Effects of Pressure on the Kinetics of Capture by Yeast Alcohol Dehydrogenase[†]

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ABSTRACT: High pressure causes biphasic effects on the oxidation of benzyl alcohol by yeast alcohol dehydrogenase as expressed in the kinetic parameter V/K which measures substrate capture. Moderate pressure increases the rate of capture of benzyl alcohol by activating the hydride transfer step. This means that the transition state for hydride transfer has a smaller volume than the free alcohol plus the capturing form of enzyme, with a $\Delta V^{\ddagger}$ of -39 ± 1 mL/mol, a value that is relatively large. This is the first physical property of an enzymatic transition state thus characterized, and it offers new possibilities for structure−activity analyses. Pressures of >1.5 kbar decrease the rate of capture of benzyl alcohol by favoring a conformation of the enzyme which binds nicotinamide adenine dinucleotide (NAD^+) less tightly. This means that the ground state for tight binding, E^*-NAD^+, has a larger volume than the collision complex, $E-NAD^+$, with a ΔV^* of 73 ± 2 mL/mol. The equilibrium constant of the conformational change K_{eq}^* is 75 ± 13 at 1 atm. The effects of pressure on the capture of NAD^+ have no activation phase because the conformational change is now being expressed kinetically instead of thermodynamically, together with but in opposition to hydride transfer, causing the effects to cancel. For yeast alcohol dehydrogenase, this conformational change had not been detected previously, but similar conformational changes have been found by spectroscopic means in other dehydrogenases, and some of them are also sensitive to pressure. The opposite signs for the volume change of tighter binding and hydride transfer run contrary to Pauling's hypothesis that substrates are bound more tightly in the transition state than in the Michaelian reactant state.

The effects of high hydrostatic pressure on the kinetics of enzymatic reactions have a rather frustrating history that can be traced to the overly simplistic paradigm that dominated enzyme kinetics for most of the century (*1*). This paradigm invokes the rapid-equilibrium hypotheses that equates Michaelis−Menten constants, K_m, to dissociation constants, K_d. Kinetic interpretations were frequently split between effects on binding, i.e., on K_m and K_d, or simply K, and effects on catalytic turnover, i.e., represented variously as k_{cat}, V_{max}, or V. We now know that the two fundamental kinetic parameters are V and V/K (*2*), that specific kinetic phenomena such as isotope effects cannot be interpreted in terms of K (*3*), or even V sometimes (*4*), and that V/K is never a measure of the rate of any product formation but rather is a measure of the rate of *capture* of substrate into complexes that are destined to yield product at some later time (*5*). Moreover, rates of capture are frequently modulated by the availability of active enzyme in the proper isomeric form (*6*), a subject not previously addressed within the confines of pressure studies. In addition, concerns have been raised about high pressure causing enzyme proteins to unfold or its constituent oligomers to dissociate. Such nonkinetic events remove active enzyme from the system and thereby complicate interpretations of high-pressure kinetics.

Morild (*7*) performed a very thorough and rigorous kinetic analysis of the effects of pressure on both liver and yeast alcohol dehydrogenase (YADH).[1] His analysis of the latter was hindered not only by the simplistic paradigm described above but also by using a highly pressure-sensitive buffer (phosphate) or a temperature-sensitive buffer (tris) in the absence of a thermostated pressure cell, and he obtained different results with the different buffers. At the time of his experiments, the probable presence of a pressure-sensitive conformational change similar to the one found in liver alcohol dehydrogenase (*8*) was not known. An attempt to deconstruct Morild's kinetics proved to be overly difficult. Rather, high-pressure kinetic measurements were repeated with (a) a nearly pressure-insensitive buffer in (b) a precisely thermostated pressure cell using (c) a substrate with kinetics of capture simpler than those of ethanol within (d) an experimental design that focused specifically on the kinetics of capture, thereby offering a reasonable chance of precise data acquisition and clear interpretation. Benzyl alcohol was chosen as the substrate because Cha et al. (*9*) had demonstrated that an intrinsic deuterium isotope effect arising from dideuterobenzyl alcohol was fully expressed on capture. Therefore, at low to moderate pressures, changes in the rate of capture of benzyl alcohol must be solely and linearly dependent upon the changes in the rate of hydride transfer.

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[1] Abbreviations: YADH, yeast alcohol dehydrogenase (EC 1.1.1.1); NAD^+, nicotinamide adenine dinucleotide.

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Dallet and Degoy (*10*) extended the studies of Morild to *Thermanaerobium brockii* alcohol dehydrogenase, a thermostable tetrameric isozyme, using YADH as a control. They subjected both enzymes to chromatography at low and high pressures, in the presence and absence of urea, and concluded that neither oligomeric enzyme dissociated at pressures of up to 2 kbar. However, unlike Morild, they did not obtain linear logarithmic plots with ethanol and had difficulty coping with biphasic functions, which were even more pronounced when pressure studies were extended to alternate substrates 2-propanol, 2-pentanol, and cyclopentanol.

THEORY

Hydrostatic pressure affects chemical systems by shifting pre-existing equilibria as a function of pressure as shown in eq 1:

$$K_p = K_o e^{-\Delta V p/RT} \tag{1}$$

where K_o is an equilibrium constant at either atmospheric or zero pressure (the difference is insignificant), ΔV is the change in partial molar volumes of products and reagents in milliliters per mole (i.e., $V_{\text{prod}} - V_{\text{reac}}$, which is a state property, like free energy and entropy, and thus independent of the pathway of the change), p is the pressure in bars (0.98692 standard atmosphere), R is the gas constant (82.0578 mL·bar/mol·K), and T is the temperature in kelvin. In absolute rate theory (*11*), this same treatment is applied to the quasi-equilibrium between a reactant state and a transition state, resulting in a pressure-dependent rate constant:

$$k_p = k_o e^{-\Delta V^{\ddagger} p/RT} \tag{2}$$

where k_o is the rate constant and $\Delta V^{\ddagger}$ represents the difference between the volume of the reactants and their volume in the transition state of the reaction and is referred to as the activation volume.

Elements of Substrate Capture. Following Klinman and Matthews (*12*), a general expression for V/K can be written as (*5*)

$$V/K = k_1\left(\frac{\prod_{j=3}^{j=i} k_j/k_{j-1}}{1 + C_f + C_r}\right) \tag{3}$$

where i is the subscript for an isotopically sensitive step in the forward direction, using the notation of Cleland (*13*), and C_f and C_r are the forward and reverse *commitments to catalysis*, respectively (*14*). Applying the pressure function of eq 2 to collection of rate constants of eq 3 yields

$$|V/K|_p = k_1\left(\frac{R_0 e^{-\Delta V_0 p/RT}}{1 + C_f e^{-\Delta V_{Cf} p/RT} + C_r e^{-\Delta V_{Cr} p/RT}}\right) \tag{4}$$

Equation 4 assumes no pressure effect on k_1 because pressure effects on diffusion-controlled processes are mediated by changes in viscosity, and Bett and Cappi (*15*) have shown that the viscosity of water is pressure-independent near 30 °C up to approximately 2 kbar. This means that there is no volume change between the reactants and the first transition state, or between the last transition state and the products.[2] It then follows that the volume change associated with the numerator is equal to the volume difference between the isotopically sensitive transition state and the free reactants, or $\Delta V_0 = \Delta V^{\ddagger}$. The ratio of rate constants in the numerator, R_0, is equal to the most complex component of the forward commitment to catalysis (see below), suggesting that the volume change of the forward commitment to catalysis approximates $\Delta V^{\ddagger}$ as well. For a single-substrate, single-product reaction, the difference between volume changes of the commitments is equal to the volume change of the equilibrium constant, ΔV_{eq}, which can be determined independently. This equality would appear to be a reasonable approximation for multisubstrate reactions, also, except perhaps for ping-pong mechanisms. Therefore, a minimal general equation for substrate capture becomes

$$|V/K|_p = k_1\left[\frac{R_0 e^{-\Delta V^{\ddagger} p/RT}}{1 + C_f e^{-\Delta V^{\ddagger} p/RT} + C_r e^{-(\Delta V^{\ddagger} - \Delta V_{\text{eq}})p/RT}}\right] \tag{5}$$

Equation 5 is biphasic with respect to pressure and offers a first attempt to address data such as that obtained by Dallet and Degoy (*10*).

When the reverse commitment to catalysis is very small, eq 5 reduces to

$$|V/K|_p = k_1\left(\frac{R_0 e^{-\Delta V^{\ddagger} p/RT}}{1 + C_f e^{-\Delta V^{\ddagger} p/RT}}\right) \tag{6}$$

which is monophasic with respect to pressure, but would still be nonlinear in a logarithmic plot if the forward commitment to catalysis had a value of ~1. When both forward and reverse commitments to catalysis are small and the intrinsic isotope effect is fully expressed on V/K, eq 7 becomes

$$|V/K|_p = k_1 R_0 e^{-\Delta V^{\ddagger} p/RT} \tag{7}$$

which is monophasic and linear.

Isomerizations of Enzyme. Overlooked in previous examinations of the effects of pressure on enzyme kinetics is the possibility of pressure causing small conformational changes or changes in states of protonation, far short of protein unfolding or oligomers dissociating, that nevertheless alter the amount of enzyme that is available for binding the substrate. Equations for addressing such changes have recently been explored within the confines of iso-mechanisms

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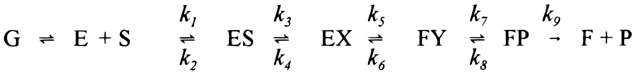
[2] An astute reviewer challenged this point. Our reading of the literature found no preceding discussion or measurement regarding an effect of pressure on a diffusion-controlled rate constant, so we believe Occam's razor obtains. The assumption depends on how one defines k_1, and eq 4 presumes a microscopic k_1 dependent upon the solvent medium only; other formulations employ a macroscopic k_1 which may include conformational changes (or the reviewer's ionization step) which, of course, may be pressure-dependent, but these may just as well be defined as part of k_2 and k_3 and formally excluded from k_1. This simplifying approximation is applied similarly throughout the literature of kinetic isotope effects, but an isotopic change in mass *must* change rates of diffusion, especially in very small molecules such as $^{15}NH_3$ (see footnote 3 of ref *32*). If the approximation is allowed in the literature of isotope effects where we know its never absolutely true, it ought to be allowed here until there is evidence to the contrary.

Scheme 1



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

(*6*). Scheme 1 addresses this phenomenon in which $G \rightleftharpoons E$, governed by the equilibrium constant $K_{G/E} = [G]/[E]$, represents such an isomerization.

Converting F back to G to complete a turnover might involve additional steps and reactants which may also be sensitive to pressure, but these are of no concern to V/K as only those steps up to and including the first irreversible step contribute to substrate capture. The general form for V/K can be expressed in terms of the net rate constant k_1' for collisions of substrate and enzyme which generate productive turnovers plus the equilibrium distribution of the isomeric form of enzyme to which the substrate binds:

$$V/K = \frac{k_1'}{1 + K_{G/E}} \tag{8}$$

Combining eqs 1, 7, and 8 gives the biphasic pressure function:

$$|V/K|_p = \frac{k_1 R_0 e^{-\Delta V^{\ddagger} p/RT}}{1 + K_{G/E} e^{-\Delta V_{G/E} p/RT}} \tag{9}$$

where R_0 is k_3k_5/k_2k_4 in Scheme 1. Equation 8 can also be combined with eq 5 for a more general pressure-dependent function:

$$|V/K|_p = \left(\frac{k_1}{1 + K_{G/E} e^{-\Delta V_{G/E} p/RT}}\right) \times \left[\frac{R_0 e^{-\Delta V^{\ddagger} p/RT}}{1 + C_f e^{-\Delta V^{\ddagger} p/RT} + C_r e^{-(\Delta V^{\ddagger} - \Delta V_{Keq}) p/RT}}\right] \tag{10}$$

Equation 10 is triphasic with respect to pressure, which may prove to be difficult to resolve with experimental data, depending upon the level of precision and the volume change of the equilibrium constant. However, the added complexity of free enzyme isomerization, when present and significant, might be resolved in a separate experiment using a slower, or nonsticky, substrate which has very low commitments to catalysis, to employ eq 9. Because free enzyme is independent of the identity of the substrate, values for $K_{G/E}$ and $\Delta V_{G/E}$ determined with the less sticky substrate could then be inserted as fixed constants in eq 10 during analyses on the original substrate of interest.

Kinetic Complexity. Commitments to catalysis become complex when there are multiple steps in a catalytic sequence. They consist of a series of nested ratios of rate constants (*14*):

$$C_f = 1 + k_i/k_{i-1}(1 + k_{i-2}/k_{i-3}(1 + k_{i-4}/k_{i-5}... \tag{11}$$

For Scheme 1, the forward commitment is

$$C_f = 1 + k_5/k_4 + k_3k_5/k_2k_4 = 1 + R_1 + R_2 \tag{12}$$

The most complex term of the forward commitment, R_2, is identical to the numerator ratio R_0. Therefore, an expanded form of eq 4 consistent with the reaction sequence in Scheme 1 can be written as

$$|V/K|_p = \frac{k_1 R_2 e^{-\Delta V_2^{\ddagger} p/RT}}{1 + R_1 e^{-\Delta V_1^{\ddagger} p/RT} + R_2 e^{-\Delta V_2^{\ddagger} p/RT} + R_3 e^{-\Delta V_3^{\ddagger} p/RT} + R_4 e^{-\Delta V_4^{\ddagger} p/RT}} \tag{13}$$

With four exponentials, eq 13 is not likely to be resolved with experimental data. Nevertheless, despite such kinetic complexity, it may be possible to extract some useful parameters. The volume change associated with $\Delta V_1^{\ddagger}$ is the volume difference between the second and third transition states; $\Delta V_2^{\ddagger}$ is the difference between the first and third, $\Delta V_3^{\ddagger}$ between the third and fourth, and $\Delta V_4^{\ddagger}$ between the third and last. (More reactive steps would add more terms to the denominator as indicated by this series.) Notably, the volumes of intermediate ground states such as ES or EY are not expressed in V/K as they are in V_{max}, which greatly simplifies the kinetics of capture. It is important to note that all volume changes are system changes, and most of the volume changes occur in solvent reorganizations such as electrostriction (*16*). It is therefore likely that the internal volume changes, $\Delta V_1^{\ddagger}$ and $\Delta V_3^{\ddagger}$, will be smaller than the most external ones, $\Delta V_2^{\ddagger}$ and $\Delta V_4^{\ddagger}$, respectively, so the values of $R_1 e^{-\Delta Vp/RT}$ and $R_3 e^{-\Delta Vp/RT}$ will change less than, and likely be masked by, the larger apparent changes in $R_2 e^{-\Delta Vp/RT}$ and $R_4 e^{-\Delta Vp/RT}$. Hence, the R_1 and R_3 terms (and other ratios in even more complex mechanisms) may be combined to give a constant term, C_p, whose numerical value is of no particular interest. Via combination of these restrictions and earlier simplifications, eq 13 becomes

$$|V/K|_p = \left(\frac{k_1}{1 + K_{G/E} e^{-\Delta V_{G/E} p/RT}}\right) \times \left[\frac{R_2 e^{-\Delta V^{\ddagger} p/RT}}{1 + C_p + R_2 e^{-\Delta V^{\ddagger} p/RT} + R_4 e^{-(\Delta V^{\ddagger} - \Delta V_{eq}) p/RT}}\right] \tag{14}$$

MATERIALS AND METHODS

Yeast alcohol dehydrogenase and NAD^+ were purchased from Boehringer, and benzyl alcohol was purchased from Sigma. Concentrations of benzyl alcohol were determined at 25 °C by enzymatic oxidation at saturating NAD^+ concentrations. The concentrations of benzyl alcohol and NAD^+ were varied from 0.83 to 12.4 mM ($K_m = 3.1$ mM) and from 0.75 to 15.1 mM ($K_m = 1$ mM), respectively. The concentration of alcohol dehydrogenase was 3.19 μM. Tris-HCl (pH 8.5) buffer was used to prevent a pressure-dependent change in pH at high pressure due to electrostriction. 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 monochrometer provided 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 by an OLIS spectroscopy operating system from which absorbancies were calculated and stored on computer.

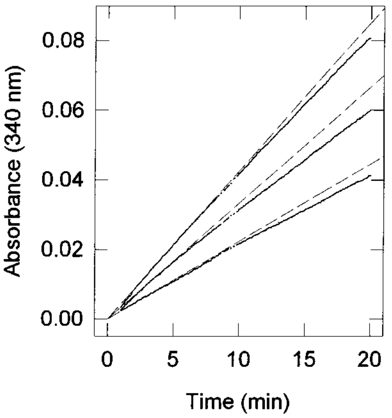


FIGURE 1: Progress curves of the oxidation of benzyl alcohol by yeast alcohol dehydrogenase as reflected in the formation and absorbance of NADH at 340 nm. Reaction rates increased as pressure increased from 1 to 269 to 539 bar. The dashed lines represent initial velocities calculated as asymptotes to the curves obtained by fitting data to an integrated form of the Michaelis−Menten rate equation.

Enzymatic activity was determined by the absorbance change at 340 nm associated with the formation of NADH during oxidation of benzyl alcohol to benzaldehyde. Because several minutes were required to mix reagents, place them in the 1.5 mL cuvette bottle in the pressure cell, and then raise the pressure of the system to a desired value, and a very limiting level of enzyme was used such that reaction velocities could be measured over a period of 20 min or more with only a nominal change in the substrate concentration prior to data collection. Nevertheless, reaction velocities did decrease with time during all assays. Therefore, partial progress curves were collected and fitted to an integrated form of the Michaelis−Menten equation (*17*) to compute the initial velocities extrapolated to zero time at a given pressure. To illustrate, three initial velocity measurements at fixed concentrations of reactants but different pressures are shown in Figure 1. Despite the awkwardness of the apparatus for determining kinetics, it can be seen that reliable rate data can be collected from which initial velocities can be calculated. Sets of initial velocities in which both the alcohol substrate and nucleotide coenzyme were varied were then fit to the rate equation for a sequential bi-bi kinetic mechanism (*18*). Only V/K values were extracted from these fittings because, due to the choices in reactant concentrations, values of V were accompanied by large standard errors. Standard errors on V/K values averaged less than 4%.

RESULTS AND DISCUSSION

Plots of relative values of V/K_A for benzyl alcohol, and V/K_N for NAD$^+$, as a function of pressure are shown in Figure 2. The rate of capture of benzyl alcohol increases immediately with slight increases in pressure, rising nearly 4-fold as pressure is increased further to 1.5 kbar, and then decreases even more precipitously at higher pressures. These data could not be fitted to eq 5 as that equation could not account for the precipitous drop at high pressures; i.e., the change in sign is dependent solely upon ΔV_{eq}, which for an alcohol oxidation to an aldehyde is only about −13 mL/mol (*7*). In contrast, nonlinear regression of these data with eq 9 converged rapidly to the following parameter values: $\Delta V^{\ddagger} = -39.9 \pm 1.0$, $K_{G/E} = 0.0134 \pm 0.0024$, and $\Delta V_{G/E} = -72.56 \pm 1.44$ mL/mol. In contrast, relative values of the capture of nucleotide did not increase as a function of pressure but remained relatively constant during moderate increases of pressure, and then decreased as the pressure exceeded 1 kbar. A fit of these data to eq 6 converged to the lower line shown in Figure 2, but with most standard errors greatly exceeding the parameters. The exception was a $\Delta V^{\ddagger}$ of 88.0 ± 31 mL/mol, a value not inconsistent with the number associated with the $-\Delta V_{G/E}$ given above (the signs appear to be opposite because the functions face in the forward and reverse directions of a turnover, respectively).

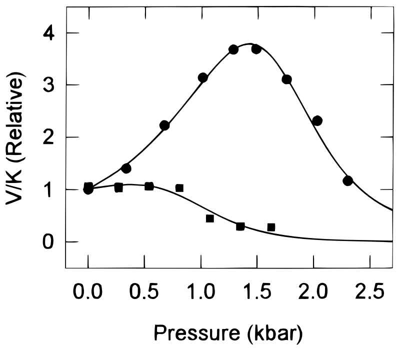


FIGURE 2: Relative values of V/K for yeast alcohol dehydrogenase as a function of pressure for benzyl alcohol (●) and NAD$^+$ (■). Each data point represents a complete initial velocity pattern. The solid lines were constructed from fits of the data to eqs 9 and 6, respectively.

Although cast in the nomenclature of iso-mechanisms, eqs 9−14 are not restricted to only “free” forms of enzyme, such as G and E, when applied to a specific V/K for a bi-substrate enzymatic reaction. Rather, the isomeric forms must be complexes of enzyme saturated with the second substrate or coenzyme, because these extrapolated to infinity in the fitting process. The isomerization detected by eq 9 must be, therefore, a conformational change that follows binding of NAD$^+$ to the enzyme:

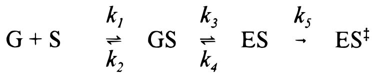
$$\mathrm{E{-}NAD^+ \rightleftharpoons E^*{-}NAD^+} \tag{15}$$

where the conformational change is denoted with an asterisk. The specific parameters describing eq 15 in terms used in eq 9 are as follows: $\Delta V^* = -\Delta V_{G/E} = 73 \pm 2$ mL/mol and $K_{eq}{}^* = -K_{G/E} = 75 \pm 13$. The equilibrium lies far to the right, indicating much tighter binding in the E*−NAD$^+$ complex, that is accompanied by a very large increase in volume. In similar studies of the effects of pressure on YADH oxidation of ethanol, Morild (*7*) calculated a volume change of −75 ± 23 mL/mol on the “dissociation constant” for NAD$^+$. Morild’s volume is in close agreement with the $\Delta V_{G/E}$ described above and, moreover, is consistent with the supposition that most of the volume change that accompanies binding comes after the collision complex is formed and consists largely of solvent reorganization during the conformational change (*16*).

The conformational change of eq 15 is also consistent with findings for other dehydrogenases, beginning with lactate dehydrogenase (*19*) and including liver alcohol dehydrogenase (*20*), but never before demonstrated with YADH despite considerable efforts to do so (*21*). It is noteworthy that the conformational change associated with nucleotide binding has been shown to be pressure-sensitive in other dehydrogenases as well. Davis and Gutfreund (*22*) observed an increase in the 340 nm absorbance of the nucleotide in an NADH complex of lactate dehydrogenase following a sudden drop in pressure. Similarly, Coates et al. (*8*) observed a decrease in the fluorescence of the protein in an NAD complex of liver alcohol dehydrogenase, also after a sudden drop in pressure. Following up on the kinetics of Coates et al., Sekhar and Plapp (*23*) obtained an equilibrium constant K_{eq}* of 290, somewhat larger than the above value for YADH.

This assignment of the large positive activation volume in the capture of benzyl alcohol also accounts for the absence of a large negative volume change in the capture of nucleotide. At saturating alcohol and limiting nucleotide levels, a revised and truncated Scheme 1 takes the form

Scheme 2



$$G + S \underset{k_2}{\overset{k_1}{\rightleftharpoons}} GS \underset{k_4}{\overset{k_3}{\rightleftharpoons}} ES \xrightarrow{k_5} ES^{\ddagger}$$

where G now represents YADH saturated with benzyl alcohol and S is the nucleotide. The conformational change, GS ⇌ ES, now occurs *after* substrate binding and before the hydride transfer, represented by ES → $ES^{\ddagger}$. As written here, pressure favors moving the former to the left and the latter to the right; hence, changes in the two effects cancel in the capture of nucleotide during pressure changes below 1 kbar. Note that the kinetic mechanism before hydride transfer is different for nucleotide capture than it is for alcohol capture, but must remain the same after hydride transfer. It follows that, if eq 7 (applied to V/K_A) with both forward and reverse commitments to catalysis removed is appropriate for alcohol capture at low pressure, then eq 6 (applied to V/K_N) with only the reverse commitment to catalysis removed ought to be appropriate for nucleotide capture. The similarity of the values for $-\Delta V_{G/E}$ and $\Delta V_1^{\ddagger}$ within the two fittings, respectively, is consistent with this mechanistic assignment because these volumes ought to be related and therefore similar. The former reflects the difference in volume of the ground states of E−NAD^+ and E*−NAD^+, while the latter reflects the difference in volume of the transition states of the conformational change and hydride transfer; i.e., R_1 of eq 13 represents k_5/k_4 of Scheme 1. Both are large positive values, and their numerical similarity shows that the volume of the tight-binding ternary complex, E*−NAD−A, must be significantly larger than in the transition state of its formation; if it were not, pressure would favor hydride transfer and not a reversal of the conformational change, leading to an increase in the rate of nucleotide capture at moderate pressure, which is not observed. This juxtaposition of opposing volume changes leads to the surprising conclusion that pressure favors the catalytic transition state while at the same time it weakens the binding of reactants, which runs contrary to Pauling's hypothesis (*24*, *25*) that substrates are bound more tightly in the transition state than in the Michaelian reactant state (which here would be the ternary complex preceding the conformational change, E−NAD−A).

The goodness of fit of the data for benzyl alcohol capture to eq 9 is also in agreement with the conclusion of Cha et al. (*9*) that the commitments to catalysis are insignificant in the oxidation of benzyl alcohol by YADH, which means that eq 7 sufficiently accounts for capture at atmospheric pressure. It follows that the increase in the rate of capture with a moderate pressure increase is a direct and proportional result of increasing the rate of hydride transfer. The activation volume must therefore be negative, and is found to be surprisingly large. As points of reference, consider the following (*22*). (a) The electrostriction associated with the ionization of acetic acid is −10.7 mL/mol, and the pK_2 of phosphoric acid is −24 mL/mol. (b) The solvent reorganization surrounding methane as it is transferred from water to a nonpolar organic solvent is 22.7 mL/mol. (c) Unfolding of chymotrypsin during denaturation is −43 mL/mol. (d) A molecule of water, of course, occupies a volume of 18 mL/mol. The question then is what to make of the reduction in volume by a magnitude of more than two water molecules while going from the free reactant state of benzyl alcohol in water to the hydride transfer transition state? Charge separations are accompanied by negative changes in volume, but it is difficult to believe that a possible dipole in the YADH transition state would generate a larger charge separation than a dissociation of phosphoric acid. Most of the solvent reorganization in YADH appears to be associated with nucleotide binding, and that is not included in the activation volume of alcohol capture; to the extent that benzyl alcohol is desolvated after binding, one expects from (b) above that the associated change in volume should be positive, not negative.

This is the first time that a pressure effect on an enzyme-catalyzed reaction can be specifically assigned to volume change within a transition state of a chemical step. Understanding its origin, sign, and magnitude will be of great interest to mechanistic enzymology, but at this time, one can only speculate on this single, large, negative number. Suffice it to say that in absolute terms, the volume is much too large to ascribe to anything associated with the hydride ion itself. Something more must come into play. A portion of the large volume change might be ascribed to hydrogen tunneling because Cha et al. (*9*) concluded that significant hydrogen tunneling must be present in the oxidation of benzyl alcohol by YADH, as evidenced by a breakdown in the Swain−Schaad relationship (*26*) in a direction opposite of that predicted when isotope effects are not fully expressed on V/K because of large commitments to catalysis (*3*, *14*). Moreover, that hydrogen tunneling is sensitive to pressure was demonstrated by Isaacs (*27*), who described eight chemical reactions suspected of tunneling, including hydrogen transfer, hydride transfer, and proton transfer, for which an observed k_H/k_D decreased with increasing pressure accompanied by activation volumes mostly in the range of −14 to −18 mL/mol. An exception with a $\Delta V^{\ddagger}$ of −25 mL/mol is the hydride transfer between leuco-crystal violet and chloranil (*28*) where tunneling accounts for 33 ± 1% of the observed deuterium isotope effect at atmospheric pressure (*29*). That means that tunneling might account for approximately one-third of the apparent activation volume. Because much smaller Swain−Schaad breakdowns have been found or calculated for chemical reactions (*30*, *31*) than for this enzymatic reaction, a larger portion of the activation volume of enzymatic reactions may originate in tunneling.

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