Effect of Pressure on Deuterium Isotope Effects of Formate Dehydrogenase†

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ABSTRACT: High pressure causes biphasic effects on the oxidation of formate by yeast formate dehydrogenase as expressed on the kinetic parameter *V*/*K*, which measures substrate capture. Moderate pressure increases capture by accelerating hydride transfer. The transition state for hydride transfer has a smaller volume than the free formate plus the capturing form of enzyme, with $\Delta V^{\ddagger} = -9.7 \pm 1.0$ mL/mol. Pressures above 1.5 kbar decrease capture, reminiscent of effects on the conformational change associated with the binding of nicotinamide adenine dinucleotide (NAD^+) to yeast alcohol dehydrogenase [Northrop, D. B., and Y. K. Cho (2000) *Biochemistry 39*, 2406−2412]. The collision complex, E-NAD^+, has a smaller volume than the more tightly bound reactant-state complex, E*-NAD^+, with $\Delta V^* = +83.4 \pm 5.2$ mL/mol. A comparison of the effects of pressure on the oxidation of normal and deuteroformate shows that the entire isotope effect on hydride transfer, 2.73 ± 0.20, arises solely from transition-state phenomena, as was also observed previously with yeast alcohol dehydrogense. In contrast, normal primary isotope effects arise solely from different zero-point energies in reactant states, and those that express hydrogen tunneling arise from a mixture of both reactant-state and transition-state phenomena. Moreover, pressure increases the primary intrinsic deuterium isotope effect, the opposite of what was observed with yeast alcohol dehydrogense. The lack of a decrease in the isotope effect is also contrary to empirical precedents from chemical reactions suspected of tunneling and to theoretical constructs of vibrationally enhanced tunneling in enzymatic reactions. Hence, this new experimental design penetrates transition states of enzymatic catalysis as never before, reveals the presence of phenomena foreign to chemical kinetics, and calls for explanations of how enzymes work beyond the tenants of physical organic chemistry.

The effects of high hydrostatic pressure on the kinetics of enzymatic reactions have only recently been explored in a manner parallel to the developments of isotope and pH effects (*1*−*3*). The major caveat hindering the use of pressure to study enzyme kinetics, i.e., whether protein denaturation and subunit dissociation might occur coincidentally with changes in catalysis, has been overcome by casting rate equations in a form originally constructed for isomechanisms (*4*). Denaturation and dissociation cause *removal* of individual molecules of enzyme from participating in catalysis, similar to an isomerization from a substrate form of free enzyme to a product form; in contrast, changes in catalytic rate constants cause a *modulation* of catalysis by all molecules of enzyme. Because an isotope effect reports back only from the latter, it can be used to distinguish between a loss of activity due to removal and a loss due to modulation (*5*). A second caveat is what to do with effects of pressure directly on intrinsic isotope effects themselves, as opposed to modulation of their expression in kinetic parameters. Fortunately, a general equation has been derived to address this unexpected eventuality (*6*) and incorporated into rate equations for enzymes with isomechanisms (*3*).

The oxidation of formate by yeast formate dehydrogenase (YFDH)[1] was chosen as a test system because Blanchard and Cleland (*7*) had demonstrated an intrinsic deuterium isotope effect arising from deuteroformate was fully expressed on the kinetic parameter, *V*/*K*, which measures substrate capture[2] (*8*, *9*). It was later shown that isotope effects on capture by YFDH vary with the redox potential of analogue pyridine nucleotides, with the increases correlating with changes from early to late transition-state structures (*10*). The enzyme catalyzes the oxidation of formate ion to carbon dioxide coupled to the reduction of NAD^+ to NADH, without involvement of water or a proton transfer. As such, it is the simplest of the dehydrogenases. Its kinetic mechanism is ordered with the nucleotide binding first, and its structure is a dimer of 42 kDa subunits (*7*).

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[1] Abbreviations: YFDH, yeast formate dehydrogenase; YADH, yeast alcohol dehydrogenase; NAD^+, nicotinamide adenine dinucleotide; thio-NAD, thionicotinamide adenine dinucleotide.

[2] Substrate capture is a new and more descriptive name for the kinetic parameter *V*/*K*. It stresses the facts that the parameter is a single entity and not a ratio, that it is the apparent rate constant for the coming together of substrate and enzyme to form captured complexes destined to yield product formation at some later time, but that no significant enzymatic catalysis is taking place and no measurable product is being formed. *V*/*K* has been widely misinterpreted as an alternative measure of enzymatic catalysis for many years, and the misconceptions derived therefrom can be avoided by using this new name.

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THEORY

The effect of pressure on the kinetics of capture can be described by (*2*)

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

where k_1 is the diffusion-controlled rate constant for the combination of enzyme (E) and substrate (S); R_0 is the product ratio of forward and reverse enzymatic rate constants up to and including a designated isotopically sensitive step;[3] $\Delta V^{\ddagger}$ is the volume of activation between reactants E + S (E*-NAD^+ and formate) and the transition state of the isotopically sensitive step; p is the pressure in bar (0.98692 atm); R is the gas constant (82.0578 mL·bar/mol·K); T is the temperature (298 K); $K_{G/E}$ is the equilibrium constant between free enzyme forms E and G (i.e., E*-NAD^+ and E-NAD^+, respectively, for formate capture where the asterisk designates a conformational change to a form of enzyme that binds the nucleotide more tightly and allows the binding of substrate); $\Delta V_{G/E}$ is the volume difference between the forms; C_f and C_r are the forward and reverse commitments to catalysis, respectively; and ΔV_{eq} is formally the volume change between reactants (E + S) and products (F + P) but assumed to be between S and P only. When ΔV_{eq} is small, commitments may be approximated by combining them as in

$$|V/K|_p = \left(\frac{k_1}{1 + K_{G/E}e^{-\Delta V_{G/E}p/RT}}\right)\left(\frac{R_0 e^{-\Delta V^{\ddagger}p/RT}}{1 + (C_f + C_r)e^{-\Delta V^{\ddagger}p/RT}}\right) \quad (2)$$

In the absence of significant commitments, eq 1 reduces to

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

Given an intrinsic isotope effect on hydride transfer, ${}^{D}k$, = k_H/k_D, a global form of eq 1 takes the form (*3*)

$$|V/K|_p = \left(\frac{k_1}{1 + K_{G/E}e^{-\Delta V_{G/E}p/RT}}\right) \times \left(\frac{R_0 e^{-\Delta V^{\ddagger}p/RT}}{1 + C_i[{}^{D}k - 1] + C_f e^{-\Delta V^{\ddagger}p/RT} + C_r e^{-(\Delta V^{\ddagger} - \Delta V_{eq})p/RT}}\right) \quad (4)$$

where C_i is the fraction of isotopic labeling (0.98). If the intrinsic isotope effect is pressure-dependent as well, eq 4 expands to

$$|V/K|_p = \left(\frac{k_1}{1 + K_{G/E}e^{-\Delta V_{G/E}p/RT}}\right) \times \left(\frac{R_0 e^{-\Delta V^{\ddagger}p/RT}}{1 + C_i[{}^{D}k({}^{D}Q - 1)e^{-\Delta V_Q p/RT} + {}^{D}k - 1] + C_f e^{-\Delta V^{\ddagger}p/RT} + C_r e^{-(\Delta V^{\ddagger} - \Delta V_{eq})p/RT}}\right) \quad (5)$$

where ${}^{D}Q$ is the ratio of Bell tunneling corrections, Q_H/Q_D (*12*) and represents the portion of an isotope effect originating in the transition state and ΔV_Q is the apparent volume difference between a hydride and deuteride transfer. When ΔV_{eq} is small, eq 5 reduces to

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

Finally, when commitments are small, eq 5 reduces to

$$|V/K|_p = \left(\frac{k_1}{1 + K_{G/E}e^{-\Delta V_{G/E}p/RT}}\right) \times \left(\frac{R_0 e^{-\Delta V^{\ddagger}p/RT}}{1 + C_i[{}^{D}k({}^{D}Q - 1)e^{-\Delta V_Q p/RT} + {}^{D}k - 1]}\right) \quad (7)$$

At atmospheric (or zero) pressure and given a small $K_{G/E}$, k_1R_0 will equal the value of V/K in eqs 3 and 7, and it can be replaced by an empirical constant during regression analysis.

MATERIALS AND METHODS

Formate dehydrogenase isolated from *Candida boidinii* and NAD^+ (Li salt) were purchased from Boehringer-Mannheim. Sodium deuteroformate (98 atom % deuterium) and 2,4,6-collidine (trimethylpyridine) were from Aldrich. Initial velocities were measured by monitoring the absorbance at 340 nm using 10 mM NAD^+. The concentration of formate was varied between 0.5 and 10 mM. Substrate concentrations were determined by mass. Assays were performed at 25 °C and pH 7.5 using 100 mM 2,4,6-collidine as buffer. This buffer was chosen because of its desirable pK (7.39) and small volume (0.2 mL/mol) of ionization (*13*). 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 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 absorbencies were calculated and stored on computer. Partial progress curves were collected and fitted to an integrated form of the Michaelis−Menten

[3] For example, if a reactive step governed by k_5 using the Cleland (*11*) nomenclature in a linear mechanism were designated the isotopically sensitive step, then $k_1R_0 = k_1k_3k_5/k_2k_4$, while V/K itself extends further, well beyond k_5 to encompass the first irreversible step. If a different step is designated, then the product ratio and the commitments to catalysis will change in definition, but V/K will remain the same algebraically in terms of rate constants. The utility of this construction is that when the designated isotopically sensitive step passes over the highest point in an activation energy diagram, what some label as "rate-limiting", then the commitments become insignificant and V/K truncates to just k_1R_0. This is useful. The label however is misleading because substrate capture does not reflect any rate associated with enzymatic turnovers, and as can be seen by the example of k_1R_0 given above, all of the rate constants from k_1 through the isotopically sensitive step are equally important, with none more limiting than any other.

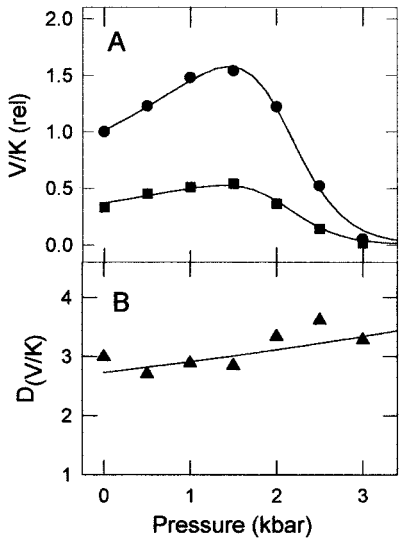

FIGURE 1: (A) Effect of pressure on the capture of formate (●) and deuteroformate (■) by YFDH. (B) Deuterium isotope effects (▲) calculated from the data in panel A. Relative values of *V*/*K* were fit to eq 7 to determine the solid lines in both panels.

Table 1: Pressure Effects and Isotope Effects on the Capture of Formate by YFDH

parameter	$(V/K)_H$[a]	$(V/K)_D$[a]	$^D(V/K)$[b]
k_1R_0	1.00 ± 0.03	0.35 ± 0.02	(2.995)[c]
$\Delta V^‡$ (mL/mol)	−9.3 ± 1.1	−10.3 ± 1.9	−9.7 ± 1.0[d]
$\Delta V_{G/E}$ (mL/mol)	−84 ± 6	−81.7 ± 8.1	−83.4 ± 5.2
$K_{G/E}$	0.0009 ± 0.0006	0.0018 ± 0.0015	0.001 ± 0.0005
^Dk			1.02 ± 0.03
^DQ			2.73 ± 0.20
ΔV_Q (mL/mol)			−2.5 ± 2.2

[a] Relative values of capture were fit to eq 3. [b] Fit to eq 7. [c] Calculated from the ratio of $(V/K)_H$ and $(V/K)_D$ measured at atmospheric pressure. [d] A value of −7.2 ± 2.3 mL/mol was obtained for $\Delta V_D^‡$ when ΔV_Q in eq 7 was replaced by $(\Delta V_H^‡ - \Delta V_D^‡)$.

equation (*14*) in order to compute the initial velocities extrapolated to zero time at a given pressure. Initial velocities were then fitted to the Michaelis−Menten equation to determine *V*/*K* for formate, and values of *V*/*K* at different pressures were then fit to the equations above using the BASIC computer program of Duggleby (*15*) assuming constant variance for weighting.

RESULTS

Figure 1A shows the biphasic pressure dependence of the capture of normal formate (upper curve) and of deuterated formate (lower curve). The results appear very similar to the capture of benzyl alcohol by YADH reported by Cho and Northrop (*2*), except the increases at low to moderate pressure are less. Separate fits of the upper and lower data points to eq 3 give the parameters shown in the second and third columns of Table 1. A global fit of both sets of data points to eq 7 gives the parameters listed in the fourth column of Table 1. A similar global fit to eq 6 returned parameters not significantly different from those in Table 1 but with an additional value for the sum of the commitments of $C_f + C_r = 1.7 \times 10^{-9} \pm 11700$, which is not significantly different from zero. If the commitments were significant and changed with pressure, then the data in column 4 would differ from those in columns 2 and 3 (*3*). This result, a difference of degree, substantiates the conclusion of Blanchard and Cleland (*7*) that the commitments of YFDH are small.

Figure 1B shows the ratio of the two sets of data points and the calculated line from the fit to eq 7. The increase in the deuterium isotope effect is direct evidence that pressure is changing the intrinsic isotope effect and is not merely changing its expression by changing commitments as described by eqs 2 and 4. In order for a change in commitments to cause an increase in an apparent isotope effect on capture, the commitments must be significant at atmospheric pressure and then decrease with increasing pressure. This result, a difference in kind, verifies the conclusion of Blanchard and Cleland that commitments of YFDH are very small.

DISCUSSION

Biphasic effects of pressure on capture have now been found for YFDH and YADH. Both show an initial increase in capture at modest pressure followed by a decrease at high pressure. These changes are due to the first volume changes associated with enzymatic catalysis that can be assigned to specific steps of a kinetic mechanism—because of the inclusion of the deuterium isotope effect in the experimental design. The volume change dominant at low pressure can be assigned to hydride transfer in both dehydrogenases, as examples of modulation of enzymatic activity, the result of changes in a specific rate constant. The activation volume of hydride transfer in both enzymatic reactions is negative, which may be significant; should this be a general phenomenon,[4] it may become a useful arbiter of mechanistic hypotheses, some of which suggest positive volumes of activation, notably electrostatic stabilization and solvent preorganization (*3*). The activation volume for hydride transfer in YFDH is significantly less than that in YADH. This difference may be a random variation given their low primary structure homology (*17*), or it may be a functional variation given that YFDH has a late transition state (*10*) while YADH has an early one (*18*, *19*). To test the latter possibility, we propose to examine the effects of pressure on YFDH using thio-NAD^+ because it has an earlier transition state than NAD^+ given its more positive redox potential (*9*).

The decreases in capture at high pressure is consistent with a positive volume change accompanying the change in conformation that follows the binding of pyridine nucleotide (*2*):

$$E + NAD^+ \rightleftharpoons E\text{-}NAD^+ \overset{K^*}{\rightleftharpoons} E^*\text{-}NAD^+ \qquad (8)$$

The more tightly bound binary complex, E*-NAD^+, has an apparent volume considerably larger than the collision

[4] It should be noted that, because of the incorporation of an isotope effect in the experimental design, these two negative values are the only activation volumes that can be assigned to a chemical step in enzymatic reactions. Other laboratories have reported both positive and negative volumes for other enzymatic reactions, e.g., Morita (*16*) obtained a volume change of +20 mL/mol on a reaction velocity of formate dehydrogenase from *Escherichia coli*, but we have no way of knowing what reactive steps this or other previously published volume changes come from. Similarly, the positive volume changes associated with binding NAD^+ in both YFDH and YADH are the only volume changes that can be assigned to specific conformational changes.

complex, E-NAD$^+$, and comparable to the 73 mL/mol found for alcohol dehydrogenase. The equilibrium constant, $K^* = 1/K_{G/E} = 1/(0.001 \pm 0.0005) = 1000 \pm 500$ appears larger than the 75 ± 13 found for YADH but is less well-determined. This conformational change has been detected in a variety of dehydrogenases (*20*) and characterized with high resolution by X-ray crystallography of YFDH by Lamzin et al. (*17*), who describe it as follows: "NAD binding causes movement of the catalytic domain and ordering of the C terminus, where a new helix appears"; the movement is "essentially a rotation by about 7.5° around hinges connecting residues 146−147 and 340−341."

The origin of the deuterium isotope effect is solely within the transition state, as indicated by the null value for the semiclassical component of the intrinsic isotope effect together with its small standard error, as was also the case with YADH. The value obtained for the isotope effect on the tunneling correction factor is in close agreement with the 2.8 ± 0.1 reported by Blanchard and Cleland (*7*). The most surprising result is the *increase* in the deuterium isotope effect with increasing pressure, illustrated in the fitted line of Figure 1B and in the negative sign found for ΔV_Q in Table 1. This sign is opposite that found for YADH and also opposite to the data of Isaacs (*21*), who examined eight nonenzymatic reactions suspected of tunneling. These empirical findings argue against hydrogen tunneling as the transition-state phenomenon giving rise to the deuterium isotope effect on capture. Also, Bruno and Bailec (*22*) developed a theoretical basis for "vibrationally enhanced tunneling" to address the proposed tunneling catalyzed by YADH (*17*), and that model predicts that if hydride transfer is accelerated by pressure, then its associative deuterium isotope effect must decrease.[5] Hence, even if the large standard error were taken into account and ΔV_Q were deemed not significantly different from zero, this result would remain inconsistent with hydrogen tunneling.

The finding that pressure favors simultaneously both the transition state for hydride transfer and the weaker binding form of enzyme-NAD$^+$ would appear to run contrary to Pauling's hypothesis that substrates are bound more tightly in a transition state than in a reactant state during enzymatic catalysis (*23*, *24*). If Pauling's hypothesis holds despite this evidence to the contrary, then it follows that the tighter binding in the transition state must have a different origin than the tighter binding of the conformational change in eq 8. This proposition is not difficult to imagine because desolvation is considered a major component of the conformational change (*25*) and ligand binding in general (*26*−*28*), and desolvation is not likely to be part of the quasi-equilibrium between reactant state and transition state. Moreover, the proposition can be tested by examining temperature dependencies because desolvation ought to have a larger entropic component.

Evidence for isotope effects that originate solely in transition states is unprecedented. Semiclassical isotope effects originate in zero-point energies due to differences in vibrational frequencies of protium and deuterium in reactant states as described by the Bigeleisen equation (*29*). Isaacs examined whether the vibrational frequencies were sensitive to pressure and concluded that their sensitivities are small and likely to be insignificant within the experimental range of pressure available to kinetic studies (*21*). This conclusion was later verified experimentally using a variety of chemical reactions displaying normal isotope effects. Tunneling does provide a transition-state component to observed isotope effects, but its contribution in chemical reactions is generally regarded as less than the differences in zero-point energies, and in fact contributes only 33% in the one case that has been quantified (*6*). Enzymatic reactions, however, are providing striking exceptions to this generality (*30*). Most relevant to the present discussion are the isotopic studies with alcohol dehydrogenases by Klinman and co-workers (*18*, *30*−*36*) wherein substantial tunneling is proposed for hydride transfer together with coupled motion between the transferrable hydride and the α proton of alcohol, which suppresses most of the contribution from zero-point energies. Even more extreme however is Scrutton and co-workers' proposal (*37*−*40*) that ground-state tunneling takes place in enzymatic reactions, which renders the barrier height—and thus differences in zero-point energies—completely insignificant. This leaves tunneling as the sole source of deuterium isotope effects in enzymatic reactions. However, their analyses are based on the Bruno and Bailec (*22*) model for vibrationally enhanced tunneling, which again is inconsistent with negative values for both $\Delta V^{\ddagger}$ and ΔV_Q. Finally, if tunneling is ruled out and the isotope effect nevertheless originates in the transition state, then it follows that some sort of motion (i.e., vibrational, coupled, or coordinated) must contribute to the bond-breaking transition state in order to account for the importance of the mass of the hydride. This, in turn, supports mechanical models of enzymatic catalysis of the kind described by Lumry (*41*−*43*).

[5] William J. Bruno, personal communication. This interpretation does not however discredit the Bell formalism of tunneling used in eqs 5−7 as that pressure function is general (*6*).

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This paper is dedicated to Rufus Worth Lumry, II, on the occasion of his 80th birthday. Professor Emeritus Lumry was probably the first to propose a mechanical model of enzymatic catalysis. Throughout the latter half of the 20th century and now into the 21st, he has pursued mechanical models while the mainstream explored models based on physical organic chemistry.

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