# Isotopic Analysis of the Reaction Catalyzed by Glycerol Dehydrogenase†

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ABSTRACT: Glycerol dehydrogenase catalyzes the reversible $NAD^+$-dependent oxidation of glycerol to form dihydroxyacetone. Initial velocity, product, and dead-end inhibition studies performed for the forward and reverse reactions support an ordered kinetic mechanism with $NAD^+$ binding first and NADH released last. A monovalent cation is required for enzymatic activity and glycerol binding, with $K^+$ having the highest activity as measured by $V$. The pH dependence of the kinetic parameters $V$ and $V/K_{glycerol}$, as well as the temperature dependence of the $V$ pH profile, suggested that an enzymic carboxylate group functions as a base in catalysis. The pH dependence of the primary deuterium kinetic isotope effect shows that $^DV/K_{glycerol}$ increases from a pH-independent value of 1.15 at high pH values to a pH-independent value of 2.44 at low pH values. $^DV$ exhibits a similar pH dependence, increasing from a pH-independent value of 2.57 at high pH values to a pH independent value of 4.88 at low pH values. A chemical mechanism for enzymatic glycerol oxidation is proposed based on the data.

Glycerol dehydrogenase (EC 1.1.1.6) catalyzes the $NAD^+$-linked[1] oxidation of glycerol to dihydroxyacetone and provides microorganisms an effective, ATP-independent, metabolic pathway for glycerol utilization. This enzyme has been isolated from a variety of bacterial sources including *Escherichia coli* (Asnis & Brodie, 1953), *Aerobacter aerogenes* (Burton & Kaplan, 1953; Rush et al., 1957), *Klebsiella aerogenes* (Lin & Magasanik, 1960), *Bacillus megaterium* (Scharschmidt *et al.*, 1984), and *Bacillus stearothermophilus* (Spencer *et al.*, 1989). The enzyme from *Cellulomonas* sp. NT3060 has been purified and extensively characterized (Yamada et al., 1982a,b; Nishise et al., 1984).

All glycerol dehydrogenases characterized to date exhibit an absolute requirement for a monovalent cation, with potassium having the highest activity. This unique property of glycerol dehydrogenases encouraged us to investigate the pH dependence of the kinetic parameters and primary deuterium kinetic isotope effects with 2-deuterated glycerol for comparison with other secondary alcohol dehydrogenases. These results are readily analyzed using previously developed theories of the pH dependence of kinetic isotope effects that have been used to describe the detailed chemical mechanisms of other secondary alcohol dehydrogenases, including malic enzyme (Cook & Cleland, 1981b) and both yeast and liver alcohol dehydrogenases (Cook & Cleland, 1981b,c). We propose a chemical mechanism of glycerol oxidation by glycerol dehydrogenase, which involves initial base-assisted proton abstraction from the C2 hydroxyl group and subsequent collapse of the alkoxide with hydride transfer to $NAD^+$.

## MATERIALS AND METHODS

*Materials.* Pyruvate kinase from rabbit muscle was purchased from Boehringer Mannheim. Glycerol dehydrogenase, glucose-6-phosphate dehydrogenase from *Leuconostoc mesenteroides* (Type XXIV), glycerokinase, NADH, ATP, glucose 6-phosphate, phosphoenolpyruvate, dihydroxyacetone, $tNAD^+$, $3\text{-}PAAD^+$, $3\text{-}APAD^+$, and all buffers were purchased from Sigma. $D_2O$ (>99.8 atom % excess) was from Cambridge Isotope Laboratories and was distilled before use. All glycerol analogs were from Aldrich except *R*- and *S*-1-amino-2-propanol, which were from Fluka. Monovalent cations were purchased as their chloride salts from Fisher.

[2-$^2$H]Glycerol was synthesized in a 20-mL solution containing 400 mM dihydroxyacetone titrated to pH 8.0 with NaOH, by the addition of sodium borodeuteride (0.42 g) at 0 °C. After constant stirring for 60 min, the reaction was quenched by the slow addition of 2 M HCl. The mixture was taken to dryness, redissolved in anhydrous methanol, and then rotary evaporated after methanol addition three times. The residue was dissolved in $H_2O$ and then applied onto a mixed bed resin of AG-1(Cl-form) and AG-50 (H+form). The [2-$^2$H]glycerol was eluted with $H_2O$, concentrated, redissolved in $H_2O$, and filtered. [2-$^2$H]Glycerol concentrations were determined by endpoint assays as described below.

*Enzyme Preparation.* Glycerol dehydrogenase from *Cellulomonas* sp. (Sigma) was not homogeneous as commercially supplied and was further purified by applying 1 mL of a solution containing 20 mg of enzyme to a Pharmacia Mono Q HR 5/5 anion exchange column equilibrated in 10 mM TEA-HCl, pH 7.8. Glycerol dehydrogenase was eluted from the column using a 200-mL linear salt gradient (0.0−1.0 M KCl) in 10 mM TEA-HCl, pH 7.8. The enzyme eluted at approximately 0.4 M KCl, and the active fractions were pooled and concentrated to 1 mL via ultrafiltration (Amicon PM10). The concentrated protein solution was then applied

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[1] Abbreviations: $NAD^+$, β-nicotinamide adenine dinucleotide; NADH, reduced β-nicotinamide adenine dinucleotide; MgATP, magnesium adenosine triphosphate; FPLC, fast protein liquid chromatography; TEA, triethanolamine, TAPS, 3-[{tris(hydroxymethyl)methyl}amino]propanesulfonic acid; BES, *N,N*-bis(2-hydroxyethyl)-2-aminoethanesulfonic acid; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; MES, 2-(*N*-morpholino)ethanesulfonic acid; PIPES, 2,2′-piperazine-1,4-diylbis(ethanesulfonic acid); CHES, 2-(cyclohexylamino)ethanesulfonic acid; CAPS, 3-(cyclohexylamino)propanesulfonic acid.

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to two Superose 12 columns (Pharmacia) connnected in series and equilibrated in 10 mM potassium HEPES buffer containing 50 mM KCl, pH 7.8. Glycerol dehydrogenase eluted as a symmetric peak at an apparent native molecular mass of 324 000 daltons. Active fractions were pooled and concentrated by ultrafiltration. The enzyme appeared homogeneous on SDS−PAGE after staining with Coomassie Blue G-250, exhibiting a monomer molecular mass of 39 500 daltons.

*Steady-State Kinetics.* Glycerol dehydrogenase reaction rates were determined by measuring the appearance or disappearance of NADH spectrophotometrically at 340 nm ($\epsilon$ = 6220 $M^{-1}$ $cm^{-1}$) in a Gilford 260 spectrophotometer equipped with thermospacers attached to a constant temperature circulating water bath maintained at the desired temperature, generally 25 °C. Assays were carried out in 1-cm pathlength quartz cuvettes in a total volume of 3 mL. The reactions were initiated by the addition of a small amount of enzyme (<20 $\mu$L). Initial velocities were determined in the forward direction by varying the concentration of glycerol at several fixed concentrations of $NAD^+$ in 100 mM TAPS, pH 8.8. In the reverse reaction, initial velocities were determined by varying the concentration of dihydroxyacetone at several fixed concentrations of NADH in 100 mM BES, pH 7.0. In both cases, the buffers were adjusted to the desired pH with KOH. Product and dead-end inhibition studies were performed at several fixed concentrations of the product or dead-end inhibitor and at variable substrate concentrations and saturating concentrations of the other substrates.

Solutions (1 mM−1 M) of glycerol analogs were prepared volumetrically in water and titrated to pH 8.8 with HCl or KOH. The maximum velocity and steady-state $K_m$ values of the oxidizable substrates were determined by monitoring the rate of formation of NADH at 340 nm, under saturating concentrations of $NAD^+$ (1.4 mM) and potassium (100 mM). Solutions of $NAD^+$, NADH, and DHA were calibrated by enzymatic spectrophotometric end-point assays using *L. mesenteroides* glucose-6-phosphate dehydrogenase and excess glucose 6-phosphate, *Streptococcus faecalis* NADH peroxidase and excess $H_2O_2$, and glycerol dehydrogenase and excess NADH, respectively. Glycerol solutions were calibrated by enzymatic spectrophotometric end-point assays using glycerokinase, pyruvate kinase, and lactate dehydrogenase and excess phosphoenolpyruvate, ATP, and NADH.

*pH Studies.* pH studies were performed as described by Wong and Blanchard (1989). Buffers were prepared by titrating their acid forms, dissolved in double-distilled water, to the desired pH value with KOH and were filtered through a Millipore 0.22-$\mu$m filter. Buffers were used at a final concentration of 100 mM at the stated pH values to allow for overlap: acetic (5.2−5.5), MES (5.5−6.4), PIPES (6.2−7.2), HEPES (7.2−8.2), TAPS (8.1−9.0), CHES (8.8−9.9), and CAPS (9.9−10.7).

The kinetic parameters $V$ and $V/K$ for glycerol were determined at each pH value by varying the concentration of glycerol between 1.0 and 50 mM at saturating concentrations of $K^+$ (100 mM) and $NAD^+$ (1.4 mM). The log values determined from fits to eq 1 were plotted against pH, determined by insertion of a microelectrode into the reaction cuvette after the initial velocity assays were performed.

*Primary Deuterium and Solvent Kinetic Isotope Effect Studies.* The pH dependence of the primary deuterium kinetic isotope effect on $V$ and $V/K_{glycerol}$ was determined by comparing the initial rates for the oxidation of [2-$^1$H]glycerol and [2-$^2$H]glycerol at saturating concentrations of $K^+$ (100 mM) and $NAD^+$ (1.4 mM) at various pH values.

*Data Analysis.* Reciprocal initial rates were plotted against reciprocal substrate concentrations, and the data were fitted to the appropriate equations using the Fortran programs of Cleland (1979). Initial velocities obtained by varying one substrate in the presence of a fixed, saturating level of the other substrates were fitted to eq 1. Initial velocities obtained

$$v = VA/(K + A) \quad (1)$$

$$v = VAB/(K_aB + K_bA + AB + K_{ia}K_b) \quad (2)$$

$$v = VA/[K(1 + I/K_i) + A] \quad (3)$$

$$v = VA/[K + A(1 + I/K_i)] \quad (4)$$

$$v = VA/[K(1 + I/K_{is}) + A(1 + I/K_{ii})] \quad (5)$$

by varying one substrate in the presence of several fixed levels of a second substrate were fitted to eq 2. Inhibition data were fitted to eqs 3−5, which describe competitive, uncompetitive, and noncompetitive inhibition, respectively.

Data for pH profiles in which the log of the parameter plotted decreased below p$K_1$ with a slope of +1 were fitted to eq 6, while data for pH profiles in which the log of the parameter plotted decreased below p$K_1$ with a slope of +1, and below p$K_2$ with a slope of +2, were fitted to eq 7, assuming independent p$K$ values. In eqs 6 and 7, $Y$ is the

$$\log Y = \log[C/1 + H/K_1)] \quad (6)$$

$$\log Y = \log[C/(1 + H/K_1 + H^2/K_1K_2)] \quad (7)$$

parameter being fitted and $C$ is the pH-independent plateau value.

Primary deuterium kinetic isotope effect data were fitted to eq 8 which assumes different isotope effects on $V$ and

$$v = VA/[K(1.0 + F_iE_{V/K}) + A(1.0 + F_iE_V)] \quad (8)$$

$$\log Y = \log[Y_L + Y_H(K/H^+)/(1.0 + K/H^+)] \quad (9)$$

$V/K$. $F_i$ is the fraction of deuterium label ($F_i$ = 0.0 and 1.0 for hydrogen- and deuterium-containing glycerol), $E_{V/K}$ is the isotope effect minus one on $V/K$, and $E_V$ is the isotope effect minus one on $V$. The pH dependence of the primary deuterium kinetic isotope effects were fitted to eq 9 where $Y$ is the parameter whose pH dependence is being determined and $Y_L$ and $Y_H$ are the values of $Y$ at low and high pH values, respectively.

Solvent kinetic isotope effect data were individually fitted to eq 1 and the extrapolated $V$ and $V/K$ values were plotted against mole fraction $D_2O$. A linear regression analysis of these data provided the solvent kinetic isotope effects on $V$ and $V/K$.

## RESULTS

*Enzyme Preparation.* Glycerol dehydrogenase prepared as described under Materials and Methods appears homogeneous as determined by SDS−PAGE. It has a subunit molecular mass of 39 500 daltons as determined by sedi-

Table 1: Kinetic Parameters Measured for Glycerol Analogs with Glycerol Dehydrogenase[a]

| compound $CH_2(R_1)$–$CH(R_2)$–$CH_2(R_3)$ | $R_1$ | $R_2$ | $R_3$ | $K_m$ (mM) | rel (%) $V_{max}$ | rel $V/K$ |
|---|---|---|---|---|---|---|
| glycerol | OH | OH | OH | 5.1 ± 0.14 | 100 | 19.6 |
| 3-mercapto-1,2-propanediol | OH | OH | SH | 4.0 ± 0.4 | 155 ± 14 | 38.4 |
| 3-chloro-1,2-propanediol | OH | OH | Cl | 6.0 ± 0.4 | 130 ± 7 | 21.7 |
| 3-bromo-1,2-propanediol | OH | OH | Br | 6.1 ± 0.4 | 109 ± 3 | 17.9 |
| 3-amino-1,2-propanediol | OH | OH | $NH_2$ | 180 ± 20 | 104 ± 11 | 0.6 |
| 1,2-propanediol | OH | OH | H | 0.06 ± 0.01 | 105 ± 6 | 1750 |
| *R*-1-amino-2-propanol | $NH_2$ | $OH_{(R)}$ | H | 4.4 ± 0.1 | 33 ± 1 | 7.5 |
| *S*-1-amino-2-propanol | $NH_2$ | $OH_{(S)}$ | H | 500 ± 30 | 9 ± 1 | 0.002 |

[a] All data were obtained in 100 mM potassium TAPS buffer, pH 8.8, containing 1 mM $NAD^+$ at 25 °C. Errors in the determined parameters were less than 10% in all cases. No significant oxidation of the following compounds was observed at 500 mM: 2-propanol, 1-mercapto-2-propanol, 1-chloro-2-propanol, 1,3-dichloro-2-propanol, and 2-amino-1,3-propanediol.

mentation equilibrium in 8 M guanidine-HCl (data not shown) as well as by SDS–PAGE. Gel filtration indicates that the native protein has a molecular mass of 324 000 daltons, suggesting that it is an octamer of identical subunits.

*Kinetic Mechanism.* When $NAD^+$ is varied at several fixed levels of glycerol, the double-reciprocal plot consists of a family of lines intersecting to the left of the ordinate, indicating that the two substrates bind to the enzyme in a sequential manner (data not shown). An intersecting pattern is also observed for the reverse reaction when NADH is varied at fixed levels of dihydroxyacetone.[2] To confirm these initial velocity results, we performed product and dead end inhibition studies (data not shown). In the direction of glycerol oxidation, NADH is a competitive inhibitor versus $NAD^+$ and a noncompetitive inhibitor versus glycerol. The dead-end inhibitor, 1,3-propanediol, is a competitive inhibitor versus glycerol and an uncompetitive inhibitor versus $NAD^+$. In the reverse direction, 1,3-propanediol is a competitive inhibitor versus dihydroxyacetone and an uncompetitive inhibitor versus NADH. The kinetic mechanism for glycerol dehydrogenase-catalyzed oxidation of glycerol is thus steady-state ordered.

*Substrate Specificity.* The kinetic parameters of seven glycerol analogs for glycerol dehydrogenase were determined at saturating concentrations of $NAD^+$ and are shown in Table 1. Relative maximum velocities and $V/K$ values were determined by comparing the maximum velocities and $V/K$ value for each analog to that of glycerol, determined at the same time under identical experimental conditions. 2-Propanol, 1-mercapto-2-propanol, 1-chloro-2-propanol, and 1,3-dichloro-2-propanol were tested as substrates for glycerol dehydrogenase at 500 mM concentrations of these compounds, but no detectable activity was observed.

*pH Profiles.* The pH dependence of the kinetic parameters were determined by varying the concentration of one of the substrates and using a saturating concentration of the other substrates and activating metal. All experiments were performed using $K^+$ as the required monovalent cation. $V/K_{K+}$ is pH-independent at high pH values but decreases as two groups exhibiting p$K$ values of 7.90 ± 0.07 and 6.19 ± 0.07 are protonated (data not shown). $V/K_{NAD+}$ and $V/K_{glycerol}$ (Figure 1) are also pH-independent at high pH values but decrease as single groups exhibiting p$K$ values of 7.19 ± 0.28 and 7.84 ± 0.16, respectively, are protonated. When the pH dependence of the maximal velocity is determined (Figure 1), a similar pH dependence is observed, with $V$ decreasing as the result of the protonation of a single group exhibiting a p$K$ value of 6.67 ± 0.18. In an attempt to identify the chemical nature of this group, the pH dependence of $V$ was determined at 18, 23, 29, and 33 °C. As seen in Figure 2, the p$K$ value determined at these temperatures did not vary significantly with temperature over this range and yielded a calculated enthalpy of ionization value of 1.6 ± 4.1 kcal/mol. Replotting the pH-independent value of log $V$ versus the reciprocal of the absolute temperature allows the calculation of a value of 15.0 ± 0.9 kcal/mol for the energy of activation.

*Primary Deuterium Kinetic Isotope Effects.* The pH dependence of the primary deuterium kinetic isotope effect on $V/K_{glycerol}$ and $V$ were determined by comparing the rate of oxidation of [2-$^1$H]- and [2-$^2$H]glycerol at concentrations of $K^+$ and $NAD^+$ shown to be saturating at pH values between 5.6 and 9.9. As shown in Figure 3, $^DV/K_{glycerol}$ exhibits its maximal value of 2.44 ± 0.04 at low pH values and decreases to a value of 1.15 ± 0.04 at high pH values as a group exhibiting a p$K$ value of 7.31 ± 0.25 is titrated. Similarly, the primary deuterium kinetic isotope effect on $V$ is maximal at low pH values, exhibiting a value of 4.88 ± 0.04, and decreases to a value of 2.57 ± 0.15 as a group exhibiting a similar p$K$ value of 7.29 ± 0.56 is titrated. The similarity of the p$K$ value of the group observed in the $V/K_{glycerol}$ (p$K$ = 7.8), $V$ (p$K$ = 6.7), $^D(V/K)_{glycerol}$ (p$K$ = 7.3), and $^DV$ (p$K$ = 7.3) pH profiles suggests that all pH-dependent behavior is influenced by the protonation state of the base.

## DISCUSSION

Glycerol dehydrogenase is unique among pyridine nucleotide-dependent dehydrogenases oxidizing secondary alcohols in requiring a monovalent cation for activity. The enzymes from *E. coli* (Tang et al., 1979), *A. aerogenes* (Lin & Magasnik, 1960; McGregor et al., 1974), and *Cellulomonas* (Yamada et al., 1982b) all have been shown to require monovalent cations for maximal activity with potassium showing the highest activity. In contrast, other secondary alcohol dehydrogenases either have no metal ion dependence (malate and lactate dehydrogenases) or use a tightly bound zinc atom (yeast and liver alcohol dehydrogenases). Because of the unusual metal ion dependence of this enzyme, we have

[2] The steady-state $K_m$ values for substrates in the forward direction at pH 8.8 are $K^+$ 0.38 ± 0.02 mM; $NAD^+$, 0.16 ± 0.05 mM; glycerol, 1.5 ± 0.5 mM. The steady-state $K_m$ values for substrates in the reverse direction at pH 7.0 are NADH, 0.034 ± 0.008 mM; dihydroxyacetone, 0.32 ± 0.06 mM.

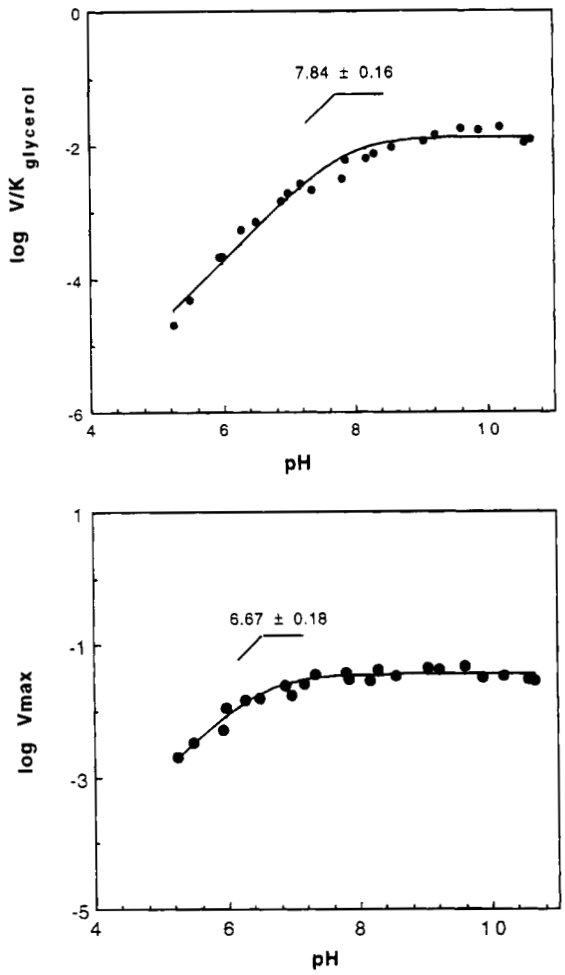



FIGURE 1: Effect of pH on the kinetic parameters $V$ and $V/K_{glycerol}$. The experimental points are fits of the data to eq 1, and the smooth curves for the $V$ and $V/K_{glycerol}$ pH profiles are fits of the data to eq 6. (Upper panel) The $V/K_{glycerol}$ pH profile decreases as a single group exhibiting a p$K$ value of 7.84 ± 0.16 is protonated. (Bottom panel) The $V$ pH profile decreases as a single group exhibiting a p$K$ value of 6.67 ± 0.18 is protonated.

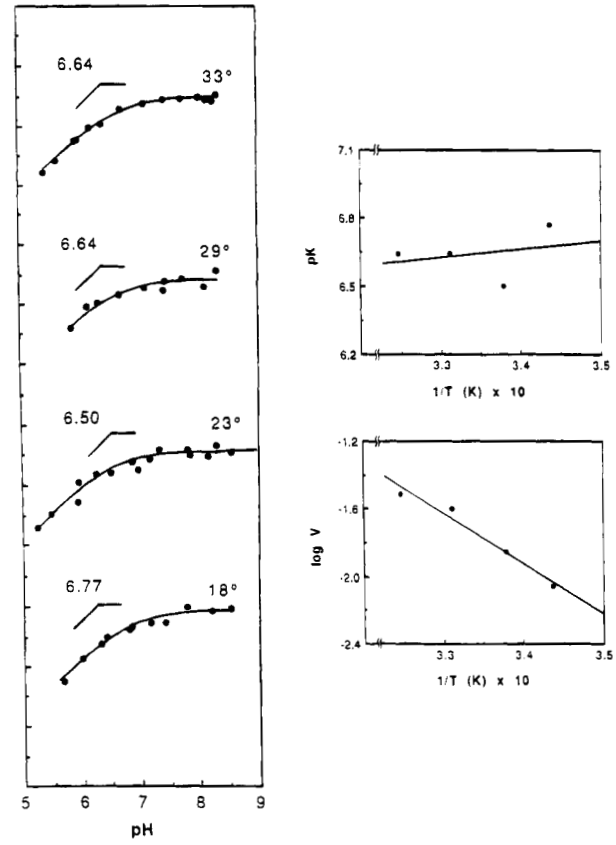



FIGURE 2: Effect of temperature on the kinetic parameter $V$ determined with glycerol dehydrogenase. (Left panel) The experimental points are fits of the data to eq 1, and the smooth curves for the $V$ pH profiles are fits of the data to eq 6. The $V$ pH profiles decrease as single groups exhibiting p$K$ values of 6.77 ± 0.14, 6.50 ± 0.14, 6.64 ± 0.21, and 6.64 ± 0.07 at 18, 23, 29, and 33 °C, respectively, are protonated. (Upper right panel) A plot of reciprocal absolute temperature against p$K$ yields a line whose slope equals $\Delta H_{ion}/2.303R$. The calculated enthalpy of ionization for this group is 1.6 ± 4.1 kcal/mol. (Lower right panel) A plot of reciprocal absolute temperature against log $V$ yields a line whose slope equals $E_{act}/2.303R$. The calculated energy of activation is 15.0 ± 0.9 kcal/mol.

further characterized this enzyme and compared it to the yeast and liver alcohol dehydrogenases.

*Kinetic Mechanism.* Most pyridine nucleotide linked dehydrogenases catalyze their respective reactions via sequential mechanisms. In our initial velocity studies with glycerol dehydrogenase, intersecting Lineweaver–Burk plots were obtained in both the forward and reverse directions, suggestive of a sequential kinetic sequence. Product and dead-end inhibition studies supported a kinetic mechanism in which $NAD^+$ binds prior to glycerol binding, and NADH is released following dihydroxyacetone release. This steady-state ordered mechanism is consistent with the kinetic mechanism proposed by Nishise et al. (1984) for the *Cellulomonas* sp. NT3060 glycerol dehydrogenase. All subsequent studies were performed using a saturating concentration of potassium as monvalent cation activator.

*Carbon Substrate Specificity.* Glycerol dehydrogenase from *Cellulomonas* resembles the enzymes from *E. coli* (Asnis & Brodie, 1953) and *A. aerogenes* (Lin & Magasanik, 1960) in its relatively broad carbon substrate specificity. As shown in Table 1, the maximal velocities exhibited by glycerol analogs are similar to that of glycerol. Interestingly, glycerol dehydrogenase exhibits the largest relative $V/K$ value for 1,2-propanediol, a result previously observed for the glycerol dehydrogenase from *E. coli* (Tang et al., 1979). These data suggest that the enzyme poorly accommodates large functional groups such as -OH, -SH, -Cl, and -Br at the three position. The binding of 3-amino-1,2-propanediol is even poorer with a relative $V/K$ value that is over 60-fold lower than that of glycerol.

All of the glycerol analogs mentioned above as substrates are chiral at the $C_2$ position, but the kinetic parameters were determined for the racemic mixtures. To determine the chiral selectivity of glycerol dehydrogenase, the kinetic constants for the $R$ and $S$ forms of 1-amino-2-propanol were determined individually. The relative $V/K$ value for the $R$ form is over 4000 times higher than that of the $S$ form, and the maximal velocity is three times greater, suggesting that the $R$ form is preferentially bound and oxidized by the enzyme.

*pH Dependence of the Kinetic Parameters.* Any complete analysis of the pH dependence of primary kinetic isotope effects relies on the prior determination of the pH dependence of the kinetic parameters, $V/K$ for substrates and activators, and $V$. We have determined the pH dependence of the kinetic parameters for all substrates and $K^+$ but will restrict this discussion only to $V/K$ glycerol and $V$, since these are relevant to the isotope effect analysis.

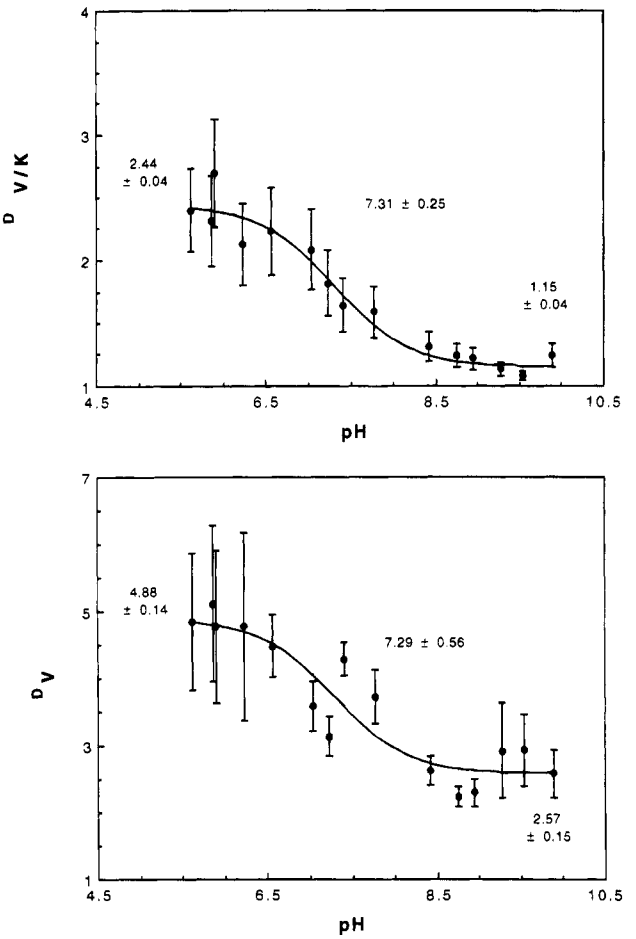



FIGURE 3: Effect of pH on the primary deuterium kinetic isotope efffects on $V$ and $V/K_{glycerol}$. Individual points are the fits of the data to eq 8, and the smooth curves through the data are fits of the data to eq 9. (Top panel) The magnitude of $^{D}V/K$ is constant at high pH values and is equal to 1.15 ± 0.04 but increases at low pH values to a value of 2.44 ± 0.04 as a group exhibiting a p$K$ value of 7.31 ± 0.25 is protonated. (Bottom panel) The magnitude of $^{D}V$ is constant at high pH values and is equal to 2.57 ± 0.15 but increases at low pH values to a value of 4.88 ± 0.14 as a group exhibiting a p$K$ value of 7.29 ± 0.56 is protonated.

The pH dependence of the $V/K$ value for glycerol demonstrates that the protonation of a group exhibiting a p$K$ value of 7.84 abolishes binding of glycerol. Since glycerol has no titratable groups in this region, this pH behavior represents the ionization behavior of an enzymic group in the E·K$^+$·NAD$^+$ complex. This p$K$ value cannot be assumed to be a thermodynamic p$K$ but rather an apparent p$K$ whose value is influenced by the ratio of rates of steps occurring after substrate binding, including chemistry, and substrate dissociation.

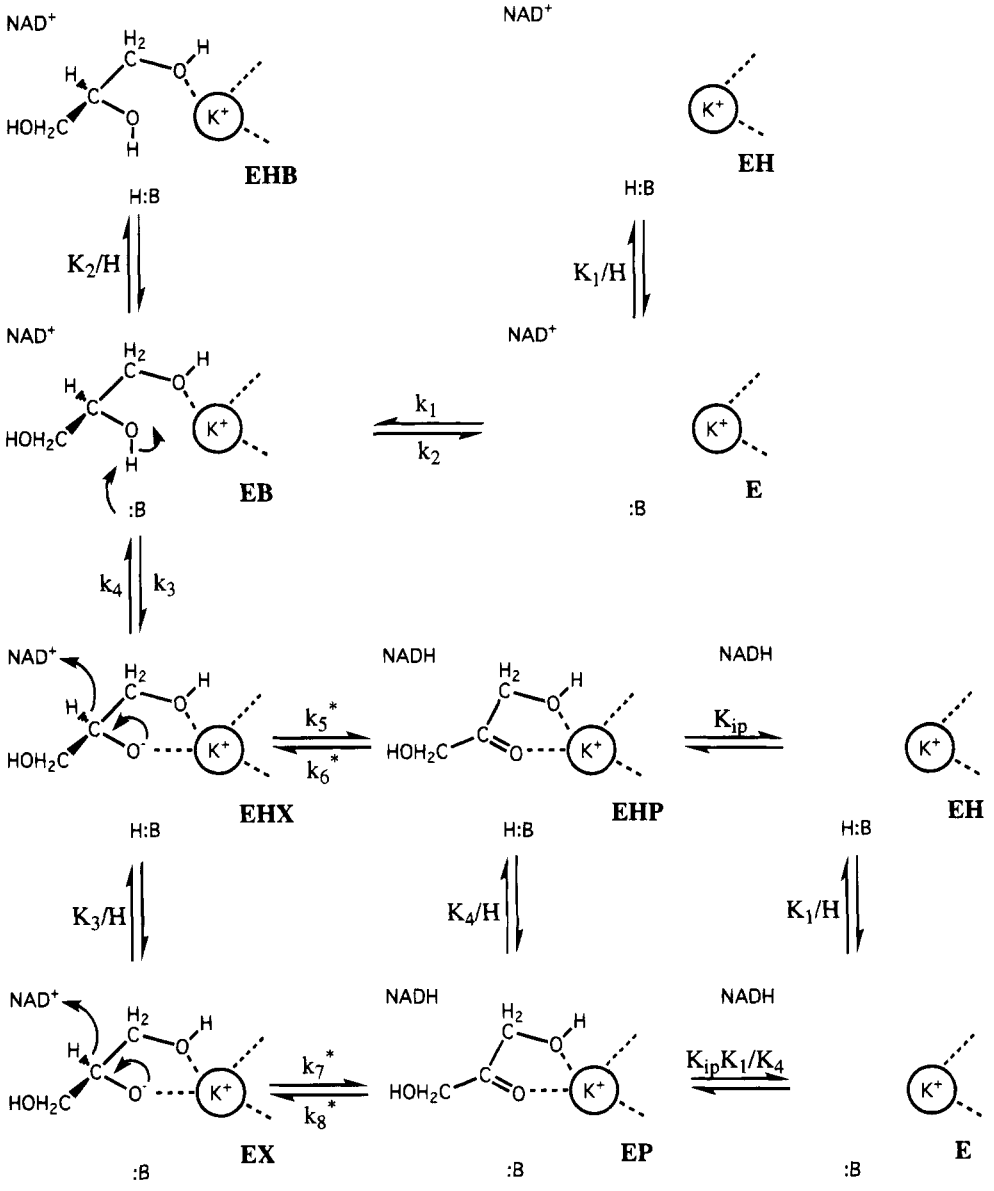
The pH dependence of the maximum velocity is dependent on the ionization behavior of enzymic groups which assist or perform catalysis. In the present case, the protonation of a group exhibiting a p$K$ value of 6.67 causes the velocity to decrease. This p$K$ value is 1 pH unit lower than observed in the $V/K$ glycerol profile, possibly due to the interaction of this group with glycerol, which would have the effect of making it more difficult to protonate. The chemical identity of this base has been investigated by analyzing the temperature dependence of the p$K$ value. The low value of the enthalpy of ionization supports, but does not prove, a conclusion that this base is the carboxyl side chain of a glutamate or aspartate residue which hydrogen bonds to the C2 hydroxyl group of glycerol.

*pH Dependence of the Kinetic Isotope Effects.* The pH dependence of kinetic isotope effects has been measured for several pyridine nucleotide-linked dehydrogenases, including formate dehydrogenase (Blanchard & Cleland, 1980), alanine dehydrogenase (Grimshaw et al., 1981), and both yeast and horse liver alcohol dehydrogenase (Cook & Cleland, 1981c). The data obtained with glycerol dehydrogenase and presented in Figure 3 conform to a model (Cook & Cleland, 1981c) in which a pH-dependent step, base-assisted proton abstraction, precedes the isotope-sensitive step in the direction of glycerol oxidation. These results suggest that glycerol exhibits a high commitment to catalysis, and the observed $V/K$ isotope effect at high pH values is reduced from the value for the intrinsic isotope effect on the hydride transfer step. As the pH is lowered, both the maximal velocity for glycerol oxidation and $V/K_{glycerol}$ decrease, and the isotope effect on $V/K$ increases. This behavior requires that the commitment factor for glycerol decrease with decreasing pH, as has been observed with alanine dehydrogenase and both alcohol dehydrogenases (Grimshaw et al., 1981; Cook & Cleland, 1981c). $^{D}V/K_{glycerol}$ only attains a value of 2.44 at the low pH asymptote, which cannot be the intrinsic isotope effect based on the measured value of $^{D}V$ at these same pH values (see below). Thus, even at low pH values, glycerol retains a finite value of the commitment factor. Theory predicts that the $V$ and $V/K$ isotope effects should both approach the intrinsic isotope effect on hydride transfer (Cook & Cleland, 1981b) when all external and internal commitments approach zero, which is clearly not the case here.

The primary deuterium kinetic isotope effect on the maximum velocity of glycerol oxidation catalyzed by glycerol dehydrogenase exhibits a pH dependence similar to that observed on $^{D}V/K_{glycerol}$; however, the asymptotic values at high and low pH values are different. The increasing magnitude of $^{D}V$ with decreasing pH is similarly the result of the pH-dependent change in the commitment factor, $c_{Vf}$, which determines the extent to which the intrinsic isotope effect on the hydride transfer step is observed in these steady-state experiments.

*Model and Evaluation of Commitment Factors.* The model used here to describe the pH dependence of the kinetic parameters and kinetic isotope effects on those parameters is essentially identical to that previously used for the analysis of the liver alcohol dehydrogenase (Cook & Cleland, 1981c) and is shown in Scheme 1. This mechanism neglects the ordered binding of K$^+$ and NAD$^+$, and the "free" enzyme form E in Scheme 1 is actually the E·K$^+$·NAD$^+$ complex. We propose that glycerol binds to the enzyme via contacts to a participating monovalent cation which is bound to the enzyme and precedes the first chemical event, the removal of the C2 hydroxyl proton by the base to form the alkoxide and the protonated enzyme, EH·alkoxide. The indicated stereochemical configuration around glycerol is suggested by the greater than 4000-fold preference for the $R$ stereoisomer of 1-amino-2-propanol. Protonation of the E·glycerol complex results in a catalytically inert EH·glycerol complex, EHB, and is the cause of the decrease in $V$ below pH 7. The rate constants $k_3$ and $k_4$ represent the rate of formation of the glycerol alkoxide and the rate of its protonation, respectively. At low pH values, EH·alkoxide decomposes with stereospecific transfer of a hydride ion to the *re* face

Scheme 1. Model and Proposed Chemical Mechanism of the Glycerol Dehydrogenase-Catalyzed Reaction




of $NAD^+$ (Spencer et al., 1989) to form the protonated product complex EH·dihdroxyacetone. At high pH values, the protonated base in EH·alkoxide loses its proton to form E·alkoxide, which is also catalytically competent to transfer the hydride ion to $NAD^+$ to form the unprotonated product complex, E·DHA. The starred rate constants in Scheme 1 are sensitive to isotopic substitution at the C2 position of glycerol and result in the primary deuterium kinetic isotope effects observed on $V$ and $V/K$.

Equations for the commitment factors which control the degree to which the intrinsic kinetic isotope effect is expressed have been derived for this mechanism (Cook & Cleland, 1981c). The general forms of the equation which describes the dependence of the observed isotope effect are

$$^{D}V/K = (^{D}k_{cat} + c_f + c_r{}^{D}K_{eq})/(1.0 + c_f + c_r) \quad (10)$$

$$^{D}V = (^{D}k_{cat} + c_{Vf} + c_r{}^{D}K_{eq})/(1.0 + c_{Vf} + c_r) \quad (11)$$

where $^{D}k_{cat}$ is the intrinsic primary deuterium kinetic isotope effect on the hydride transfer reaction, $^{D}K_{eq}$ is the isotope effect on the equilibrium constant, $c_r$ is the reverse commitment factor, and $c_f$ and $c_{Vf}$ are the forward commitment factors which appear in the expressions for $^{D}V/K$ and $^{D}V$, respectively. Cook et al. (1980) have previously determined the magnitude of $^{D}K_{eq}$ to be 1.18 for the $NAD^+$-coupled oxidation of secondary alcohols. The reverse commitment, $c_r$, is unlikely to be a large term, since it appears in the expressions for both $^{D}V/K$ and $^{D}V$. If the reverse commitment was large relative to either forward commitment, $^{D}V$ would be equal to $^{D}V/K$ at high pH values, which is clearly not the case. We thus have made all subsequent interpretations using only the forward commitment factors as variables.

The expression for $c_f$ is

$$c_f = (k_5/k_4)(1.0 + k_7K_3/k_5H) \quad (12)$$

and will be pH-dependent because of the presence of the $K_3$/H term, where $K_3$ is the dissociation constant of the base in the EH·alkoxide complex. At pH values above p$K_3$, $c_f$ will asymptotically approach a limiting value described by the ratio $k_7/k_4$. Assuming a value of 4.88 for $^{D}k_5$ (i.e., the

largest measured value of the primary kinetic isotope effect),[3] we can solve for the value of $k_7/k_4$ using the value of 1.15 for the high pH limiting value of $^DV/K$. The value of 25 obtained shows that hydride transfer from the unprotonated enzyme−alkoxide, E·X, is 25 times faster than the protonation of the alkoxide at high pH values.

The expression for $c_{Vf}$ is

$$c_{Vf} = \frac{k_7(1.0 + k_5H/k_7K_3)(1.0 + H/K_2)}{k_4H/K_3(1.0 + H/K_2) + k_3(1.0 + H/K_3)} \quad (13)$$

At pH values higher than p$K_2$ or p$K_3$, this expression will asymptotically approach a limiting value described by the ratio $k_7/k_3$. Again, the value of $k_7/k_3$ can be numerically solved using eq 11, with a value of 2.57 as the high pH limiting value of $^DV$, and using a value of 4.88 for $^Dk_7$. The value of 1.5 for $k_7/k_3$ suggests that hydride transfer[4] from E·alkoxide is 1.5 times faster than the rate of formation of EH·alkoxide from E·glycerol. This numerical analysis allows an estimation of the equilibrium constant for alkoxide formation, $k_3/k_4$, since values for $k_7/k_4$ and $k_7/k_3$ have been calculated. The value for the equilibrium constant of 17 suggests that the alkoxide is stabilized on the enzyme surface. Rapid kinetic studies on the oxidation of a series of primary alcohols by horse liver alcohol dehydrogenase yielded values for this equilibrium constant of 6 (Sekhar & Plapp, 1990).

Both the primary deuterium kinetic isotope effects on $V$ and $V/K$ attain pH-independent values at low pH values. The commitment factor which determines the magnitude of $^DV/K_{glycerol}$ ($c_f$, see eq 12) at low pH values has as its limiting value the ratio of the rate of hydride transfer from the protonated alkoxide complex, $k_5$, and the rate of protonation of the alkoxide, $k_4$. As before, we can calculate this ratio $k_5/k_4$ to be 1.7, suggesting that hydride transfer through the protonated pathway is only 1.7 times faster than alkoxide protonation. This value may be compared to the ratio of the rate of hydride transfer from the unprotonated alkoxide and the rate of alkoxide protonation of 25. Since $k_4$ is pH-independent, the ratio of rates of hydride transfer to $NAD^+$ from the unprotonated and protonated enzyme−alkoxide complexes, E·alkoxide and EH·alkoxide, respectively, is 15 (i.e., $k_7/k_5 = 15$). This may be compared with the corresponding ratio of 28 for the oxidation of benzyl alcohol (Dworschack & Plapp, 1977).

$^DV$ increases to a pH-independent value of 4.88 as a group exhibiting a p$K$ value of 7.3 is protonated. The pH-dependence of $^DV$ is a result of the presence in the denominator of the expression for $c_{Vf}$ of a $k_4$ term that is multiplied by $H^2/K_2K_3$ and a $k_3$ term that is multiplied by $H/K_3$. Thus at the low pH extreme, the $k_4$ term will dominate, and $c_{Vf}$ will approach $k_5/k_4$, which is the same commitment factor as that which controls the expression of $c_f$. This predicts that $^DV$ and $^DV/K_{glycerol}$ will become equal at low pH values, and this is not observed experimentally. However, the $k_3$ term will contribute more to the denominator term for 1.2 pH units below the p$K$ value of the base since it is 17 times greater than $k_4$. Only at the very lowest pH values (<5.5) will $c_{Vf} = c_f$ and will $^DV$ approach $^DV/K_{glycerol}$. Experimentally, it is not possible to obtain supporting data in the appropriate pH range for several reasons, including the rapidly increasing values for both $K_{m,NAD^+}$ and $K_{m,glycerol}$ (over 300 mM at pH 5.5) and the extremely unfavorable equilibrium constant.

[3] Literature values for the intrinsic primary deuterium kinetic isotope effects on hydride transfers from secondary alcohols to $NAD^+$ include 5.7 for yeast alcohol dehydrogenase catalyzing isopropanol oxidation (Cook & Cleland, 1981b), 5.6 for chicken liver malic enzyme catalyzing L-malate oxidation (Grissom & Cleland, 1988), and 5.1 for tartrate dehydrogenase catalyzing D-malate oxidation (Tipton, 1993).

[4] We assume throughout this discussion that the intrinsic deuterium kinetic isotope effect for hydride transfer from the glycerol alkoxide to NAD is the same whether the carboxylate is protonated or unprotonated ($^Dk_5 = {}^Dk_7$).

## CONCLUSIONS

Our original interest in the detailed comparative examination of glycerol dehydrogenase has revealed a number of interesting features. Glycerol dehydrogenases use a monovalent cation to stabilize the developing charge of the alkoxide intermediate, while alcohol dehydrogenases use a tightly bound zinc atom. The *Cellulomonas* glycerol dehydrogenase apparently uses a carboxylic base to deprotonate the C2 alcohol, while an imidazole-paired serine fulfills this function in alcohol dehydrogenase. Yet the pH dependence of the kinetic parameters and kinetic isotope effects are remarkably similar and can be interpreted within a mechanism previously described for alcohol dehydrogenase (Cook & Cleland, 1980b). These similarities extend to the equilibrium constants for alkoxide formation on the enzyme surface and relative rates of hydride transfer from the alkoxide to $NAD^+$ via protonated and unprotonated pathways. It is noteworthy that these two secondary alcohol dehydrogenases have arrived at a common catalytic solution in spite of their different active site components.

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