corrected for 1.3% H contamination in [1,1-$^2H_2$,*ring*-^{14}C(U)]benzyl alcohol by using an integrated expression of products arising from substrate which has H contamination (Cha et al., 1989; Grant & Klinman, 1989).

RESULTS AND DISCUSSION

LADH was chosen for this structure–function study because of the availability of X-ray crystallographic structures, including one of the productive ternary complex of *p*-bromobenzyl alcohol·NAD^+·LADH (Eklund et al., 1982). Unlike the oxidation of benzyl alcohol catalyzed by YADH, which has a rate-limiting chemical step (Klinman, 1976), the LADH reaction is partially limited in rate by product benzaldehyde dissociation (Sekhar & Plapp, 1990). The influence of kinetic complexity on the magnitude of observed tritium isotope effects (k_H/k_T and k_D/k_T) is described in eqs 2 and 3:

$$k_H/k_T = [(k_H/k_T)_{int} + C_H]/[1 + C_H] \quad (2)$$

$$k_D/k_T = [(k_D/k_T)_{int} + C_D]/[1 + C_D] \quad (3)$$

where $(k_H/k_T)_{int}$ and $(k_D/k_T)_{int}$ are the intrinsic tritium kinetic

Table I: Kinetic Constants, Tritium Isotope Effects, and the Exponential Relationship between k_D/k_T and k_H/k_T Isotope Effects for the Oxidation of Benzyl Alcohol Catalyzed by Mutant Forms of LADH[a]

		larger alcohol pocket		smaller alcohol pocket	
	wild-type	ESE	Leu^{57}→Val	Leu^{57}→Phe	Phe^{93}→Trp
V_{max} (s^{-1})[b]	0.32	1.7	0.095	0.24	0.13
V_{max}/K_m (mM^{-1} s^{-1})[b]	8.8	3.3	3.5	8.6	4.7
primary k_H/k_T[c]	7.149 ± 0.025 (34)	7.593 ± 0.036 (21)	7.550 ± 0.030 (13)	7.308 ± 0.048 (13)	7.755 ± 0.034 (24)
primary k_D/k_T[c]	1.894 ± 0.013 (13)	1.872 ± 0.006 (24)	1.902 ± 0.021 (11)	1.827 ± 0.008 (16)	1.858 ± 0.005 (20)
secondary k_H/k_T[c]	1.335 ± 0.003 (36)	1.332 ± 0.004 (21)	1.332 ± 0.003 (14)	1.318 ± 0.007 (14)	1.333 ± 0.004 (24)
secondary k_D/k_T[c]	1.073 ± 0.008 (13)	1.075 ± 0.003 (24)	1.065 ± 0.011 (11)	1.033 ± 0.004 (17)	1.048 ± 0.004 (19)
primary exponent[d]	3.08 ± 0.04	3.23 ± 0.02	3.14 ± 0.05	3.30 ± 0.03	3.31 ± 0.02
secondary exponent[d]	4.10 ± 0.44	3.96 ± 0.16	4.55 ± 0.75	8.50 ± 0.99	6.13 ± 0.50

[a] Measured at pH 7.0, 25 °C, with 10 mM NAD^+, 300 mM semicarbazide hydrochloride, and 300 mM potassium phosphate. [b] Standard errors are less than 15% of the reported value. [c] The error is reported as the standard error of the mean, and the number of determinations is in parentheses. [d] The exponent relating $(k_D/k_T)^{exp} = k_H/k_T$. This exponent's semiclassical upper limit is 3.26–3.34 (Swain et al., 1958; Saunders, 1985). The error of the exponent was calculated as follows: error = $\exp[\{\partial \ln(k_H/k_T)/\ln(k_H/k_T)\}^2 + \{\partial \ln(k_D/k_T)/\ln(k_D/k_T)\}^2]^{1/2}$ (Grant, 1989).

isotope effects and C_H and C_D, the commitments to catalysis, are the ratio of rate constants for the chemical step versus dissociation of substrate or product from the enzyme (Northrop, 1977). As discussed previously (Cha et al., 1989), kinetic complexity in a reaction leads to a greater reduction in the observed k_H/k_T isotope effect relative to its intrinsic value than in the observed k_D/k_T isotope effect relative to its intrinsic value. This leads to a breakdown in the Swain–Schaad relationship (eq 4) which is opposite to that predicted for tunneling (eq 5):

$$\text{kinetic complexity:} \quad k_H/k_T < (k_D/k_T)^{3.26-3.34} \tag{4}$$

$$\text{tunneling:} \quad k_H/k_T > (k_D/k_T)^{3.26-3.34} \tag{5}$$

For the case in which both kinetic complexity and hydrogen tunneling exist in a reaction, it is possible that a canceling of effects on the exponential breakdown takes place, such that one fortuitously observes the semiclassical relationship of $k_H/k_T \approx (k_D/k_T)^{3.26-3.34}$ (Swain et al., 1958; Saunders, 1985).

In the present study, we first measured tritium isotope effects using the wild-type form of LADH. As shown in Table I, exponents relating k_H/k_T and k_D/k_T isotope effects do not deviate greatly from 3.26 to 3.34. In light of the known property of slow aldehyde release from LADH, this result most likely reflects opposing influences of tunneling and kinetic complexity. We reasoned that an increase in the rate of product release would lead to inflated exponents relating tritium isotope effects and, in this manner, direct evidence for H-tunneling. With the goal of testing this hypothesis, site-directed mutagenesis was used to either increase or decrease the size of the alcohol binding pocket.

Using the structure of the ternary complex as a guide, hydrophobic residues that are within 4 Å (C–C distance) of the bound alcohol were selected as targets for mutagenesis. The LADH mutants that have been examined are Leu^{57}→Val, Leu^{57}→Phe, Phe^{93}→Trp, and a chimeric combination of the E- and S-isozymes of LADH (ESE), which differs from the wild-type E-isozyme by Thr^{94}→Ile, Arg^{101}→Ser, Phe^{110}→Leu, and the deletion of Asp^{115} (Park & Plapp, 1992). This deletion produces an enzyme active on a steroid alcohol and is believed to make a larger alcohol binding pocket by shifting Leu^{116} away from the bound alcohol. The positions of these residues are displayed in Figure 1 relative to the bound *p*-bromobenzyl alcohol, NAD^+, and the catalytic Zn.

The steady-state parameters V_{max} and V_{max}/K_m for expressed LADH enzymes were measured under identical conditions to isotope effect measurements and are reported in Table I. The kinetic parameter that is directly compared in the present study by competitive isotope effects is V_{max}/K_m, which has

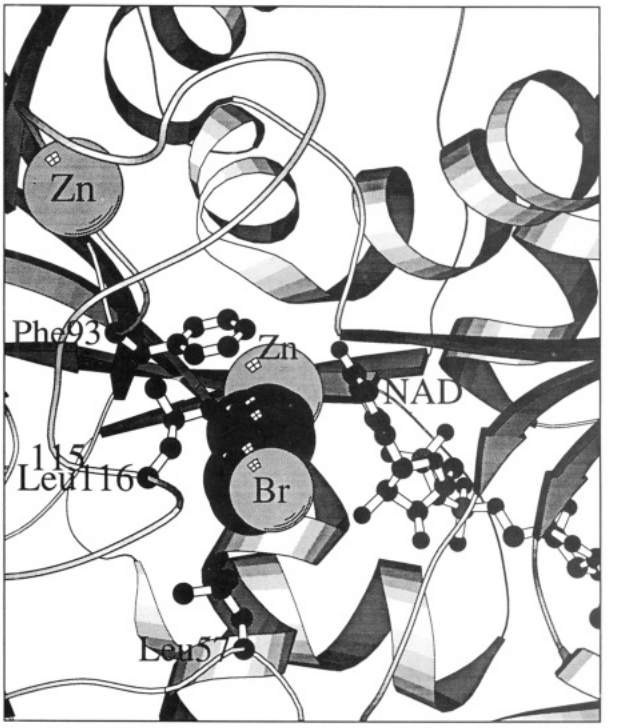

FIGURE 1: Ribbon structure of LADH displaying the positions of Leu^{57}, Phe^{93}, Leu^{116}, and residue 115 relative to the bound *p*-bromobenzyl alcohol, the cofactor NAD^+, and the structural and catalytic Zn atoms of the ternary complex crystallographic structure (Eklund et al., 1982). Residue 115 is deleted in the ESE enzyme, and this change is believed to shift Leu^{116} away from the bound alcohol.

values of 38%, 40%, and 53% of the wild-type value for ESE, Leu^{57}→Val, and Phe^{93}→Trp enzymes, respectively. Surprisingly, the Leu^{57}→Phe and wild-type enzymes have virtually identical V_{max}/K_m values.

Comparison of k_H/k_T and k_D/k_T isotope effects and their exponential relationship among the wild-type and mutant forms of enzyme (Table I) indicates different trends, depending on whether mutagenesis has led to an increase or decrease in the size of the substrate binding pocket. The isotope effects measured for enzyme with a larger alcohol binding pocket (ESE and Leu^{57}→Val) are similar to the wild-type values. The k_D/k_T isotope effects, which are the most sensitive probes of transition-state structure since they approximate intrinsic values (Grant & Klinman, 1989; Rucker et al., 1992), differ only within experimental error for these enzymes. This suggests that features of the hydride transfer such as the extent of tunneling are unchanged among the wild-type, ESE, and Leu^{57}→Val forms of LADH. The primary k_H/k_T values for

ESE and Leu^{57}→Val mutants are measurably larger than the wild-type value and are consistent with decreased kinetic complexity. The simplest conclusion from these data is that enlarging the alcohol binding pocket has slightly reduced the kinetic complexity of the reaction, without altering the intrinsic isotope effects, transition-state structure, or degree of hydrogen tunneling.

By contrast, isotope effects measured for mutants with a smaller alcohol binding pocket (Leu^{57}→Phe and Phe^{93}→Trp) are significantly different from those of the wild-type values and lead to a breakdown in the exponential relationship between k_D/k_T and k_H/k_T isotope effects that is in the direction anticipated for tunneling. As with the mutants with a larger alcohol pocket, the primary k_H/k_T values for Leu^{57}→Phe and Phe^{93}→Trp are measurably larger than the wild-type value, consistent with decreased kinetic complexity. Additionally, however, these mutants have primary and secondary k_D/k_T isotope effects that are significantly less than the wild-type values. This leads to exponents relating secondary isotope effects that are greatly inflated relative to the semiclassical limit of 3.26–3.34 (Swain et al., 1958; Saunders, 1985). The values for exponents of 8.50 and 6.13 for Leu^{57}→Phe and Phe^{93}→Trp enzymes, respectively, provide clear-cut evidence for tunneling. The isotope effects and, in particular, their exponential relationships for the Leu^{57}→Phe mutant are very similar to values previously measured for YADH (Cha et al., 1989), which contains Trp at positions 57 and 93 (Ganzhorn et al., 1987). It appears that the reduction in size of the alcohol binding pocket has converted LADH to a form that resembles YADH, leading to increased dissociation rates for alcohol substrate and aldehyde product, a concomitant reduction in kinetic complexity, and an increased ability to detect tunneling.

Mutations with a smaller alcohol binding pocket have k_D/k_T isotope effects that are less than the wild-type values, suggesting that the potential surface for the hydride transfer has changed compared to the wild-type form of enzyme. The reduction of the primary and secondary k_D/k_T isotope effects for the Leu^{57}→Phe and Phe^{93}→Trp enzymes may be the result of an earlier transition state, less inherent deuterium tunneling, or a combination of changes occurring to the reaction potential energy surface. In order to assess possible changes of inherent tunneling, the temperature dependencies of isotope effects were compared for the ESE and Phe^{93}→Trp enzymes. The ESE enzyme was chosen as a model for the temperature dependence of isotope effects of the wild-type enzyme because the ESE enzyme has less kinetic complexity, while it is suggested that the intrinsic isotope effects are identical. Figure 2 shows Arrhenius plots of $\ln(k_L/k_T)$ versus the reciprocal of temperature between 0 and 45 °C for primary isotope effects of the ESE and Phe^{93}→Trp enzymes. Extrapolation to infinite temperature provides the magnitude of isotope effects on preexponential Arrhenius parameters A_H/A_T and A_D/A_T, (Table II).

The observation of values for A_L/A_T which fall below their semiclassical limit can provide unambiguous evidence for tunneling when the hydrogen-transfer step is known to be rate limiting [as reviewed in Klinman (1991)]. As a probe of tunneling, the temperature dependence of k_D/k_T isotope effects has an advantage over k_H/k_T isotope effects, due to the reduction or elimination of kinetic complexity from k_D/k_T measurements (Grant & Klinman, 1989; Rucker et al., 1992). Turning to the ESE enzyme, deuterium tunneling is suggested by the primary A_D/A_T = 0.61, which is well below the semiclassical lower limit of A_D/A_T = 0.9 (Schneider & Stern, 1972). Since greater tunneling is expected with H than D, the similarity of A_H/A_T and A_D/A_T is consistent with kinetic complexity in the former parameter, which leads to an elevation of the observed value of A_H/A_T toward unity. The Phe^{93}→Trp enzyme has a primary A_D/A_T = 0.86, which is within experimental error of the semiclassical range, whereas the primary A_H/A_T = 0.49 is significantly below the semiclassical lower limit of A_H/A_T = 0.6 (Schneider & Stern, 1972). In this case the trends in A_H/A_T and A_D/A_T are in the direction expected for H-tunneling, confirming a reduction in kinetic complexity relative to the ESE and wild-type enzyme forms. Of particular interest is the lower value of A_D/A_T for ESE than for Phe^{93}→Trp, suggesting greater inherent tunneling in the ESE enzyme. We suggest that the wild-type and ESE enzyme forms are actually characterized by a greater degree of tunneling than Phe^{93}→Trp and Leu^{57}→Phe enzymes but that this is obscured in the protium case by slow release of products.

In closing, we emphasize that one of the mutated enzymes (Leu^{57}→Phe) shows V_{max} and V_{max}/K_m values virtually identical to those of the wild-type enzyme. This is in contrast to demonstrable changes in isotope effects and extents of

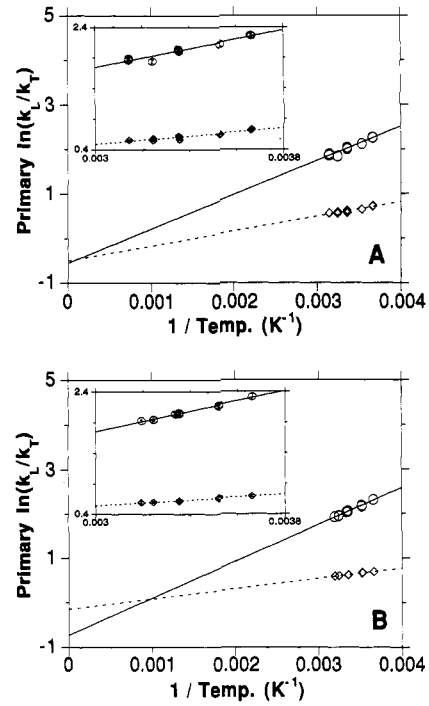

FIGURE 2: Arrhenius plots for the temperature dependence of primary k_H/k_T (○) and k_D/k_T (◇) isotope effects for the reactions catalyzed by ESE (A) and Phe^{93}→Trp (B) enzymes over the temperature range of 0–45 °C. Each data point represents a single experiment with five to eight determinations, and the error bars shown in the insert are the standard deviation of each experiment. The points are fit to the Arrhenius equation $\ln(k_L/k_T) = \Delta E_{act}/RT + \ln(A_L/A_T)$ to obtain the Arrhenius parameters A_H/A_T and A_D/A_T.

Table II: Arrhenius Parameters from the Temperature Dependence of Primary Tritium Isotope Effects[a]

	ESE[b]	Phe^{93}→Trp[b]	semiclassical lower limit[c]
primary A_H/A_T	0.58 ± 0.13	0.49 ± 0.05	0.6
primary A_D/A_T	0.61 ± 0.10	0.86 ± 0.06	0.9

[a] Isotope effects were measured over the temperature range of 0–45 °C under the conditions described in Table I. [b] The reported error for Arrhenius parameters (A_L/A_T) is the standard error of the y-intercept of a least squares linear fit of the Arrhenius equation $\ln(k_L/k_T) = \Delta E_{act}/RT + \ln(A_L/A_T)$. [c] The theoretical lower limit of Arrhenius parameters for reactions without hydrogen tunneling (Schneider & Stern, 1972).

hydrogen tunneling. We conclude that isotope effects can reflect subtle changes in structure–function studies of enzyme catalysis that may not be apparent from a comparison of initial rate parameters. The generality of tunneling in enzyme-catalyzed H-transfer reactions is supported by the use of site-directed mutagenesis, which has been used to demonstrate protium tunneling in a system that normally has this phenomenon masked by kinetic complexity. Of potential importance, modifications of the protein structure which decrease the size of the alcohol binding pocket appear to decrease the degree of tunneling contributing to the hydride-transfer step. We are currently exploring other modifications of LADH, in an effort to understand the relationship between protein structure and the manifestation of tunneling in H-transfer reactions.

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