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Effect of Pressure on a Heavy-Atom Isotope Effect of Yeast Alcohol Dehydrogenase

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Abstract: Hydrostatic pressure causes a monophasic decrease in the ^{13}C primary isotope effect expressed on the oxidation of benzyl alcohol by yeast alcohol dehydrogenase. The primary isotope effect was measured by the competitive method, using whole-molecule mass spectrometry. The effect is, therefore, an expression of isotopic discrimination on the kinetic parameter *V*/*K*, which measures substrate capture. Moderate pressure increases capture by activating hydride transfer, the transition state of which must therefore have a smaller volume than the free alcohol plus the capturing form of enzyme [Cho, Y.-K.; Northrop, D. B. *Biochemistry* **1999**, *38*, 7470−7475]. The decrease in the ^{13}C isotope effect with increasing pressure means that the transition state for hydride transfer from the heavy atom must have an even smaller volume, measured here to be 13 mL·mol^{-1}. The pressure data factor the kinetic isotope effect into a semiclassical reactant-state component, with a null value of $k_{12}/k_{13} = 1$, and a transition-state component of $Q_{12}/Q_{13} = 1.028$ (borrowing Bell's nomenclature for hydrogen tunneling corrections). A similar experiment involving a deuterium isotope effect previously returned the same volume and null value, plus a pressure-sensitive isotope effect [Northrop, D. B.; Cho, Y.-K. *Biochemistry* **2000**, *39*, 2406−2412]. Consistent with precedence in the chemical literature, the latter suggested a possibility of hydrogen tunneling; however, it is unlikely that carbon can engage in significant tunneling at ambient temperature. The fact that the decrease in activation volumes for hydride transfer is equivalent when one mass unit is added to the carbon end of a scissile C−H bond and when one mass unit is added to the hydrogen end is significant and suggests a common origin.

Introduction

Hydrostatic pressure is not expected to affect normal primary kinetic isotope effects on chemical reactions because the differences in reaction rates for isotopic and nonisotopic reactants arise solely from differences in zero-point vibrational frequencies, as described by Bigeleisen and Wolfsberg[1] and illustrated in Figure 1A. Various examples of these vibrational frequencies were examined by Isaacs,[2] using infrared and Raman spectra at high pressure, who inferred that their sensitivities to hydrostatic pressure are relatively small and insignificant at the experimental range of pressure available to kinetic studies, e.g., <5 kbar. This inference was verified experimentally in multiple chemical reactions expressing normal deuterium isotope effects, including hydrogen transfer, hydride transfer, and proton transfer.

However, eight reactions with large abnormal deuterium isotope effects, for which quantum mechanical hydrogen tunneling was suspected, illustrated in Figure 1B, were subsequently shown by Isaacs to be sensitive to pressures in the low kilobar range. All of them displayed a partial decrease in measured isotope effects as pressure increased. For example, Lewis and co-workers[3] had shown that the deuterium isotope effect on the oxidation of leuco-crystal violet by chloranil (tetrachloro-*p*-benzoquinone) was abnormally large, i.e., $k_H/k_D > 12$, and had an anomalous temperature dependence. Isaacs, Javaid, and Rannala[4] therefore examined the effects of pressure on this

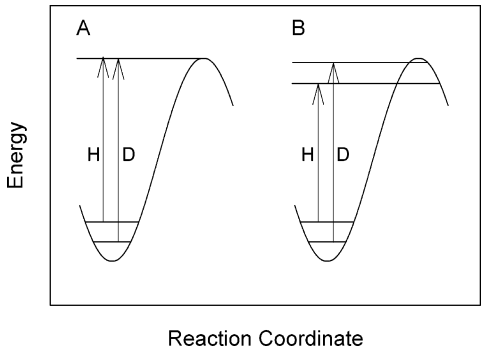

Figure 1. Reaction-coordinate diagrams depicting the origin of deuterium isotope effects. (A) The semiclassical model, wherein different energies of activation for hydrogen and deuterium originate in different zero-point energies in the reactant state. (B) The quantum mechanical model, wherein particle tunneling at different reaction barrier widths near the transition state also contributes to differences in energies of activation.

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(1) Bigeleisen, J.; Wolfsberg, M. *Adv. Phys. Chem.* **1958**, *1*, 15−76.
(2) Isaacs, N. S. In *Isotope Effects in Organic Chemistry*, *Volume 6*; Buncel, E., Lee, C. C., Eds.; Elsevier: London, UK, 1984; pp 67−105.
(3) (a) Lewis, E. S.; Robinson, J. K. *J. Am. Chem. Soc.* **1968**, *90*, 4337. (b) Lewis, E. M.; Perry, J. M.; Grinstein, *J. Am. Chem. Soc.* **1970**, *92*, 709.

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reaction and found that the isotope effect decreased asymptotically to values near $k_H/k_D = 8$. They concluded that pressure affected the tunneling correction factor described by Bell,[5] who formulated the following expression:

$$\left(\frac{k_H}{k_D}\right)_{obs} = \frac{k_H Q_H}{k_D Q_D} \tag{1}$$

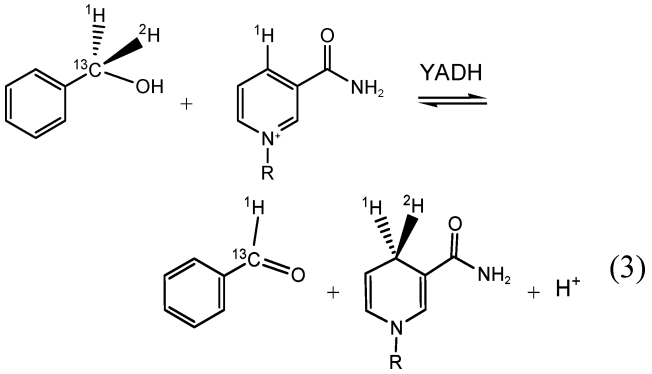
where k_H/k_D represents the portion of an isotope effect arising from differences in zero-point energies in the reactant state and Q_H/Q_D is the tunneling correction factor arising from apparent differences in the transition state.

Northrop[6] factored eq 1 into pressure-dependent and pressure-independent components, as shown in the following expression:

$$\left(\frac{k_H}{k_D}\right)_p = \frac{k_H}{k_D}\left(\frac{Q_H}{Q_D} - 1\right) e^{-\Delta V_Q p/RT} + \frac{k_H}{k_D} \tag{2}$$

where ΔV_Q is the change in partial molar volumes of hydride versus deuteride transfer, p is the pressure in bar (0.98692 standard atmospheres), R is the gas constant at 83.13 mL·bar/mol·K,[7] and T is the kelvin temperature. A fit of the data of Isaacs, Javaid, and Rannala[4] to eq 2 returned values of $\Delta V_Q = 36.5 \pm 3.0$ mL/mol, $k_H/k_D = 7.8 \pm 0.1$, and $Q_H/Q_D = 1.44 \pm 0.02$. Thus, 33% of the observed deuterium isotope effect at atmospheric pressure is sensitive to hydrostatic pressure and would appear to originate in hydrogen tunneling.

Deuterium isotope effects on the reaction catalyzed by yeast alcohol dehydrogenase (YADH, EC 1.1.1.1) were suspected of having a hydrogen tunneling component,[8] and consistent with this supposition, Northrop and Cho[9] found that high pressure caused a decrease in the primary isotope effect on hydride transfer, ^2H in eq 3, from benzyl alcohol to nicotinamide adenine dinucleotide (NAD^+). However, a fit of the data to eq 2 returned



(3)

values of $\Delta V_Q = 10.4 \pm 1.5$ mL/mol, $k_H/k_D = 0.99 \pm 0.03$, and $Q_H/Q_D = 4.99 \pm 0.37$. Thus, unlike the chemical oxidation of leuco-crystal violet, where a minor portion of the deuterium isotope effect was pressure-dependent, *all* of the observed deuterium isotope effect on the enzymatic oxidation of benzyl alcohol appears to be sensitive to hydrostatic pressure. Or, to put it another way, the entire isotope effect appears to originate in some phenomenon in the transition state, with none of it originating from differences in zero-point vibrational frequencies in the reactant state. This unusual finding has been observed subsequently with other alcohol substrates[10] and other pyridine nucleotides (unpublished results).

While hydrogen tunneling may be partially responsible for the pressure sensitivity of the primary deuterium isotope effect of YADH, it is difficult to understand how it alone can account for these results. In the Bell formulation of eq 1, reactant-state and transition-state effects are multiplied together, so the zero-point vibrational differences will not simply go away when tunneling becomes significant. Some other transition-state phenomenon must certainly be contributing to some, if not all, of the isotope effect. In an attempt to distinguish between tunneling and some other transition-state phenomenon, a determination of the effect of pressure on a ^{13}C isotope effect was initiated because tunneling does not contribute to heavy-atom isotope effects, at least not to any significant degree at ambient temperatures.[11]

Theory

Isotopic discrimination in a competitive experimental design is dependent solely upon the steady-state kinetic parameter V/K, or substrate capture.[12] It is independent of events following the first irreversible step and obeys the equation:

$$\frac{(V/K)_{12}}{(V/K)_{13}} = \frac{k_{12}/k_{13} + C}{1 + C} \tag{4}$$

where k_{12}/k_{13} is the intrinsic isotope effect and C stands for the sum of the forward and reverse commitments to catalysis.[13] If the commitments change as a function of pressure, eq 4 becomes:

$$\left(\frac{(V/K)_{12}}{(V/K)_{13}}\right)_p = \frac{k_{12}/k_{13} + C\, e^{-\Delta V^{\ddagger} p/RT}}{1 + C\, e^{-\Delta V^{\ddagger} p/RT}} \tag{5}$$

where $\Delta V^{\ddagger}$ is the volume of activation between reactants E + S, e.g., E*-NAD^+ and benzyl alcohol in the current experimental design,[9] and the transition state of the isotopically sensitive step. By analogy to eq 2, the effect of pressure on the tunneling component of a heavy-atom isotope effect of carbon should obey the equation:

$$\left(\frac{k_{12}}{k_{13}}\right)_p = \frac{k_{12}}{k_{13}}\left(\frac{Q_{12}}{Q_{13}} - 1\right) e^{-\Delta V_Q p/RT} + \frac{k_{12}}{k_{13}} \tag{6}$$

By substituting the right half of eq 6 for the intrinsic isotope effect in eq 5, a general expression for the effect of pressure on a ^{13}C isotope effect on substrate capture can be written:

(4) Isaacs, N. S.; Javaid, K.; Rannala, E. *J. Chem. Soc., Perkin Trans. 2* **1987**, 709−711.
(5) Bell, R. P. *The Tunnel Effect in Chemistry*; Chapman and Hall: London and New York, 1980.
(6) Northrop, D. B. *J. Am. Chem. Soc.* **1999**, *121*, 3521−3524.
(7) Previous papers from this laboratory incorrectly listed a value of 82.0578 in the text for the gas constant. This value applies to pressures measured in atmospheres, not bars. However, all computer regression analyses in these papers used the correct value of 83.133739.
(8) (a) Huskey, W. P.; Schowen, R. L. *J. Am. Chem. Soc.* **1983**, *105*, 5704−5706. (b) Cha, Y.; Murray, C. J.; Klinman, J. P. *Science* **1989**, *243*, 1325−1330.
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(11) Zuev, P. S.; Sheridan, R. S.; Albu, T. V.; Truhlar, D. G.; Hrovat, D. A.; Borden, W. T. *Science* **2003**, *299*, 867−870.
(12) Northrop, D. B. *J. Chem. Educ.* **1998**, *75*, 1153−1157.
(13) Northrop, D. B. In *Isotope Effects on Enzyme-Catalyzed Reactions*; Cleland, W. W., O'Leary, M. H., Northrop, D. B., Eds.; University Park Press: Baltimore, 1977; pp 122−152.

$$\left(\frac{(V/K)_{12}}{(V/K)_{13}}\right)_p = \frac{(k_{12}/k_{13})(Q_{12}/Q_{13} - 1)\, e^{-\Delta V_Q p/RT} + k_{12}/k_{13} + C\, e^{-\Delta V^{\ddagger} p/RT}}{1 + Ce^{-\Delta V^{\ddagger} p/RT}} \quad (7)$$

Results

To generate an organic ion for analysis by whole-molecule mass spectrometry, the product of the YADH reaction, benzaldehyde, was converted to benzoic acid by linking the enzymatic reaction to aldehyde dehydrogenase. The benzoate ion was extracted from the reaction mixture in the aqueous phase at alkaline pH. Figure 2 shows a representative mass spectrum of a sample of extracted benzoic acid derived from equimolar portions of α-^{12}C-benzyl alcohol and α-^{13}C-benzyl alcohol. The isotopic peaks are cleanly separated with very little overlap. As has been pointed out by Goshe and Anderson,[14] who first proposed whole-molecule electrospray mass spectrometry as a means for determining heavy-atom isotope effects, the accuracy of the method is set by the degree of overlap and capability of one's mass spectrometer to resolve the isotopic peaks.

Figure 3 shows an isotopic dilution curve in which the ratios of the isotopic peak areas vary as a function of changes in the molar portions of ^{12}C-benzoic acid and ^{13}C-benzoic acid derived from the benzyl alcohols. A fit of the data to a straight line returned an $R^2 = 0.9947$. This fit attests to the precision of the method and demonstrates that whole-molecule electrospray mass spectrometry is suitable for determining heavy-atom isotope effects of carbon by isotopic discrimination.

The solid line in Figure 4 shows the heavy-atom isotopic discrimination as expressed on substrate capture as a function of pressure. The data generate a concave monophasic curve that decreases with increasing pressure. The data were fit to eq 7 by fixing $\Delta V^{\ddagger}$ at -38 mL·mol^{-1} (the value obtained for the activation volume of hydride transfer in deuterium isotope effect experiments[9]), and the regression converged with values of $Q_{12}/Q_{13} = 1.028 \pm 0.006$, $\Delta V_Q = 13.1 \pm 4.5$ mL·mol^{-1}, $k_{12}/k_{13} = 1.00 \pm 0.01$, and $C < 0.001 \pm 0.0013$. Given that the latter is small and insignificant, the data were refit to eq 7 with the commitment term set at zero, which returned values of $Q_{12}/Q_{13} = 1.028 \pm 0.005$, $k_{12}/k_{13} = 0.999 \pm 0.006$, and $\Delta V_Q = 13.1 \pm 4.2$ mL·mol^{-1}. A final fitting to eq 7 with k_{12}/k_{13} fixed at 1.0 generated the curve shown in Figure 4, with values of $Q_{12}/Q_{13} = 1.028 \pm 0.001$ and $\Delta V_Q = 13.1 \pm 0.7$ mL·mol^{-1}.

Given the unexpected nature of this result, and for the purposes of a complete analysis, attempts were also made to force a fit of the data to eq 5. These were difficult regressions to run, in that initial estimate of parameters had to be very close to the final values in order to achieve convergence. The dashed line in Figure 4 shows one fitting to eq 5, with the intrinsic isotope effect constrained to the value observed at atmospheric pressure. The first phase of the biphasic curve is convex and overlays the concave pattern of the data points. Fitted parameters were $C = 0.13 \pm 0.22$ and $\Delta V^{\ddagger} = -36 \pm 13$ mL·mol^{-1}. This is a very poor fit, given the large standard error and the lack of agreement between point and line. Similarly, as shown in the inset to Figure 4, an unconstrained fit brings the second phase of the biphasic curve into harmony with the concave pattern of the data points, but with very large commitments to catalysis and a long convex extrapolation at negative theoretical pressures

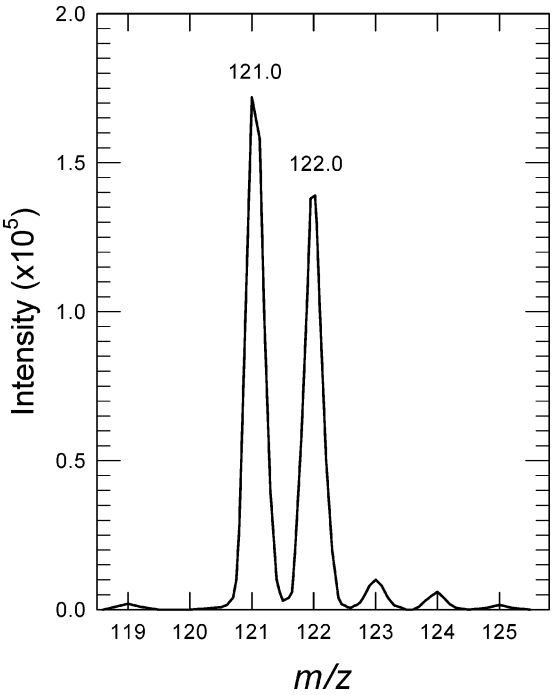

Figure 2. Mass spectrum of a sample of extracted benzoic acid. Contributions from the ^{12}C isomer to the peak at *m*/*z* 122 due to natural abundance isotopes, and from the ^{13}C isomer to the peak at *m*/*z* 121 due to less than 100% isotopic labeling, measured in separate runs to be less than 5%, were offsetting and were ignored in the calculations of isotope effects.

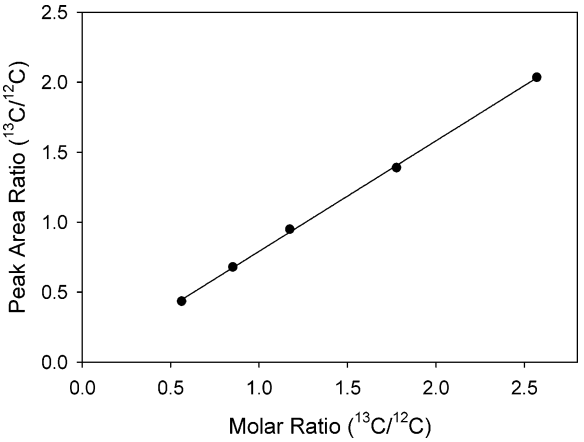

Figure 3. Isotope dilution curve. Equimolar samples containing mixtures of ^{12}C-benzyl alcohol and ^{13}C-benzyl alcohol in various proportions were subjected to oxidation, extraction, and analysis by whole-molecule electrospray mass spectrometry.

to a very large intrinsic isotope effect. Fitted parameters were $k_{12}/k_{13} = 1.16 \pm 0.61$, $C = 5 \pm 22$, and $\Delta V^{\ddagger} = -14.5 \pm 7.5$ mL·mol^{-1}. Not only is this a poor fit, given the large standard errors, but also the value for the intrinsic isotope effect is unrealistic, given that the Streitwieser semiclassical limit for isotope effects on ^{13}C−H bond cleavage is 1.02095.[15]

Conclusions

The heavy-atom isotope effects obtained here for YADH are in reasonable agreement with precedent, as Scharschmidt, Fisher, and Cleland[16] obtained a value of $(V/K)_{12}/(V/K)_{13} = 1.022$ by

(14) Goshe, M. B.; Anderson, V. E. *Anal. Biochem.* **1995**, *231*, 387−392.
(15) Huskey, P. W. In *Enzyme Mechanism from Isotope Effects*; Cook, P. F., Ed.; CRC Press: Boca Raton, FL, 1991; pp 331−338.

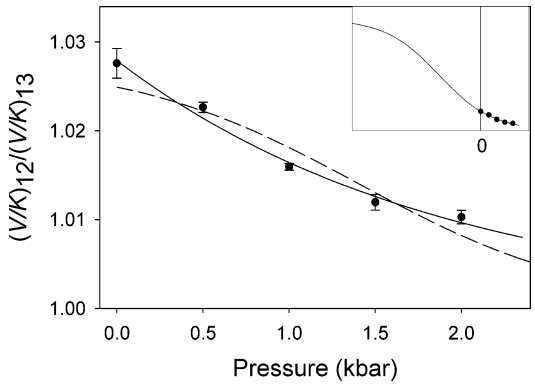

Figure 4. Effect of pressure on values of $(V/K)_{12}/(V/K)_{13}$ fit to eq 7. The dashed line represents a fit of the data to eq 5, with a fixed value for the intrinsic isotope effect of $k_{12}/k_{13} = 1.028$. The inset is a fit of the data to eq 5 without any constraint on the intrinsic isotope effect.

the natural abundance method using dideuteriobenzyl alcohol at pH 8.0. Both accuracy and precision appear well-served by the whole-molecule electrospray mass spectrometry method, in addition to the ease and simplicity afforded by not having to degrade the molecule and isolate the heavy atom of interest.

The primary finding is the large decrease in measured isotopic discrimination with increasing hydrostatic pressure. That in itself is unprecedented and unexpected, and it constitutes new information about isotope effects and enzymatic reactions. Moreover, the decrease is consistent with a kinetic model in which it originates solely from a direct reduction of the intrinsic isotope effect itself, and not from a change in the expression of the isotope effect due to increasing commitments to catalysis. The fit to eq 6 using a partial regression is consistent with only very small values for the commitments throughout the entire pressure range (e.g., $C = 0.0037$ at atmospheric pressure increases to only 0.079 at 2 kbar), in agreement with similar results of the effect of pressure on the deuterium isotope effect, which returned $C < 0.001 \pm 0.012$.[9] By definition, the commitments must be exactly the same in both experimental designs. The fit of the competitive heavy-atom isotope effects to eq 6 with negligible commitments, shown in Figure 4, gives a simple convex decrease as a function of increasing pressure, with excellent agreement between point and line.

This pressure function is highly reminiscent of the results obtained when noncompetitive deuterium isotope effects were subjected to high pressures; in both cases, a monophasic decrease was observed approaching an asymptotic value not significantly different from one, denoting the absence of a semiclassical isotope effect originating in differences in zero-point vibrational frequencies. Both the heavy-atom isotope effect and the deuterium isotope effect are sensitive to pressure, and both are completely pressure-dependent. Moreover, the volume changes associated with both decreases are virtually identical, $\Delta V_Q = 13.1 \pm 0.7$ mL·mol^{-1} versus $\Delta V_Q = 10.4 \pm 1.5$ mL·mol^{-1}, respectively. In other words, adding an extra mass unit to either end of the scissile C−H bond, illustrated as ^{13}C−^{2}H in eq 3, has the same effect on the pressure dependence. This strongly suggests that the two measured volume changes have a common origin. In contrast, semiclassical rate theory assumes different zero-point energies as the origin for heavy-atom and deuterium isotope effects.

Hydrogen tunneling in an enzyme-catalyzed reaction was first proposed by Cha, Murray, and Klinman,[8b] who noted a breakdown in the Swain−Schaad relationship[17] in a comparison between k_D/k_T and k_H/k_T isotope effects. They argued that H had a much greater probability of tunneling than D or T, based on de Broglie wavelengths which they calculated to be 0.63, 0.45, and 0.36 Å, respectively, for particles with an energy of 20 kJ·mol^{-1}. They also calculated the de Broglie wavelength for C under the same conditions and obtained a much smaller value of 0.18 Å. The probability of carbon tunneling is obviously very much lower than that for the heavy isotopes of hydrogen; to put it another way, if a barrier for hydride transfer were thin enough for carbon to tunnel to a significant degree, then H, D, and T would all tunnel with virtually the same probability, and there would be no breakdown in the Swain−Schaad relationship. Therefore, if the commonality of volume changes reflects a common origin, and if carbon does not undergo significant tunneling, then it follows that tunneling is not the transition-state phenomenon responsible for the pressure dependence of the deuterium isotope effect.

The Born−Oppenheimer approximation holds that the energy surface for isotopic and nonisotopic reactions is virtually the same, and isotopic differences in chemical reactivity originate in vibrational differences of atoms with different masses. These vibrational differences cause the atoms to populate portions of that energy surface differently. As illustrated most simply in Figure 1A, deuterium vibrates at a lower level in an energy well than does protium in the reactant state at ambient temperature, but both occupy equivalent energy levels in the transition state, as both are in flight during a reaction yielding a primary isotope effect. Applied to the current data, wherein enzymatic isotope effects appear to originate solely within a transition state, this view requires that YADH contains a necessary and highly significant vibrational component within the transition state itself, a component not found in ordinary chemical reactions and not described by the physical organic chemistry of small molecules. Lumry and co-workers[18] have long held the view that enzymatic catalysis has a dominant mechanical component which is the primary source of rate accelerations. This mechanical component consists of protein domains that are paired "in a dyad arrangement" connected by a "hinge" and "dynamically matched to oscillate like a tuning fork so oriented as to raise by transient compression the potential energy" of the enzyme−substrate reactant state.[18d] It is a difficult concept to grasp because we lack the conceptual tools to deal with motion. For example, a crucial assumption of the quasi equilibrium hypothesis in absolute rate theory is that motion along the reaction coordinate is separated from, and independent of, all other motions, and reaction coordinate diagrams have no provision for incorporating a motion component. As pointed

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out by Lowry and Richardson:[19] "Detailed calculations for very simple reactions...indicate that the error introduced by the separability assumption into a rate constant calculated from a known potential energy surface is significant." This is supported by numerous recent examples from the application of computational chemistry to enzymatic reactions, including dehydrogenases,[20] but experimental data have not been forthcoming. Crystallographic evidence for domain movements in proteins has been known for some time,[21] with the postulated roles for domain motion in enzymatic catalysis limited to the exclusion of water from the active site, positioning of catalytic groups around the reactants, and prevention of the escape of reaction intermediates. Hard experimental evidence for a role in rate acceleration remained elusive until disulfide bridges were engineered between two domains of a nucleotide phosphatase, rendering it almost inactive in comparison to the reduced form of the engineered enzyme.[22] This recent report finally gives direct support to the hypothesis that domain motion can contribute to the rate enhancement of chemical steps of enzymatic catalysis.

Other anomalies in isotope effects on enzymatic reactions, particularly their temperature dependencies, have been attributed to hydrogen tunneling, and when data do not agree with Bell's formulation, a motion-driven vibrational component is often proposed to drive and thereby enhance hydrogen tunneling.[23] But any formulation in which mass becomes important in a transition state should by itself lead to breakdowns in Swain−Schaad relationships and to greater complexity in temperature dependencies, so the coupling of hydrogen tunneling to a vibrational component seems redundant. Moreover, adding one or more components to the semiclassical reaction rate theory will not remove the contribution of zero-point vibrational frequencies to measured isotope effect, but only lessen its importance, because the reactant-state and transition-state components are multiplicative, as in eq 1. The absence of a semiclassical reactant-state component in the pressure dependence of a heavy-atom isotope effect reported here, and in deuterium isotope effects reported elsewhere, suggests that motion in enzymatic transition states is not only the origin of the anomalies in isotope effects on enzyme-catalyzed reactions, but also the origin of the isotope effects themselves. If domain motion plays a significant role in enzymatic rate accelerations, then mass and momentum contribute to attaining the transition state. This nullifies the quasi equilibrium assumption, which in turn erases any "memory" of the reactant state.[24]

Experimental Section

Yeast alcohol dehydrogenase was purchased from Boehringer, and aldehyde dehydrogenase, NAD^+, glutathione, and benzyl alcohol were purchased from Sigma. Benzyl-α-^{13}C alcohol (99% ^{13}C) was purchased from Aldrich. Heavy-atom isotope effects were determined using equimolar mixtures of ^{12}C- and ^{13}C-labeled alcohols. The alcohol dehydrogenase-catalyzed oxidation of benzyl alcohol to benzaldehyde at pH 7.5 and 25 °C was investigated by coupling the reaction to the aldehyde dehydrogenase-catalyzed oxidation of benzaldehyde to benzoic acid to generate an ionizable product. Reaction mixtures subjected to high pressures contained 2 mM glutathione, 100 mM KCl, 5 mM NAD^+, 2 mM benzyl alcohol, 200 units of yeast alcohol dehydrogenase, and 0.5 unit aldehyde dehydrogenase in 40 mM *N*-tris(hydroxymethyl)-methyl-2-aminoethanesulfonic acid buffer, which has an ionization volume of -1.5 mL· mol^{-1} and a p*K* of 7.48.[25]

High pressures were generated with a computer-controlled, automated, screw-drive pump with feedback pressure sensor obtained from Advanced Pressure Products. Samples of reactants were mixed together to initiate reactions and placed in a 1.5 mL bottle inside an ISS HP-200 high-pressure cell. The pressure chamber was sealed and brought to pressure in less than a minute. Reactions proceeded for upward of 6 h. Reactions were quenched with HCl at approximately 20% and 100% conversions, based on the absorbance at 340 nm measured with an Olis model DW-2 dual-beam spectrophotometer using a reference at 420 nm. The aqueous samples were extracted with ethyl ether at an acidic pH, followed by a back extraction of the organic phase with 5% NH_4OH solution at an alkaline pH.

Mass spectra of the extracted samples were obtained in profile mode on an Agilent 1100 series LC/MSD SL trap mass spectrometer using a KD Scientific model 100 infusion pump operating at 5 μL/min. The nebulizer was operated with nitrogen at 15 psi, 4 L/min, and 325 °C. The instrument was operated in negative-ion mode, scanning from 50 to 300 *m*/*z* with an ion charge control target of 30 000 and a maximum accumulation time of 50 ms. The capillary voltage was 3500 V. Each spectrum in the total ion current profile was an average of 10 spectra, with two rolling averages. Spectra were collected for approximately 2 min after the total ion current profile had plateaued, and then they were averaged to produce the final mass spectrum. Integration of the peaks of the mass spectrum was performed using the Data Analysis 2.2 package of the LC MSD Trap 4.2 software.

Heavy-atom isotope effects were calculated from the ratios of peak areas for *m*/*z* 121.0 and 122.0, illustrated in the example of Figure 2, using the following form[26] of the Bigeleisen−Wolfsberg equation:[1]

$$\frac{(V/K)_{12}}{(V/K)_{13}} = \frac{\log(1-f)}{\log(1-f\,R_p/R_o)} \tag{8}$$

where f is the fractional conversion of the reaction, R_p is the $^{13}C/^{12}C$ isotope peak ratio of the product at fractional conversion f, and R_0 is the $^{13}C/^{12}C$ isotope peak ratio of the initial substrate or, as measured here, the final product at $f = 1.0$. Assays were run in triplicate.

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(24) We thank an anonymous reviewer for raising this point. A metaphor for the importance of momentum in the transition state might be throwing stones at a glass window. With soft throws the stones bounce off the window, but stones thrown hard enough to break the window do not return to the thrower. Instead, their momentum propels a continued forward motion through the broken glass. Lumry[18f] refers to this phenomenon as an "overshoot". The same reviewer suggested that, because pressure drives water into the interior of proteins, pressure may cause the dynamics in proteins to be more pronounced. For example, some of the inserted water will solvate buried ions, so some salt bridges could be weakened or broken under pressure, making the protein more flexible.
(25) Kitamura, Y.; Itoh, T. *J. Sol. Chem.* **1987**, *16*, 715−725.
(26) Equation 8 was derived for trace labeled substrates. When dealing with comparable levels of labeled and unlabeled substrates, a more complex form of eq 4 applies (see eq 55 of Cleland, W. W. In *Investigations of Rates and Mechanisms of Reactions, Vol. 6*; Bernasconi, C. F., Ed.; John Wiley & Sons: New York, 1986; pp 791−870). However, the differences are insignificant with small isotope effects of 5% or less (W. W. Cleland, personal communication).