Inverse Solvent Isotope Effects in the NAD-Malic Enzyme Reaction Are the Result of the Viscosity Difference between D_2O and H_2O: Implications for Solvent Isotope Effect Studies

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Abstract: Solvent isotope effect studies were undertaken with the NAD-malic enzyme from *Ascaris suum*. With different varied substrates and divalent metal ion activators, nearly all the measured isotope effects are inverse, with the effect on *V/K* more inverse than the effect on *V*. The usual explanations for inverse solvent isotope effects, such as medium effects, involvement of a thiol group, or dissociation constant of a metal-chelated water, were unsatisfactory in the NAD-malic enzyme reaction. The observation of a close correspondence between changes in kinetic parameters induced by increased solvent viscosity with glycerol as viscosogen and the inverse solvent isotope effects has led to the conclusion that solvent effects are the result of the difference in viscosity between D_2O and H_2O.

Introduction

The NAD-malic enzyme from *Ascaris suum* catalyzes the oxidative decarboxylation of malate to yield pyruvate, CO_2, and NADH. With NAD as substrate, the enzyme catalyzes the reaction in two distinct steps, with oxidation of malate to an oxalacetate intermediate preceding the slower decarboxylation of the intermediate to enolpyruvate.[1,2] Multiple primary deuterium/primary ^{13}C isotope effects suggest that a change to a concerted mechanism occurs when alternative dinucleotides such as 3-APAD are used as substrates.[3,4] A switch to a concerted mechanism may also occur when a thiol group near the malate binding site is modified.[3,5] An alternative explanation, still adhered to by some, suggests that a secondary ^{13}C isotope effect is manifest on the hydride transfer step in a sequential mechanism that results from hyperconjugative weakening of the C-3 to C-4 bond of enzyme-bound oxalacetate when C-4 is held out of the C-3 to C-4 plane. The authors believe that this mechanism has been ruled out.[3−5]

The transfer of a hydride from C-2 of malate to C-4 of the nicotinamide ring of NAD and the abstraction of a proton from the 2-hydroxyl of malate by an active site general base occur in the oxidation step. These two processes could occur via a concerted or a stepwise mechanism.

Solvent isotope effect studies were undertaken in order to further investigate the NAD-malic enzyme reaction. Nearly all the measured isotope effects are inverse. A close correspondence between the solvent effects and the effects on the kinetic parameters of increasing solvent viscosity, and the absence of any other satisfactory explanation, have led to the conclusion that the solvent effects are due to viscosity changes in D_2O. The results are discussed in terms of the implications for conducting solvent isotope effect studies.

Materials and Methods

Chemicals and Enzyme. Mitochondrial NAD-malic enzyme from *A. suum* was purified according to the procedure of Allen and Harris[6] or Karsten and Cook.[3] The enzyme had a final specific activity of 32 units/mg assayed in the direction of oxidative decarboxylation with 100 mM Hepes, pH 7.5, 1 mM DTT,[7] 153.4 mM malate (28 mM when corrected for the Mg−malate complex), 13.5 mM NAD (2 mM when corrected for the Mg−NAD complex), and 249 mM $MgSO_4$ (112 mM when corrected for the Mg−malate and Mg−NAD chelate complexes). Enzyme was stored at −20 °C in a buffer containing 10 mM Hepes, pH 7.5, 10 mM DTT, 1 mM EDTA, and 10% glycerol. Preparation and storage of the SCN-modified enzyme are identical to those used previously.[5]

Malate, APAD, sucrose, and DTT were from Sigma, while NAD was purchased from Boehringer-Mannheim or Sigma. D_2O (99 atom % D) was from Sigma or MSD Isotopes, while the glycerol was from Fisher. All other reagents and chemicals represented the highest quality available from commercial sources.

Substrate Calibration. The precision of solvent isotope *V/K* effects measured by the method of direct comparison requires that one accurately know the concentration of substrates.[8] Therefore, all substrate concentrations were calibrated enzymatically by endpoint analysis.[9] Concentrations were determined using 2 units of chicken liver malic enzyme in 100 mM Taps, pH 9. All assays contained the following: NADP, 1 mM; $MgSO_4$, 2 mM; and DTT, 0.2 mM. The concentrations from several determinations were in agreement within 1%. The concentration of NAD was determined spectrophotometrically using an extinction coefficient of 17 800 M^{-1} cm^{-1} at 259 nm.

Metal Chelate Correction. Since metal−ligand chelate complexes are not reactants for the NAD-malic enzyme reaction, the concentration of reactants and inhibitors added to the reaction mixture were corrected

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(7) Abbreviations: APAD, 3-acetylpyridine adenine dinucleotide; Ches, 2-(cyclohexylamino)ethanesulfonic acid; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Mes, 2-(*N*-morpholino)ethanesulfonic acid; Taps, 3-[[tris(hydroxymethyl)methyl]amino]propanesulfonic acid.

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for the concentration of the chelate complexes.[10] The following dissociation constants were used in the corrections: Mg−malate, 25.1 mM; Mn−malate, 5.8 mM; Cd−malate, 4.4 mM; Mg−NAD, 19.5 mM; Mg−Mes, 160 mM.[11-13] The dissociation constants for metal−APAD were assumed to be identical to that for metal−NAD. All other reaction components gave negligible corrections under the conditions used.

Stability Constants Measurements. The absorption spectrum of 8-hydroxyquinoline was measured as a function of pH(D) in H_2O or D_2O with 50 mM of a suitable buffer system (Mes, 5.5−6.5; Hepes, 7.0−8.0; Taps, 8.5−9.0; Ches, 9.5−10.0). The measurement of the stability constant for Mg−malate in H_2O (pH 7.5) and D_2O (pD 7.5) was then carried out by adding a small amount of a concentrated solution of Mg^{2+} to a solution containing 1.5 mM 8-hydroxyquinoline in 50 mM Hepes, in the presence and absence of malate.[14,15] The measurement of the stability constant was also performed in H_2O in the presence of 9% glycerol. The solution was maintained at constant temperature, and the spectral change at 360 nm was recorded using a Hewlett-Packard 8452A diode array spectrophotometer.

Stopped-Flow Kinetic Studies. Experiments were performed with a temperature-controlled Hi-Tech SF-51 stopped-flow apparatus equipped with a sample handling unit (SHU-51) and a spectrometer unit (SU-40) and interfaced with a Hewlett-Packard Series 3000 computer. The observation cell of the SHU-51 accommodates a high-efficiency mixer and a 10 mm optical path length. The dead time of the instrument is <2 ms. All experiments were carried out with a high concentration of malic enzyme (30 μM) at 25 °C, pH 6.9 (100 mM Hepes). Negligible pH changes were observed before and after the reaction. The final concentrations of reactants used were 13.5 mM NAD (2 mM when corrected for Mg−NAD), 50 mM L-malate (27.2 mM when corrected for Mg−malate), and 47 mM $MgSO_4$ (30.2 mM when corrected for Mg−NAD and Mg−malate), and these are high enough to represent near-saturating conditions. The progress of the reaction was followed by monitoring the NADH absorbance at 340 nm.

Isotope Effect Studies. Solvent deuterium isotope effects were obtained at pH(D) 7, 2 pH units above and below, respectively, the pK values observed in the V/K_{malate} pH profile to eliminate from consideration isotope effects on acid-dissociable groups.[16] For reactions run in 100% D_2O, buffers and reactants were prepared in 100% D_2O, rotary evaporated to near dryness, and redissolved in 100% D_2O. A typical assay contained 100 mM Hepes, pH 7.0, 1 mM DTT, 160 mM Mg^{2+}, 2 mM NAD, variable concentrations of malate, and 0.01 unit of malic enzyme. Enzyme was added from a concentrated stock solution in H_2O using volumes of 0.005 mL or less. Thus, the isotope effect was corrected for the 0.5% water. Isotope effects were obtained by direct comparison of initial velocities in H_2O and D_2O. Slow or irreversible changes in enzyme activity as a result of the presence of D_2O were ruled out by the linear time courses obtained in H_2O and D_2O and by assaying identical stock solutions of enzyme prepared in H_2O and D_2O in H_2O after incubation for 12 h.

Viscosity Studies. Kinetic parameters were determined in H_2O at a relative viscosity of 1.24 at pH 7.0 and 25 °C. Assays contained 9% glycerol (w/v) as the viscosogen and typically contained 100 mM Hepes, pH 7.0, 20 mM uncomplexed Mg^{2+}, 0.5 mM uncomplexed NAD, and variable concentrations of malate. The relative viscosity used in the assays is the same as the relative viscosity of D_2O at 25 °C.[16] The amount of glycerol required to achieve a relative viscosity of 1.24 was determined by constructing a standard curve of the viscosity vs percent glycerol data presented in Bazelyansky et al.[17] and determining the required amount of glycerol from the linear standard curve.

Data Processing. Reciprocal initial velocities were plotted as a function of reciprocal substrate concentrations, and all plots were linear. Data were analyzed via the appropriate rate equations and whenever possible by using BASIC versions of the Fortran programs developed by Cleland.[18] Data obtained by varying the concentration of one substrate in H_2O (or D_2O) at saturating levels of the other substrates were fitted using eq 1. Individual saturation curves were fitted using eq 2.

$$v = VA/[K_a(1 + F_iE_{V/K}) + A(1 + F_iE_V)] \quad (1)$$

$$v = VA/(K_a + A) \quad (2)$$

In eqs 1 and 2, v and V are the initial and maximum velocities, respectively, A is the concentration of malate, K_a is the Michaelis constant for malate, and F_i is the fraction of D_2O in the solvent. E_V and $E_{V/K}$ are the isotope effects minus 1 on V and V/K, respectively.

Results

Stability Constant Measurements. There are no significant changes in the shape of the absorption spectra for 8-hydroxyquinoline in H_2O or D_2O, but there is a decrease in the ϵ_{360} in D_2O (data not shown). These results are consistent over the pH(D) range 5−10. The method for the stability constant determination[14,15] is based on the titration of malate with Mg^{2+} in the presence of a low concentration (1.5 mM) of 8-hydroxyquinoline, which acts as a spectrophotometric indicator of free Mg^{2+}. Titrations are made at different malate concentrations and plotted as A_{360} vs $[Mg^{2+}]$. The initial slopes of such plots can be used to calculate the K_d from $[Mg^{2+}]_{free}/[Mg^{2+}]_{total} = 1/(M_t/K_d + 1)$, where M_t is the total malate. The calculated apparent stability constant for Mg−malate in H_2O (pH 7.3) is 24.2 ± 2.7 mM and in D_2O (pD 7.3) is 22.1 ± 2.8 mM, measured by the competition method, which compare favorably to the published K_d value of 25.1 mM.[11] The inverse kinetic solvent deuterium isotope effects discussed below cannot be a result of a change in the Mg−malate stability constant in D_2O. The Mg−malate stability constant is 22 ± 4 mM in the presence of 9% glycerol, as determined by the same competition method.

Pre-Steady-State Kinetic Studies. A lag in the pre-steady-state time course for the NAD-malic enzyme reaction has been reported and results from a slow isomerization of the E:NAD complex.[19] As a result, the time course was measured at near-saturating substrate conditions in H_2O or D_2O to determine whether the isomerization was solvent deuterium sensitive. No detectable difference was observed in the isomerization rate constant when the time course was measured in the two solvents (data not shown).

Solvent Isotope and Viscosity Effect Measurements. Solvent deuterium isotope effects were obtained for malic enzyme and are reported in Table 1. In most cases, the solvent effects are inverse, with the effect on V/K more inverse than the effect on V. The solvent effects are equal for both malate and NAD with Mg^{2+} as the divalent metal ion activator. Fumarate, an allosteric activator of malic enzyme, has no effect on the size of the isotope effect. The solvent isotope effects on V/K and V are equal in the reverse reaction direction with pyruvate as the varied substrate. Finally, the SCN-malic enzyme displays similar inverse solvent isotope effects as the native enzyme.

With glycerol as viscosogen, an increase in solvent viscosity ($\eta_{rel} = 1.24$) generally results in an increase in V/K and V. When expressed as a ratio of the kinetic parameter determined in H_2O to the value determined in the presence of viscosogen, the

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Table 1. Comparison of Solvent and Viscosity Effects on the Kinetic Parameters of the NAD-Malic Enzyme from *A. suum*

		D_2O		relative viscosity[a]	
fixed substrate(s) activator	varied substrate	V/K[b]	V	V/K	V
NAD, Mg^{2+}	malate	0.5 ± 0.1[c]	0.76 ± 0.02	0.6 ± 0.2	0.85 ± 0.07
malate, Mg^{2+}	NAD	0.57 ± 0.02	0.81 ± 0.06	0.689 ± 0.001	0.891 ± 0.001
NAD, Mg^{2+}, fumarate	malate	0.47 ± 0.07	0.80 ± 0.02	0.46 ± 0.03	0.84 ± 0.01
APAD, Mg^{2+}	malate	0.49 ± 0.06	0.91 ± 0.02	0.62 ± 0.06	0.82 ± 0.06
NAD, Cd^{2+}	malate	0.37 ± 0.06	1.0 ± 0.2	0.61 ± 0.02	0.86 ± 0.08
NAD, Mn^{2+}	malate	0.5 ± 0.3	1.272 ± 0.006	0.62 ± 0.05	1.0 ± 0.1
APAD, Mn^{2+}	malate	0.76 ± 0.03	0.99 ± 0.01	0.57 ± 0.01	0.78 ± 0.04
APAD, Mn^{2+}, fumarate	malate	0.81 ± 0.03	1.07 ± 0.02	0.68 ± 0.02	0.85 ± 0.03
NADH, CO_2, Mg^{2+}	pyruvate	0.42 ± 0.01	0.35 ± 0.08	0.41 ± 0.02	0.44 ± 0.07
		SCN-Modified NAD-Malic Enzyme			
NAD, Mg^{2+}	malate	0.61 ± 0.04	0.97 ± 0.06	nd[d]	nd
APAD, Mg^{2+}	malate	0.43 ± 0.03	0.97 ± 0.05	nd	nd

[a] Glycerol was used as viscosogen to adjust viscosity to that of D_2O. [b] Kinetic parameters are reported as a ratio of values [H_2O/D_2O or (η_{rel} = 1)/(η_{rel} = 1.24)]. [c] Values reported in the table are the averages of at least two separate determinations. [d] Not determined.

resulting value is <1 (Table 1). In nearly every case, the ratio of values of V/K and V are, within error, equal to the corresponding solvent isotope effect values. A few exceptions are the data obtained with NAD and Cd^{2+} and with APAD and Mn^{2+}, where the solvent effect on V/K is about 0.2 less inverse than the corresponding viscosity effect. There is also no solvent isotope effect on V, which is about 0.2 larger than the corresponding viscosity effect value on V. In order to compare the effect of different viscosogens at a similar relative viscosity as was used to obtain the results with glycerol, the kinetic parameters were determined with sucrose or the macroviscosogen ficoll as the viscosogen. With Mg^{2+} as metal ion activator and NAD as dinucleotide substrate, the viscosity effect is 0.8 on V/K and 0.9 on V with sucrose as viscosogen. Kinetic parameters determined in the presence of ficoll are identical within error to those determined in the absence of ficoll.

When the concentration of glycerol is doubled to 18%, the ratios of V and V/K_{malate} in H_2O to that in the presence of viscosogen decrease from the values of 0.8 and 0.57 using 9% glycerol to 0.5 ± 0.2 and 0.2 ± 0.1. Similarly, the ratios measured in the presence of 9% glycerol in D_2O are 0.46 ± 0.08 and 0.28 ± 0.07, respectively. These data are consistent with the effects of glycerol and D_2O reflecting changes in the relative viscosity of the solution.

The presence of glycerol, in addition to having an effect on viscosity, will also change the dielectric constant of the assay solution, which thus raises the possibility that the effects observed on the kinetic parameters are the result of the dielectric constant difference between assay mixes that contain glycerol and those that do not. Consequently, the effect of the presence of 9% ethanol on the kinetic parameters was investigated. Ethanol has a significantly lower dielectric constant than glycerol,[20] and consequently, the presence of 9% ethanol will lower the dielectric constant of the solution well below that of an assay containing 9% glycerol. The presence of ethanol was found to have no effect on malic enzyme initial rates compared to control assays without ethanol, consistent with the premise that changes in the kinetic parameters observed in the presence of glycerol are the result of differences in solvent viscosity.

Discussion

As indicated in the Results section, there is no change in the metal−malate dissociation constant in D_2O compared to H_2O, nor is there any observed difference in the rate constant for the isomerization of the E:NAD complex measured in the two solvents. Neither of these possibilities can account for the inverse solvent isotope effects. When an inverse solvent effect has been observed in an enzyme-catalyzed reaction, it has usually been attributed to (A) the acid dissociation constant of a thiol, (B) medium effects, or (C) dissociation of a metal-chelated water. Each of these possibilities will be discussed for the malic enzyme reaction.

(A) The Acid Dissociation Constant of a Thiol Group. The sulfhydryl group is the only functional group of those present in the naturally occurring 20 amino acids that has an exchangeable hydrogen with a fractionation factor less than unity (~0.5).[16] Thus, when experiments have indicated that the fractionation factor of a catalytic group on enzyme was ~0.5, the supposition has been that these are sulfhydryl groups. The small change in the observed pK of a thiol in D_2O (~0.2−0.3)[16] has been used to assign an inverse solvent isotope effect to thiol ionization. A nitrogen- or oxygen-containing group will give an increase in pK of 0.4−0.6 in D_2O compared to H_2O. A pK value of 4.7 ± 0.3 has been reported for the general base catalyst in the NAD-malic enzyme reaction based on the pH dependence of V/K_{malate},[21] while a pK of 5.5 ± 0.2 is obtained from the pD dependence of V/K_{malate} (data not shown). The difference in pK values in the present case is about 0.8 (with a standard error close to 0.3), inconsistent with thiol ionization. In addition, a thiol group near the malate binding site in the NAD-malic enzyme can be modified to form an SCN-malic enzyme. Inverse solvent isotope effects of similar magnitude are also observed for the SCN-malic enzyme in which the active site thiol has been modified.

(B) Medium Effects. In a situation where isotope exchange between solute and solvent does not occur, solvent isotope effects that arise from medium effects are possible.[16] They are best considered as generalized solvation effects for enzymes or substrates, or as arising from a multiplicity of enzyme structural sites. Despite the fact that the exact origin of medium effects and the detailed character of the corresponding sites are not generally possible to decide, medium effects have two essential properties: a large number of protons are involved, and each generates only a small isotope effect per corresponding site. In the absence of any specific solute−solvent interaction, these effects are small and generally can be neglected. However, the recent work from Markley's group on staphylococcal nuclease indicates that these effects can be substantial and should not be ignored.[22] Medium effects would likely not result, however, from the presence of a viscosogen (see below).

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(C) Dissociation of Metal-Chelated Water. Inverse fractionation factors have been determined for both $M-OD^-$ and $M-OD_3^+$.[16] The fractionation factors for most metal–water complexes range from 0.7 to 1.0. For example, the fractionation factors for an inner-shell metal-ligated water of Co(II) carbonic anhydrase is 0.72–0.77 at pH 8.5 and 0.8–0.9 at low pH.[16] Likewise, for the hydrolysis of inorganic pyrophosphate catalyzed by magnesium ion and inorganic pyrophosphatase, the fractionation factors for the water protons have been found to be between 0.7 and 1.0.[23] Effects as large as 0.43–0.47 have been measured on the $V/K_{alcohol}$ for yeast alcohol dehydrogenase-catalyzed oxidation of *n*-butanol and attributed to displacement of a Zn-bound water upon substrate binding.[24] Since the malic enzyme reaction requires a divalent metal ion activator, the involvement of a metal-chelated water is possible. However, the inverse isotope effects observed in the NAD-malic enzyme reaction are greater than what is normally observed for the dissociation of a metal-chelated water.

None of the above provide a satisfactory explanation for the observed inverse solvent isotope effects in the NAD-malic enzyme reaction. The only satisfactory explanation left for the observed inverse solvent isotope effects consistent with the data is that a change in solvent viscosity in D_2O is the cause of the effects. The solvent isotope effects are depicted in Table 1 and are compared to the effects caused by the presence of glycerol as viscosogen at a relative viscosity identical to that in D_2O. The changes in the kinetic parameters at increased viscosity are identical in nearly all cases to those observed in D_2O, strongly suggesting they are the consequence of a change in the same physical parameter, namely, solvent viscosity.

Fumarate is an allosteric activator of the NAD-malic enzyme reaction, and the mechanism of activation has been shown to be the result of a decrease in the off-rate for malate dissociation from enzyme.[25] Fumarate binding to enzyme decreases the magnitude of the primary deuterium isotope effect on V/K by increasing the forward commitment to catalysis. If the inverse solvent isotope effect is due to an effect on a rate constant for a step present downstream from malate binding, such as in the transition state for hydride transfer, the presence of fumarate should result in a less inverse isotope effect. The presence of fumarate does not cause a change in $^{D_2O}(V/K_{malate})$. However, fumarate still activates the reaction in D_2O and also in the presence of glycerol (data not shown). The activation by fumarate in D_2O and in the presence of glycerol is consistent with the inverse solvent isotope effects being due to a change in solvent viscosity. The data further suggest that the activating effect of increased solvent viscosity may be the result of a difference in enzyme conformation in solvents of higher viscosity. The possibility of a viscosity-induced enzyme conformational change, including viscosity changes induced by D_2O, that could effect enzymatic reaction rates has been recognized previously.[26] The location of the conformation change in the present case is as yet unknown, but it may well represent a closing of the active site known to occur with many enzyme-catalyzed reactions to eliminate water and allow the reaction to occur; that is, it would represent an increase in the forward commitment factor for reactant. The change in kinetic parameters is likely similar to the increase in the microscopic rate constant associated with a precatalytic conformational change, as suggested by Grissom and Cleland[27] to explain similar viscosity effects in the reaction catalyzed by the NADP-malic enzyme from chicken liver.

The forward and reverse commitment factors are equal to zero when APAD is the dinucleotide substrate and Mn^{2+} is the divalent metal ion activator;[5] consequently, any normal isotope effect would most likely be expressed on V/K and V under these conditions. If the viscosity effects on the kinetic parameters are equal in D_2O and in the presence of glycerol, as they are in nearly all other cases presented here, then the less inverse values of about 0.2 in D_2O obtained with Mn^{2+} and APAD compared to the corresponding viscosity values could be the result of a small normal solvent isotope effect. When the viscosity effect is divided out from the solvent effect, the calculated values for the isotope effects are $^{D_2O}(V/K) = 1.33 \pm 0.06$ and $^{D_2O}V = 1.27 \pm 0.07$, and, in the presence of fumarate, $^{D_2O}(V/K) = 1.19 \pm 0.06$ and $^{D_2O}V = 1.26 \pm 0.05$. The apparently equal normal effects on V/K and V in the absence of fumarate are consistent with the commitment factors being equal to zero. The values of $^{D_2O}(V/K)$ decrease from 1.33 in the absence of fumarate to 1.19 in the presence of fumarate. This change reflects the expected trend in these values if there is a small normal isotope effect expressed with Mn^{2+} and APAD and fumarate increases the forward commitment factor. The results are therefore consistent with a small normal solvent isotope effect expressed with Mn^{2+} and APAD.

It cannot be assumed that a viscosity effect will be expressed equally in the presence of all viscosogens. For example, with sucrose as viscosogen at a comparable viscosity ($\eta_{rel} = 1.24$), the change in the kinetic parameters is less than that seen with glycerol. Similar results have been reported for the NADP-malic enzyme from chicken liver.[27] In addition, the macroviscosogen ficoll elicits no change in the kinetic parameters for the NAD-malic enzyme. Results similar to these, in which different viscosogens do not elicit equal changes in enzymic kinetic parameters, have been observed previously.[28,29] Among the possible explanations for this observation is that the Stokes–Einstein relation derived to describe the motion of molecules assumes that the particles in motion are much larger than the solvent, and in cases where the particle is of the same size or smaller than the viscosogen, deviations from the Stokes–Einstein relation are observed.[28] A second possibility for the observation of unequal viscosity effects has been attributed to the fact that the mobility of small ions and neutral molecules is only moderately impaired by the solution viscosity increase and is mostly dependent on the volume fraction of the cosolute.[28,29] The viscosity effects in the NAD-malic enzyme reaction are unlikely to result from effects on the diffusion of substrate molecules to the active site since the viscosity effects are in the opposite direction predicted by theory for viscosity effects, and, given the significant isotopes effects observed in the enzymic reaction,[2,3] there is likely to be little rate limitation in the malic enzyme reaction from diffusion. Therefore, the viscosity effects observed in the malic enzyme reaction are most probably the result of changes in enzyme conformation or effects on the rates of enzyme conformational changes that are associated with the catalytic process. Both of these possibilities couild involve very small enzyme changes or motions.

Implications for Solvent Isotope Effect Studies. Theory suggests that a change in solvent viscosity should manifest its effects on enzyme reaction rates by affecting diffusion-sensitive steps such as those involved in substrate binding or product

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release. If an enzyme reaction is limited or at least partially limited by diffusion, then an increase in solvent viscosity should slow down diffusion rates and result in a decrease in enzyme reaction rates. This will lead to a "normal" viscosity effect and values for ratios of kinetic parameters of >1 when expressed as $(V/K)_{\eta=1}/(V/K)_{\eta>1}$ or $V_{\eta=1}/V_{\eta>1}$. The viscosity effects reported here for malic enzyme could be considered "inverse" viscosity effects. In cases where the enzymic reaction is limited by chemical steps or other diffusion-insensitive steps, the reaction rates should be unaffected by a change in solvent viscosity.

The results reported here for NAD-malic enzyme have implications for the use of solvent isotope effects to study enzyme mechanisms. In cases of enzyme catalysis where the isotopically sensitive step is entirely rate limiting, the measured isotope effect on *V/K* or *V* will represent the intrinsic isotope effect value. If, for example, there is an inverse viscosity effect on *V/K* or *V* arising from the more viscous D_2O, which is also present in addition to the solvent isotope effect, the observed value of the isotope effect will be diminished by the viscosity effect. It may also be possible for a change in viscosity to express a normal effect that is not the result of an effect on diffusional processes, for instance, an effect on enzyme conformation. In a case such as this, an overestimation of the value of the isotope effect would occur.

In cases where diffusion partially limits an enzymic reaction, the contribution to the value of the observed isotope effect could be from both a classical isotope effect and the increased viscosity in D_2O affecting diffusional processes. In addition, detection of a solvent isotope effect could be masked by a significant inverse viscosity effect. Finally, an enzymic reaction that is entirely diffusion limited could be expected to display a significant normal viscosity effect. The greater relative viscosity of D_2O compared to that of H_2O could be expected to elicit changes in *V/K* or *V* which could be interpreted as arising from a significant classical solvent isotope effect.

The foregoing discussion suggests that as a precaution it would seem prudent to always perform a viscosity control when solvent isotope effect studies are to be conducted. In the NAD-malic enzyme reaction and others,[27-29] the magnitude of the viscosity effect is different depending on the identity of the viscosogen, with a larger effect observed with glycerol compared to the larger viscosogens sucrose or ficoll. This result implies that any viscosity control should employ more than one viscosogen. In most cases, the best choice for viscosogen will likely be glycerol due to its small size, but since the magnitude of the viscosity effect can vary with viscosogen, there is no guarantee that any viscosity effect expressed in the presence of glycerol will be expressed to the same extent or even at all in D_2O. Therefore, it may not be safe to assume that any observed viscosity effect can simply be subtracted from the measured solvent isotope effect to give the true isotope effect. As suggested by the data presented here for malic enzyme, effects on enzyme activity resulting from changes in solvent viscosity may contribute to the difficulties in interpretation of solvent isotope effects. However, for many enzymes, the viscosity effects on enzyme activity may be minimal or nonexistent, in which case any observed solvent isotope effect could be interpreted disregarding a viscosity component.

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