Effects of High Pressure on Solvent Isotope Effects of Yeast Alcohol Dehydrogenase

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ABSTRACT The effect of pressure on the capture of a substrate alcohol by yeast alcohol dehydrogenase is biphasic. Solvent isotope effects accompany both phases and are expressed differently at different pressures. These differences allow the extraction of an inverse intrinsic kinetic solvent isotope effect of 1.1 (i.e., $^{D_2O}V/K = 0.9$) accompanying hydride transfer and an inverse equilibrium solvent isotope effect of 2.6 (i.e., $^{D_2O}K_s = 0.4$) accompanying the binding of nucleotide, NAD^+. The value of the kinetic effect is consistent with a reactant-state E-NAD^+-Zn-OH_2 having a fractionation factor of $\phi \approx 0.5$ for the zinc-bound water in conjunction with a transition-state proton exiting a low-barrier hydrogen bond with a fractionation factor between 0.6 and 0.9. The value of the equilibrium effect is consistent with restrictions of torsional motions of multiple hydrogens of the enzyme protein during the conformational change that accompanies the binding of NAD^+. The absence of significant commitments to catalysis accompanying the kinetic solvent isotope effect means that this portion of the proton transfer occurs in the same reactive step as hydride transfer in a concerted chemical mechanism. The success of this analysis suggests that future measurements of solvent isotope effects as a function of pressure, in the presence of moderate commitments to catalysis, may yield precise estimates of intrinsic solvent isotope effects that are not fully expressed on capture at atmospheric pressure.

INTRODUCTION

Solvent isotope effects are complicated by the fact that primary effects, secondary effects, and medium effects are multiplied together, as in the following equation (Schowen, 1978):

$$\left(\frac{k_{H_2O}}{k_{D_2O}}\right)_{total} = \left(\frac{k_H}{k_D}\right)_{pri} \times \left(\frac{k_H}{k_D}\right)_{sec} \times \left(\frac{k_{H_2O}}{k_{D_2O}}\right)_{medium} \quad (1)$$

Previous attempts to factor experimental data into components of Eq. 1 center around the proton inventory technique, in which composite solvent isotope effects are plotted against the atom fraction of deuterium in mixed isotopic waters (Venkatasubban and Schowen, 1984). A linear plot represents a single origin, whereas nonlinear plots may be generated from multiple origins. To evaluate proton inventories arising from multiple states, apparent first-order rate constants are examined by fittings to the equation of Kresge (1964):

$$k_n = k_H \frac{\prod(1 - n + n\phi^T)}{\prod(1 - n + n\phi^R)} \quad (2)$$

where k_n represents the observed rate constants in mixed isotopic waters, k_H is the rate constant in H_2O, n is the fraction of deuterium in mixed isotopic waters, and ϕ^T and ϕ^R are the deuterium fractionation factors of the transition state and reactant state, respectively. Hence interpretations are difficult, not only because of the products of multiple effects of Eq. 1, but also because of the quotients of multiple reactant- and transition-state fractionation factors of Eq. 2. Typically, multiple origins can be distinguished from one origin by using proton inventories, but rarely can three origins be distinguished from two (Quinn and Sutton, 1991).

Further difficulties arise when solvent isotope effects are applied to enzyme-catalyzed reactions because of the inherent kinetic complexity of multistep mechanisms, which can distort proton inventories (Kiick, 1991) in ways that sometimes can be resolved to extract intrinsic solvent isotope effects (Quinn and Sutton, 1991). What is needed to surmount these difficulties is a means of perturbing the separate isotope effects differently. High pressure is here proposed as such a perturbant. It is an ideal system variable in that it adds nothing to the system; rather, it simply changes distributions within preexisting equilibria. The results demonstrate that effects of pressure can separate multiple isotope effects from each other, which, in turn, may make it possible to individually quantify them and assign each to specific steps within kinetic mechanisms. Such assignments are not possible at the level of Eqs. 1 and 2. Moreover, when applied in conjunction with substrate isotope effects, a primary kinetic assignment can distinguish between concerted and stepwise chemical mechanisms of enzymatic catalysis, in a manner less ambiguous than that of multiple isotope effects measured in the absence of pressure (Rendina et al., 1984).

The oxidation of benzyl alcohol by yeast alcohol dehydrogenase (YADH) was chosen as a test system because an intrinsic tritium isotope effect of $^Tk = 7$ arising from tritiated benzyl alcohol is fully expressed in V/K (Cha et al.,

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1989). It follows that at low to moderate pressures, changes in *V/K*, or *capture* (Northrop, 1998) of benzyl alcohol, were expected and later shown to be directly proportional to an increase in the rate constant for hydride transfer due to its negative activation volume (Cho and Northrop, 1999). While these experiments were being initiated, however, it was reported that the effect of pressure on YADH was biphasic (Dallet and Legoy, 1996). The subsequent decrease in capture at high pressure was then shown to be due to a positive volume change in the conformational change of an E-NAD^+ complex (Cho and Northrop, 1999). Such a conformational change involves considerable solvent reorganization (Robinson and Sligar, 1995), which, in turn, might be subject to a solvent isotope effect, but that had not previously been so demonstrated. These two assignments of pressure effects were confirmed by pressure effects on kinetic isotope effects arising from dideutero-benzyl alcohol (Northrop and Cho, 2000). Solvent isotope effects have been demonstrated previously for YADH, as well as for the closely related horse liver enzyme (LADH), using a variety of substrates (Klinman, 1975; Welsh et al., 1980; Taylor, 1983; Sekhar and Plapp, 1990; Ramaswamy et al., 1999). The results with the different enzymes and substrates were sometimes similar and sometimes different, as were their interpretations.

THEORY

The effect of multiple steps in substrate capture plus the prevention of active enzyme from participating in capture can be described within the conventions of an isomechanism (Rebholz and Northrop, 1995):

$$G + S \rightleftharpoons E + S \underset{k_2}{\overset{k_1}{\rightleftharpoons}} ES \underset{k_4}{\overset{k_3}{\rightleftharpoons}} EX \underset{k_6}{\overset{k_5}{\rightleftharpoons}} FY \underset{k_8}{\overset{k_7}{\rightleftharpoons}} FP \xrightarrow{k_9} F + P$$

Scheme 1

where G represents an inactive isomer, or possibly isomers, of free enzyme and E an active isomer. For the purpose of the present discussion, EX $\rightleftharpoons$ FY is the hydride transfer step, which in a concerted mechanism includes the proton transfer. In a stepwise mechanism, either ES $\rightleftharpoons$ EX or FY $\rightleftharpoons$ FP may represent proton transfer. It is assumed that between ES and F (the form of enzyme following the release of a first product, P) only a single proton transfer step is isotopically sensitive. Solvent isotope effects may also arise within equilibria between G and E, and through changes in viscosity that will affect k_1 and k_2. The inactive G forms of enzyme may include slightly to completely unfolded proteins; hence this kinetic design accommodates the primary concern about applying high pressure to enzymes: that high pressure may cause denaturation as well as changes in rate constants.

A general expression for the effect of pressure on substrate capture can be written as (Cho and Northrop, 1999)

$$|V/K|_p = \left(\frac{k_1}{1 + K_{G/E}e^{-\Delta V_{G/E}p/RT}}\right) \cdot \left(\frac{R_0 e^{-\Delta V^{\ddagger}p/RT}}{1 + C_f e^{-\Delta V^{\ddagger}p/RT} + C_r e^{-(\Delta V^{\ddagger} - \Delta V_{eq})p/RT}}\right) \quad (3)$$

where R_0 is the product ratio of forward and reverse enzymatic rate constants up to and including the hydride transfer step (e.g., k_3k_5/k_2k_4 in Scheme 1), $\Delta V^{\ddagger}$ is the volume of activation between reactants E + S and the transition state $EX^{\ddagger}$ of hydride transfer (in ml/mol), p is the pressure in bars (0.98692 standard atmospheres), R is the gas constant at 82.0578 ml·bar/mol·K, T is the temperature in Kelvin (298°), $K_{G/E}$ is the equilibrium constant between free enzyme forms E and G, $\Delta V_{G/E}$ is the volume difference between the forms, and C_f and C_r are the forward and reverse commitments to catalysis, k_5/k_4 (1+ k_3/k_2), and k_6/k_7 (1 + k_8/k_9), respectively (Northrop, 1977), for hydride transfer in Scheme 1.

For a proton transfer in a concerted mechanism, the product ratio and the commitments for a solvent isotope effect would be the same as for a substrate isotope effect on hydride transfer. In a stepwise mechanism, however, the definitions change even though the form of the overall equation does not. For example, if ES $\rightleftharpoons$ EX were a proton transfer step and if it were subject to a solvent isotope effect, then R_0 would be k_3/k_2, $\Delta V^{\ddagger}$ would be the volume of activation between reactants E + S and the transition state $ES^{\ddagger}$ of k_3, and C_f and C_r would be k_3/k_2 and k_4/k_5 (1 + k_6/k_7(1 + k_8/k_9)), respectively. If k_5/k_4 were small and less than 1 in k_5/k_4 (1+ k_3/k_2) with respect to hydride transfer, then k_4/k_5 would have to be large and greater than 1 by precisely the same degree in k_4/k_5 (1 + k_6/k_7(1 + k_8/k_9)) with respect to proton transfer.

To perform global fittings of primary isotopic data, an expression for an isotopic dependence of sensitive rate constants must be included. This expression has the following form (Cleland, 1977a):

$$k_1 = \frac{k_{H_2O}}{1 + C_i[^{D_2O}k - 1]} \quad (4)$$

where C_i is the gram-atom fraction of D_2O and ^{D_2O}k is an intrinsic primary solvent isotope effect. A similar expression for the isotopic dependence of an equilibrium between free enzyme forms can be written as

$$K_i = \frac{K_{H_2O}}{1 + C_i[^{D_2O}K - 1]} \quad (5)$$

where ^{D_2O}K is a solvent isotope effect on the equilibrium constant, $K_{G/E}$. Furthermore, the viscosity of D_2O is higher than that of H_2O by a factor of 1.24 at 25°C (Arnett and McKelvey, 1969), which will reduce the diffusion-con-

trolled rate constant, k_1, when commitments are large. Effects of viscosity on k_1 will be canceled by effects on k_2 of R_0 when commitments are small and V/K is far from diffusion controlled (Brouwer and Kirsch, 1982). Viscosity has only recently been addressed with regard to isotope effects (Kurz et al., 1992; Karsten et al., 1995). Substituting Eqs. 4 and 5 plus the viscosity factor into Eq. 3 yields the global rate equation for the expression of solvent isotope effects on the kinetics of capture as a function of pressure:

$$|V/K|_p = \left(\frac{k_1}{1 + \dfrac{K_{G/E}e^{-\Delta V_{G/E}p/RT}}{1 + C_i[^{D_2O}K - 1]}}\right) \cdot \left(\frac{R_0 e^{-\Delta V^{\ddagger}p/RT}}{1 + C_i[^{D_2O}k - 1] + [1 + 0.24C_i] \cdot (C_f e^{-\Delta V^{\ddagger}p/RT} + C_r e^{-(\Delta V^{\ddagger} - \Delta V_{eq})p/RT}}\right) \quad (6)$$

When ΔV_{eq}, the volume difference between E + S and F + P, is relatively small, commitments may be combined as in the following expression:

$$|V/K|_p = \left(\frac{k_1}{1 + \dfrac{K_{G/E}e^{-\Delta V_{G/E}p/RT}}{1 + C_i[^{D_2O}K - 1]}}\right) \cdot \left(\frac{R_0 e^{-\Delta V^{\ddagger}p/RT}}{1 + C_i[^{D_2O}k - 1] + [1 + 0.24C_i](C_f + C_r)e^{-(\Delta V^{\ddagger}p/RT)}}\right) \quad (7)$$

In the absence of significant commitments, Eq. 6 reduces to

$$|V/K|_p = \left(\frac{k_1}{1 + \dfrac{K_{G/E}e^{-\Delta V_{G/E}p/RT}}{1 + C_i[^{D_2O}K - 1]}}\right)\left(\frac{R_0 e^{-\Delta V^{\ddagger}p/RT}}{1 + C_i[^{D_2O}k - 1]}\right) \quad (8)$$

Equations 6–8 resemble Eqs. 1 and 2 in that they contain solvent isotope effects multiplied together but differ significantly in that changes in pressure will modulate the expression of those effects differently, making it possible to separate, quantify, and assign individual effects.

MATERIALS AND METHODS

YADH and NAD^+ were purchased from Boehringer, benzyl alcohol was purchased from Sigma, and D_2O was purchased from Cambridge Isotope Lab. Tris-HCl buffer at 80 mM and pH 8.5 was used to minimize pressure-dependent changes in pH; for pD 8.5, a value of 0.4 was added to pH meter readings (Lumry et al., 1951). Enzymatic assays under pressure were performed at 25°C to minimize pressure-dependent changes in viscosity (Bett and Cappi, 1965), using 3.19 μM alcohol dehydrogenase and 10.5 mM NAD^+ (K_m = 1 mM) and 0.83–12.4 mM benzyl alcohol (K_m = 3.1 mM). The high-pressure-generating system with a servo feedback to a computer controller was purchased from Advanced Pressure Products, and the high-pressure cell was from SLM-Aminco. A Gilford Model 240 was the light source and was placed in front of a sapphire window of the pressure cell. An end-on photomultiplier was placed in line in front of the opposite window. Photomultiplier voltages were collected and manipulated with an OLIS Spectroscopy Operating System from which absorbencies were calculated and stored on computer.

Enzymatic activity was determined by the absorbency change at 340 nm associated with the formation of NADH during oxidation of benzyl alcohol to benzaldehyde. Progress curves were collected and fitted to an integrated form of the Michaelis-Menten equation (Duggleby, 1985) to compute the initial velocities extrapolated to zero time at a given pressure. Sets of initial velocities in which the alcohol substrate was varied in the presence of excess nucleotide coenzyme were then fit to the Michaelis-Menten equation, and rate constants for capture were calculated; therefore, the "isomers of free enzyme" G and E of Scheme 1 are forms of E-NAD^+ in this particular experimental design. The percentage of D_2O was 99.4%, based upon volumes of mixing; therefore, C_i of Eqs. 6–8 was 0.994. Nonlinear regressions employed the BASIC computer program of Duggleby (1984).

RESULTS

Fig. 1 *A* shows the biphasic pressure dependence of the capture of benzyl alcohol by YADH as determined in H_2O and D_2O. The latter has a greater amplitude, indicating an inverse solvent isotope effect, and is significantly shifted to the right, suggesting different solvent isotope effects on the two phases. A fit of the data in Fig. 1 *A* to the truncated Eq. 7 generated the parameters listed in Table 1, and fits to either Eq. 7 or Eq. 8 generated the solid lines in Fig. 1 *A*. The ratio of the fitted lines is plotted in Fig. 1 *B*. The resulting sigmoidal curve shows an inverse solvent isotope

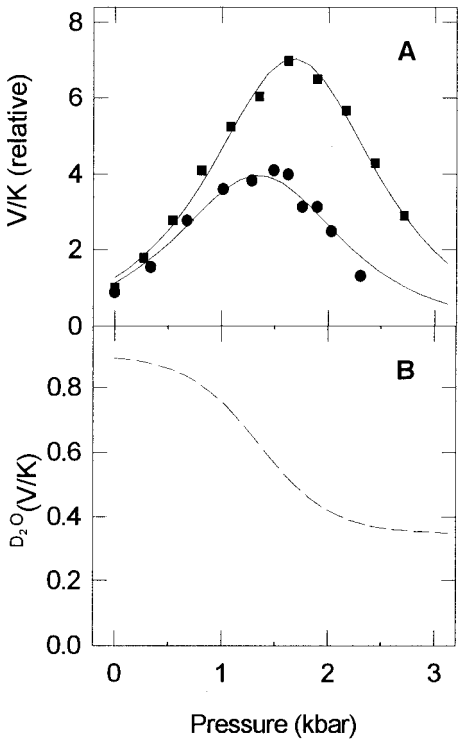

FIGURE 1 Effect of pressure on relative rate constants for the capture of benzyl alcohol by yeast alcohol dehydrogenase (*A*) in H_2O (●) and D_2O (■) and on solvent isotope effects (*B*). The solid lines are a fit to Eqs. 7 or 8. The dashed line consists of ratios of the computed points used to construct the solid lines.

effect undergoing a smooth transition from one value at low pressure to a more extreme value at high pressure. The fitted parameters in Table 1 identify the former as a kinetic solvent isotope effect on alcohol oxidation and the latter as an equilibrium solvent isotope effect between a form of enzyme that can participate in the capture of benzyl alcohol and another form, or forms, that cannot.

It should be emphasized that a volume of activation is a property of state, like free energy or entropy, and thus is independent of the pathway of the change and of specific volume changes that may occur along a particular pathway. In the present case, a volume change may or may not accompany the binding of the substrate, benzyl alcohol, because such a volume change is tacitly incorporated into $\Delta V^{\ddagger}$ as formulated in Eq. 3, i.e., $\Delta V^{\ddagger} = \Delta V_1 - \Delta V_2 + \Delta V_3 - \Delta V_4 + \Delta V_5$, and $\Delta V^{\ddagger}$ can attain a particular value whether ΔV_2 is the same as ΔV_1 or not, by compensating for volume changes associated with the other rate constants. It should also be noted that the physical origin of the volume changes is immaterial. For example, ΔV_3 might reflect a change in a pK_a such as that of the zinc-bound water (see below).

DISCUSSION

The kinetic argument for a concerted mechanism

Kinetic analyses traditionally proceed with a fitting of data to alternative rate equations and the elimination of all but one of them. Equation 6, however, is general and applies to all forms of capture. Thus alternative mechanisms differ in degree, not in kind. The extremely small sum of commitments to catalysis in Table 1 reduces Eq. 7 to Eq. 8 and unambiguously assigns the proton transfer responsible for the kinetic solvent isotope effect to the same step containing the hydride transfer, the intrinsic isotope effect of which is known to be fully expressed because of small commitments (Cha et al., 1989). If the proton transfer were associated with a step other than hydride transfer, then one of the commitments would have to be very large; they could not both have commitments less than 1 or greater than 1. Only when proton transfer and hydride transfer are both partially rate limiting, because of commitments of similar magnitude in the vicinity of 1, does any mechanistic ambiguity arise in this kinetic design.

TABLE 1 Pressure parameters of solvent isotope effects

^{D_2O}k	0.901 ± 0.075
^{D_2O}K	2.61 ± 0.34
$V^{\ddagger}$	−35.3 ± 3.2 ml/mol
$V_{G/E}$	−72.2 ± 2.5 ml/mol
$K_{G/E}$	0.0192 ± 0.0068
$C_f + C_r$	10^{-17} ± 0.0014
k_1R_o*	1.00 ± 0.09

Data points shown in Fig. 1 *A* were fitted to Eq. 7, where, assuming $C_f + C_r = 0.0014$, the sum of the commitments did not exceed 0.05 at 2.5 kbar. A fit to Eq. 8 with no commitments converged to identical values and standard errors for the remaining parameters.

*Values of data points were normalized to yield a parameter value of 1.00.

To illustrate this point, given the values in Table 1, k_5/k_4 must be less than the sum of the commitments, or $k_5/k_4 < 0.0014 < C_f + C_r$. It follows that if hydride transfer preceded proton transfer in a stepwise mechanism, then the reciprocal ratio $k_4/k_5 > 1000$, in the reverse commitment of a substrate isotope effect. Such a large commitment would suppress the expression of the isotope effect to an undetectable level, which differs in the extreme from what was reported by Cha et al. (1989). Consistent with the same step assignment is the close agreement between the activation volumes of the first phase when isotopic and nonisotopic data were fit separately to Eq. 3 (not shown).

The role of a low-barrier hydrogen bond

Gould (1988) found twofold inverse solvent isotope effects for the capture of 1-butanol by YADH, for which a normal fourfold substrate deuterium kinetic isotope effect was observed upon hydride transfer. These results were interpreted within a mechanism in which the reactant-state enzyme-NAD^+ complex has a fractionation factor of ~0.5 for a zinc-bound water that is displaced by the alcohol, and the transition state has a fractionation factor of 1.0. Subsequently, Sekhar and Plapp (1990) determined inverse solvent isotope effects of LADH by the proton inventory technique in the transient state and calculated a fractionation factor of 0.37 for the reactant state and 0.73 for the transition state. Therefore, like a proton transfer from a thiol, the proton in flight is moving from a state with a low fractionation factor to one with a higher value, which generates an inverse kinetic isotope effect (Quinn and Sutton, 1991). Based on these data and the structures of LADH complexed with NAD^+ and substituted benzyl alcohols (Ramaswamy et al., 1994), Ramaswamy et al. (1999) propose a mechanism in which a low-barrier hydrogen bond (LBHB) is formed between a serine hydroxyl and the alkoxide ion of the substrate alcohol. A notable characteristic of LBHBs is that they have low fractionation factors (Cleland et al., 1998). Movement of the proton from this bond to fully join the serine, in concert with hydride transfer, is consistent with the transient inverse effects.

Previous steady-state studies using multiple heavy atom and deuterium substrate isotope effects together with effects of pH concluded that alcohol dehydrogenase proceeds by a stepwise catalytic mechanism in which a proton dissociates from the zinc-bound alcohol and then a hydride ion is transferred from the resulting alkoxide to NAD^+ (Shore et al., 1974; Kvassman and Pettersson, 1978, 1980; Morris et al., 1980; Cook and Cleland, 1981a–c; Eklund et al., 1982; Scharschmidt et al., 1984; Pettersson, 1987). This proton is ultimately transferred to a base, His^{51}, facilitated by a hydrogen bond system that includes Ser^{48} (Thr^{48} in the yeast

enzyme) and the 2′-hydroxyl of the ribose attached to the nicotinamide ring of NAD (Eklund et al., 1982). The transfer by itself would generate a fully ionized alkoxide ion; however, an LBHB places both the substrate alcohol and the serine hydroxyl in partial or half-alkoxides. Therefore, the previous steady-state studies address the proton release from free alcohol to the LBHB coupled to protonation of the histidine, whereas the transient-state studies reflect the proton exiting the bound alcohol LBHB coupled to the hydride transfer, as illustrated in Fig. 2.

For the yeast enzyme, the inverse kinetic solvent isotope effect appears to be on the same step as hydride transfer but is more complicated than the original concept of a concerted mechanism described by Rendina et al. (1984) for substrate isotope effects. Steady-state capture with small commitments reports on the change in fractionation factors between reactant states of free E + S and the final transition state of R_O, i.e., k_5 in Scheme 1 and Fig. 2. The fractionation factor for the zinc-bound water is not known, but cobalt-bound water of carbonic anhydrase has been measured as ϕ = 0.72–0.77 (Kassebaum and Silverman, 1989). Therefore, the reactant state of "free enzyme" for YADH is E-NAD$^+$-Zn-OH$_2$ with $\phi^{Re} \approx (.75)^2 = 0.56$ for the water that is displaced by alcohol to join bulk water, where $\phi^{H_2O} = 1.0$. The reactant state of the "free substrate" is an alcohol with $\phi^{Rs} = 1.0$ (Cleland, 1980). The transition state of the proton exiting an LBHB may be assumed to be similar to LADH, with $\phi^T = 0.73$ (Sekhar and Plapp, 1990). Together, these generate a composite solvent isotope effect of $^{D_2O}k = \phi^{Re}\phi^{Rs}/\phi^{H_2O}\phi^T = (0.56)(1.0)/(1.0)(0.73) = 0.77$, according to eq 2. This value approximates ^{D_2O}k of Table 1.

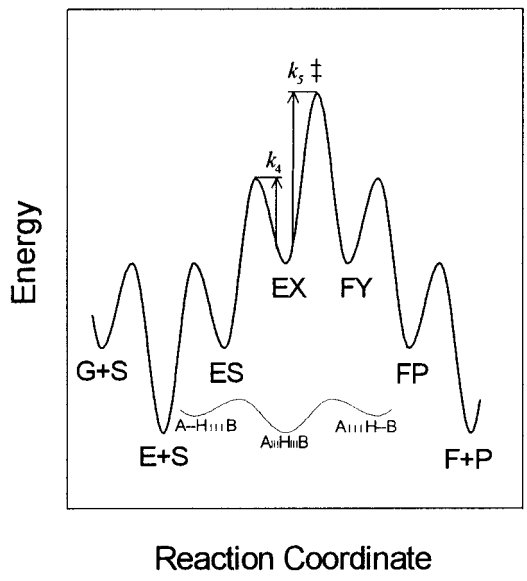

FIGURE 2 Imaginary reaction coordinate diagrams at low pressure for substrate capture described by Scheme 1 (*upper curve*): the equilibrium between G and E forms of enzyme strongly favors E; both forward and reverse commitments to catalysis with respect to hydride transfer are small, which defines the transition state for hydride transfer (‡) as the one with the highest energy. The lower curve represents the parallel steps of stepwise and concerted proton transfers: a LBHB (represented by $A_{|||}H_{|||}B$) forms before hydride transfer can occur (i.e., the stepwise mechanism) and breaks down to form a normal hydrogen bond during the hydride transfer governed by k_5 in the upper curve (i.e., the concerted mechanism).

Pressure-induced inhibition of a conformational change

The magnitude of the volume change associated with the binding of NAD$^+$, represented by the difference in volume between E and G of Scheme 1 and between E-NAD$^+$ and E*-NAD$^+$ in Cho and Northrop (1999), has a sign opposite that of the value in Table 1, or $\Delta V^* = 72$ ml/mol in the direction of catalysis. The positive volume change is consistent with a variety of ligands binding to proteins, the origins of which have been of interest for many years (Weber et al., 1974; Heremans, 1982; Weber and Drickamer, 1983; Rand et al., 1993). The primary candidates are solvent reorganizations in the form of electrostriction of water molecules surrounding ions and of icelike cages of water molecules surrounding hydrophobic groups. Both occupy a smaller volume than bulk water and thereby contribute positive volume changes to ligand bindings that employ salt bridges and hydrophobic bonds, respectively.

The thermodynamic solvent isotope effect on the binding of NAD$^+$ is the reciprocal of the value shown in Table 1, or $^{D_2O}K^* = 0.38 \pm 0.04$, a new parameter with an unusually large magnitude. Isotope effects on binding are usually quite small (LaReau et al., 1989), with a few exceptions involving covalent interactions, such as formation of a hemiketal (Stein and Trainor, 1986) or Schiff base (Bruice and Santi, 1982). However, this thermodynamic effect would itself appear small in a direct binding experiment conducted at atmospheric pressure. Because the equilibrium lies so far to the right, i.e., $K^* = 1/0.0192 = 52$, a further shift to the right by an inverse solvent isotope effect is without a noticeable effect. According to the data in Table 1, 98.1% of free enzyme is in the E form in H_2O. This increases to just 99.3% in D_2O, giving an apparent solvent isotope effect of only 0.988. Hence the effect of pressure is absolutely essential to draw out this otherwise hidden phenomenon to where it can be detected at all, let alone measured with precision.

The origin of the solvent isotope effect on ligand binding has significant implications for protein-ligand interactions. It could be associated directly with the volume change arising from electrostriction and icelike cages of water. However, given a fractionation factor of $\phi = 1.02$ between ice and water (Arnason, 1969) and assuming the same change in bond order for ice, electrostriction, and icelike cages, the resulting isotope effect should be normal, not inverse as observed. The inverse effect must therefore arise from some component other than desolvation associated with tighter binding of NAD$^+$. The magnitude of the in-

verse effect seems extreme for a secondary isotope effect; however, it is not unlike the values reported by Gould (1988) of $^{D_2O}V/K = 0.43–0.47$ for the oxidation of *n*-butanol by YADH. These extreme values could result from a large number of exchangeable hydrogens in the enzyme that become more stiffly bonded when the enzyme clamps down on the nucleotide and both undergo some desolvation. Returning to Scheme I for a model and assuming that ES $\rightleftharpoons$ EX is the clamping step and EX $\rightleftharpoons$ EY is an isotopically sensitive proton transfer fully expressed on capture not of nucleotide but of alcohol and ignoring the isomechanism, then $V/K = k_1k_3k_5/k_2k_4$ and isotope effects on any or all rate constants k_1 to k_5 will be expressed, i.e., they are all equally "rate-limiting." If only clamping and proton transfer are isotopically sensitive, then

$$^{D_2O}(V/K) = (k_1/k_2) \cdot {}^{D_2O}(k_3/k_4) \cdot {}^{D_2O}k_5 \qquad (9)$$

Stiffer bonding makes the $^{D_2O}(k_3/k_4)$ equilibrium isotope effect on clamping inverse, which is offset somewhat by a normal $^{D_2O}k_5$ kinetic isotope effect in the overall solvent isotope effect on capture. An inverse solvent isotope effect of $^{D_2O}K_i = 0.69$ was also observed in the slow onset of inhibition of pepsin by pepstatin (Cho et al., 1994). In this experimental design, there is no catalytic effect, but only the equilibrium isotope effect of clamping analogous to Eq. 9.

Similar and otherwise inexplicable inverse effects on capture have been observed for several other enzymes, notably malic enzyme with $^{D_2O}V/K = 0.50$ (Cleland, 1977b); glucokinase with 0.29 (Pollard-Knight and Cornish-Bowden, 1984); adenosine deaminase with 0.45 (Weiss et al., 1987); adenosine 5′-monophosphate deaminase with 0.33, accompanied by a curved proton inventory where $n \geq 2$ (Merkler and Schramm, 1993); β-lactamase with 0.88 (Adediran et al., 1996); and desuccinylase with 0.62 (Born et al., 1998). Cleland makes a strong case for restrictions on torsional motions—but not bending or stretching motions—as the origin of such effects, based on a series of calculated fractionation factors in which the torsional force constant of methanethiol was varied (Cleland, 1987), and on multiple experimental isotope effects (Waldrop et al., 1992; Rishavy and Cleland, 1999). The results in Table 1 confirm Cleland's hypothesis that the likely origin of large inverse solvent isotope effects is a binding phenomenon and not a catalytic one, because $^{D_2O}K_{E/G}$ is solely thermodynamic, as opposed to Eq. 9, which has the ambiguity of both thermodynamic and kinetic components. Most importantly, this hypothesis of restricted torsional motions on the way to transition states of enzymatic reactions complements the mechanical models of catalysis proposed on the basis of the effects of pressure on a substrate isotope effect (Northrop and Cho, 2000).

An inverse effect on capture can also be found in data for carboxypeptidase with $^{D_2O}V/K = 0.45$ (Lumry et al., 1951). This pioneering reference has been unfairly overlooked for two reasons. First, calculating pD from pH meter readings (see Methods and Materials) was independently rediscovered by P. K. Glasoe and F. A. Long in 1960 and published in a paper which did not cite the Lumry, Smith, and Glantz paper. Subsequent reviews cited Glasoe and Long (e.g., Schowen, 1978). The 1960s was the decade that popularized isotope effects in enzymology (Katz and Crespi, 1970), so the 1951 paper was ahead of its time. Second, Lumry et al. varied the concentration of substrate carbobenzoxyglycyl-L-tryptophan and measured the effects of deuterium on Michaelian kinetic parameters—the first of its kind in enzyme kinetics—and found that the solvent isotope effects were greater for K than for V, i.e., $\leq$3.1 versus $\leq$1.3, respectively. Subsequent reviews of kinetic isotope effects in enzyme-catalyzed reactions did not cite or discuss these data (e.g., Thompson, 1963, which nevertheless does cite the paper for its pD measurement), perhaps because an effect on the "binding" of substrate was not considered a real kinetic effect. Now we know that the Michaelis constant is more than binding (and the authors interpreted ^{D}K to mean precisely that, again far ahead of its time; see Simon and Palm, 1966). We also know that the authors' data really express an inverse solvent isotope effect on V/K or "capture," a kinetic phenomenon that has nothing to do with catalytic turnover (Northrop, 1998). Moreover, by confirming Cleland's hypothesis that restrictions on torsional motions is the origin of anomalous inverse solvent effects on V/K, the present paper and Eq. 9 provide the necessary foundation needed to reinterpret the isotopic data of Lumry et al. Thus we have come full circle. Combining the measurements of pD and inverse $^{D_2O}(V/K)$ for carboxypeptidase in a historical retrospective reveals that this overlooked but very important paper was the first definitive report of a solvent isotope effect on an enzyme-catalyzed reaction (and only the second enzymatic isotope effect to be determined). Other reports had appeared in which a reaction rate of an enzyme-catalyzed reaction was altered in D_2O, but in the absence of the proper pH correction, those alterations might have been effects of pH instead of deuterium. This possibility was also delineated by Lumry et al., who varied pH as well, showing that the two effects are intertwined—yet another first in enzymology, a demonstration of a pH-dependent isotope effect.

CONCLUDING REMARKS

Differential changes caused by high pressure allow the deconvolution of a composite solvent isotope effect into two component parts, one kinetic and one thermodynamic in origin. The nature and magnitude of the individual isotope effects allow for reasonable interpretations of their origins, namely participation of a LBHB in catalysis and restrictions on torsional motions during ligand binding, respectively. In addition, new information is obtained in the form of estimates of the commitments to catalysis. In the present anal-

ysis, commitments were very small, and only the upper limit was significant. But in future experiments using substrates such as propanol or butanol, the isotope effects of which are less fully expressed, it may be possible to extract finite values for other, more moderate commitments. One reason for optimism is the constancy of the equilibrium constant and volume change associated with the conformational change accompanying the binding of NAD^+; these are independent of the choice of alcohol substrate, and the values from Table 1 can be inserted into future nonlinear regressions as constants instead of parameters, thus ensuring a greater likelihood of convergence to the remaining parameters. With commitments in hand, precise estimates for intrinsic isotope effects will also soon be in hand.

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