Progress in Establishing the Rate-Limiting Features and Kinetic Mechanism of the Glyceraldehyde-3-phosphate Dehydrogenase Reaction†

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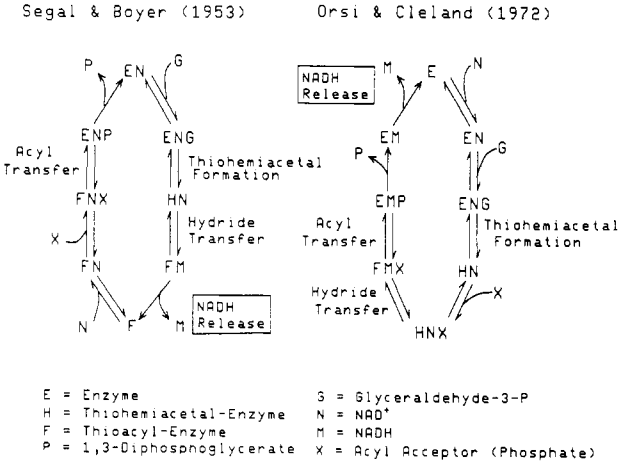
ABSTRACT: Primary hydrogen isotope effects and steady-state kinetics have been used to study the mechanism of glyceraldehyde-3-phosphate (GAP) dehydrogenase at pH 8.6. The isotope effect determined by using GAP-1d was unity and independent of arsenate (used as the acyl acceptor) and NAD^+ concentrations when the aldehyde substrate was at saturating concentrations. At low GAP concentrations (apparent V/K conditions), the primary hydrogen isotope effect (H/D) was in the range of 1.40–1.52 and independent of arsenate and NAD^+ concentrations. Apparent V/K for NAD^+ was independent of GAP concentration, and apparent V/K for GAP was independent of NAD^+ concentration. The dependence of apparent V/K for GAP on arsenate concentration was more complex but extrapolated to nonzero V/K at the zero-arsenate intercept. These observations are consistent with the general features of the Segal and Boyer (1953) mechanism for the reaction.

The kinetic mechanism of the glyceraldehyde-3-phosphate dehydrogenase (GAPDH)[1] reaction has been the subject of some controversy over a long history of mechanistic investigations [see the account by Dalziel et al. (1981)]. The enzyme uses both hydride-transfer and acyl-transfer chemistry to catalyze the oxidative phosphorylation of D-glyceraldehyde 3-phosphate (D-GAP) (Harris & Waters, 1976). A central point of contention has been the placement of NADH release in the steady-state mechanism. Without elaborating on the history of the mechanistic studies, the debate about the reaction can be cast in terms of the differences between a mechanism proposed by Segal and Boyer (1953) and the scheme suggested by Orsi and Cleland (1972) (see Scheme I).

Since 1970, transient kinetics (Trentham, 1971; Dalziel et al., 1981) and steady-state studies (Duggleby & Dennis, 1974; Meuneir & Dalziel, 1978; Crow & Wittenberger, 1979) using GAPDH from several sources have been used to support the basic features of the Segal and Boyer mechanism over the Orsi and Cleland proposal. Recently, kinetic isotope effects have been reported for GAPDH-catalyzed reactions and interpreted in terms of the Orsi and Cleland mechanism (Canellas & Cleland, 1991). These same authors found sufficiently large primary hydrogen isotope effects to suggest that hydride transfer was important in limiting the steady-state rate regardless of the level of saturation by GAP. We recently completed our own study of kinetic isotope effects on the GAPDH reaction. Our results offer support for the Segal and Boyer mechanism and suggest that hydride transfer does not play a dominant role in controlling the steady-state rate. We report here our observations of primary hydrogen kinetic isotope effects on GAPDH-catalyzed reactions as well as initial velocity studies relating to the kinetic mechanism of the enzyme.

Scheme I: Minimal Kinetic Mechanisms[a] Proposed by Segal and Boyer (1953) and Orsi and Cleland (1972)[b]



[a] The key feature distinguishing the two mechanisms is the placement of NADH release. [b] This is the Orsi and Cleland (1972) mechanism as described in Canellas and Cleland (1991).

MATERIALS AND METHODS

Synthesis and Purification of DL-*GAP-*d_1. Deuterated DL-GAP was prepared using a modification of published procedures (Serianni & Barker, 1979; Serianni et al., 1979a,b, 1982). In the first step of the synthesis, glycerol 1-phosphate was converted to glycolaldehyde phosphate. The disodium salt of DL-glycerol 1-phosphate (2.7 mmol, Sigma) was moistened with 1.0 mL of water and added, while stirring, to 40 mL of glacial acetic acid. After the salt was dissolved, 0.17 mL of 18 M sulfuric acid was added, followed by 5.4 mmol of lead tetraacetate. After 2 h, oxalic acid (5.4 mmol) was added to the reaction mixture, which was stirred for an additional 30 min. The resulting suspension was then filtered through Celite with several water washes. The filtrate was added to 5.0 mmol of barium acetate, and the mixture (kept at ca. 4 °C) was stirred for 15 min. The resulting white

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[1] Abbreviations: ADP, adenosine 5′-diphosphate; ATP adenosine 5′-triphosphate; Bis-Tris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)-1,3-propanediol; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; HEPPS, *N*-(2-hydroxyethyl)piperazine-*N*′-3-propanesulfonic acid; GAP, glyceraldehyde 3-phosphate, GAPDH, D-glyceraldehyde-3-phosphate dehydrogenase; NAD^+, nicotinamide adenine dinucleotide; NADH, reduced form of NAD^+; TES, *N*-[tris(hydroxymethyl)methyl]-2-aminoethanesulfonic acid.

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suspension was filtered through Celite, and the filtrate was treated with excess Dowex 50 (H^+) and then was subjected to an overnight continuous liquid–liquid extraction (at 4 °C) with diethyl ether. The aqueous layer was recovered and lyophilized down to about 0.8 mL.

In the next step, DL-glycerolnitrile phosphate was prepared from glycolaldehyde phosphate. A solution of KCN (2.5 mmol) in D_2O at 20 °C was added to a three-necked flask, and the pH was adjusted to 8.0 by adding drops of 0.7 M acetic acid-d_4. Glycolaldehyde phosphate from the previous step was dissolved in a small amount of D_2O and added to the KCN solution. The pH of the solution was maintained between 8.0 and 8.3 with additions of 0.7 M acetic acid-d_4 and 1.0 M NaOD. After about 25 min, the pH was lowered to 4.0 using 17 M acetic acid-d_4. A further adjustment of pH to 1.53 was made using 6 M sulfuric acid-d_2.

The final step of the synthesis was the hydrogenation of glyceronitrile phosphate to produce DL-GAP-d_1 in a Dayton Electric hydrogenation apparatus. Palladium–barium sulfate (150 mg, 5%, 65 mg per mmol of nitrile) was weighed into a pressure-resistant bottle, and 5.0 mL of D_2O was added. The system was evacuated and charged with nitrogen gas three times and then evacuated and charged with deuterium gas (D_2) at slightly higher than atmospheric pressure. After the catalyst was reduced for about 20 min at room temperature with stirring, 30 mL of DL-glycerolnitrile phosphate in D_2O was added, and the system was charged three times with nitrogen and finally with deuterium gas. The reaction was allowed to run for 10 h.

After the hydrogenation, the catalyst was removed by filtration through Celite, and the filtrate was treated with excess Dowex 50x8 (H^+) to form a very acidic solution. This solution was concentrated using lyophilization and adjusted to pH 4.5 using dilute NaOH. A DEAE–Sephadex A-25 (acetate) column (1.2 × 50 cm) equilibrated with 0.05 M sodium acetate at pH 4.5 was used to further purify the DL-GAP-d_1 product. The loaded column was developed with a linear gradient (0.05–0.8 M) of sodium acetate. The chromatogram showed three well-separated peaks in aborbance at 254 nm. The fractions containing GAP were pooled, treated with excess Dowex 50x8 (H^+), and concentrated by lyophilization. The final yield of product (concentration determined using a GAPDH assay with arsenate) was 45% based on the starting glycerol 1-phosphate. Proton NMR showed the product to be 90% deuterated (all peaks observed could be assigned to GAP or HOD). Small amounts of acetic acid that might be present in the sample should have no influence on the reaction velocity. Sodium acetate as high as 100 mM had no influence on rates measured under the conditions of our isotope-effect measurements. All isotope effects measured with this material have been corrected for the light-isotope impurity ($v_{o,D}$ [corrected] = $(v_{o,D} - 0.1 \times v_{o,H})/0.9$).

Synthesis and Purification of D-*GAP-d_1*. D-GAP-d_1 was prepared enzymatically using B-side deuterated NADH-d_1. The deuterated NADH was prepared according to the method of Schleicher and Simon (1977). D-Glucose-1-d_1 (16.2 mg, 18 mM, Merck Sharp and Dohme Isotopes), 37.8 mg (10 mM) of NAD^+, and 15 units of *B. megaterium* glucose dehydrogenase (Sigma) were allowed to react for 2 h in 50 mM, pH 8.2, Bis-Tris buffer. The enzyme was removed by adding several drops of CCl_4, mixing with a vortex mixer, and filtering the mixture. The filtrate was applied to a Bio-Gel P_2 column (1 × 15 cm), and the column was eluted with water. The fractions containing NADH were collected and pooled. The A_{260nm}/A_{340nm} ratio was 2.28, and the NADH yield was 99% based on the starting NAD^+ concentration. No glucose dehydrogenase activity was detected in the product solution.

The B-side deuterated NADH was then used to prepare D-GAP-d_1 by running the GAPDH reaction in the reductive dephosphorylation direction. D-Glycerate 3-phosphate (0.1588 g, 50 mM), 0.0781 g (10 mM) of NADH-d_1 (B-side), 0.0493 g (20 mM) of $MgSO_4$, and 0.4841 g (80 mM) of ATP were mixed with 10 mL of 200 mM, pH 7.6, TES buffer containing 5 mM EDTA. A mixture of yeast GAPDH and phosphoglycerate kinase (1.15 mg, ca. 50–140 units of each enzyme, Sigma) was then added to the reaction solution. After stirring the reaction solution for 30 min at room temperature, the proteins were removed using ultrafiltration. The yield of D-GAP at this point was 74%. The enzyme-free reaction solution was applied to a DEAE–Sephadex A-25 column (1.5 × 50 cm) equilibrated with 10 mM, pH 6.5, Bis-Tris buffer. The column was eluted at 5 °C using a sodium chloride gradient produced by 0.6 M NaCl in 10 mM, pH 6.5, Bis-Tris buffer, and the same buffer with no added NaCl. Fractions containing GAP were pooled. These fractions contained no detectable D-glycerate 3-phosphate, NADH, NAD^+, ATP, or ADP. The D-GAP-d_1 fractions were concentrated to about 1 mL using lyophilization. The NaCl in the fractions was removed using a Sephadex G-10 column.

Other Materials. All other materials were obtained from standard chemical suppliers (Fisher, Alrich, or Sigma). Water was house distilled, passed through a mixed-bed ion exchanger, and redistilled. GAPDH from rabbit muscle was obtained from Sigma as a lyophilized powder and was used without further treatment. DL-GAP and D-GAP (undeuterated) were obtained from Sigma as the diethylacetal dicyclohexylammonium) salts. The free aldehydes were prepared by adding 0.7 g of Dowex 50xB-200 ion-exchange resin and 6.0 mL of water to a test tube along with about 25 mg of the acetal. The mixture was heated in a boiling water bath for about 3 min, cooled in an ice–water bath, centrifuged (bench-top centrifuge at 9000 rpm), and then decanted followed by washing of the resin. The concentration of D-GAP was always determined by using the GAPDH reaction run to completion at pH 8.6, using arsenate as the acyl acceptor. Proton NMR spectra of samples of GAP prepared from the commercial acetals (prepared in D_2O) showed only GAP (and HOD) peaks. Comparisions of the weights of acetals used in many experiments with the results of enzyme assays for D-GAP showed the yields for the acetal hydrolyses were 95–105%.

Kinetics. Reactions were studied by monitoring absorbance at 340 nm using a Varian DMS300 UV–visible spectrophotometer. Voltages from the chart-recorder output were collected as a function of time using an analog-to-digital board in a microcomputer controlled by software written locally. After converting the voltages to absorbance, initial velocities were determined as slopes of least-squares lines through the data. Linearity in the assays was assessed by observing random patterns of residuals. For a few measurements, a Hewlett-Packard diode array spectrophotometer (Model 8452a) was used with a similar acquisition/analysis package written locally. Temperature was maintained at 25 ± 0.05 °C (accurate to ±0.15 °C) using water baths and water-jacketed cuvet holders.

Direct Arsenate Assay. Most measurements were made using a direct assay of the GAPDH reaction rate with arsenate serving as the acyl acceptor. Reactions were initiated by adding usually 20 μL of a GAPDH solution to give 1.0-mL reaction solutions.

Table I: Primary Hydrogen Isotope Effects on the GAPDH Reaction Determined Using the Direct Arsenate Assay[a]

[total arsenate], mM	[NAD+], mM	$^{D}V_G$ [b]	$^{D}(V/K)_G$ [b]
0.05 (0.5 K_m)[c]	0.715	1.018 ± 0.014	1.436 ± 0.037
0.20 (2.0 K_m)	0.715	0.995 ± 0.023	1.444 ± 0.095
3.00 (30 K_m)	0.715	0.982 ± 0.055	1.418 ± 0.089
3.00	0.030 (0.8 K_m)[c]	0.979 ± 0.034	1.526 ± 0.101
3.00	0.080 (2.0 K_m)	0.973 ± 0.029	1.396 ± 0.071
3.00	0.982 (17 K_m)	0.982 ± 0.055	1.418 ± 0.089

[a] Reaction conditions: pH 8.6, 0.1 M HEPPS buffer, 1.0 mM DTT, 0.5 mM EDTA, 1.6 μg/mL GAPDH, and 25.0 °C. [b] Isotope effects are shown as ratios of H/D parameters. V_G and $(V/K)_G$ are apparent Michaelis–Menten parameters determined by measuring initial velocity as a function of [GAP] while other substrate concentrations were held constant at values shown in the table. Error limits are least-squares estimates of standard deviations. [c] Values in parentheses show substrate concentrations in comparison to apparent K_m values determined for the present experimental conditions.

Coupled-Enzyme Assay. The assay of Canellas and Cleland (1991) was used for a few measurements. In this assay, phosphate is used as the acyl acceptor, and glycerokinase (we used the Sigma enzyme from *C. utils*) and 3-phosphoglycerate kinase (we used the Sigma enzyme from rabbit muscle) along with 10 mM glyceraldehyde (to stimulate the ATPase activity of glycerokinase) and ATP are added to remove the inhibitory 1,3-diphosphoglycerate product and to recycle phosphate. Our conditions were exactly those listed in Canellas and Cleland (1991). When the concentration of GAP was varied in a series of experiments, the validity of the assay was checked by demonstrating that the observed reaction rate was linear in GAPDH concentration at both high and low GAP concentrations. In all of our experiments there was a substantial background increase in absorbance at 340 nm. This was presumably arising from the addition of the enolate of glyceraldehyde to the NAD^+ ring. All of our reported kinetic parameters have been obtained from observed velocities minus the background velocity.

RESULTS

All kinetic parameters in this report are apparent V_{max} or apparent V_{max}/K_m obtained when one substrate concentration was varied while holding the remaining substrate concentrations constant. In each case, these two parameters [we call these V_S and $(V/K)_S$, where S identifies the substrate concentration varied] were obtained from least-squares fits of initial velocity vs substrate concentration to eq 1, a rearranged form of the conventional Michaelis–Menten equation.

$$v_o = [S]/((V/K)_S^{-1} + [S]/V_S) \quad (1)$$

Primary Hydrogen Isotope Effects. Table I shows kinetic isotope effects determined by using the direct arsenate assay with DL-GAP. We also made a few measurements using D-GAP-h_1 and D-GAP-d_1 and found $^{D}(V/K)_G = 1.590 \pm 0.020$ and $^{D}V_G = 0.985 \pm 0.015$ (0.715 mM NAD^+, 2.96 mM total arsenate, and other conditions as described in Table I). These isotope effects are much smaller than the values reported by Canellas and Cleland (1991) using the coupled assay (with DL-GAP) outlined in our Materials and Methods section. We repeated these experiments using the exact conditions and the assay described in Canellas and Cleland (1991) and obtained $^{D}(V/K)_G = 1.29 \pm 0.17$ and $^{D}V_G = 0.972 \pm 0.075$. The larger estimated uncertainties obtained using the coupled assay may arise from complications associated with the substantial background reaction (see the Materials and Methods section) in this assay system. Our isotope effects do not change much if we fail to make corrections for the background rates. We suspected that some of the differences between our measurements and those of Canellas and Cleland might stem from differences in inhibitor contamination between the protiated and deuterated substrates. We used DL-GAP-d_1 that had not been chromatographically purified to see if we could obtain large apparent isotope effects. Instead, we observed results very close to our previous measurements [$^{D}(V/K)_G = 1.78 \pm 0.41$ and $^{D}V_G = 0.72 \pm 0.20$, using the coupled assay]. Any inhibitors that might be present in our unpurified deuterated substrate seem to have sufficiently low K_i's to have little influence at their contaminating concentrations.

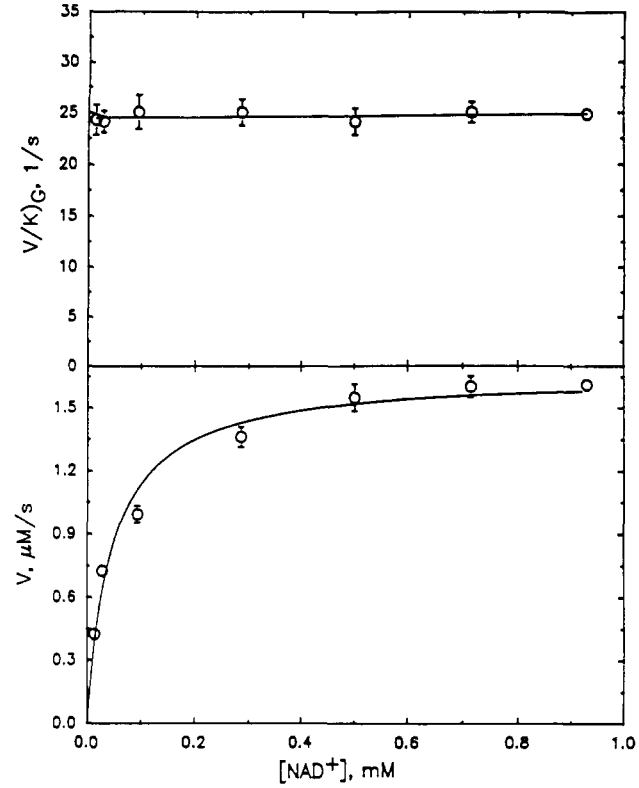

FIGURE 1: Apparent V and V/K (determined by varying [DL-GAP]) as functions of [NAD^+] in 3.00 mM total arsenate. V/K was calculated based on the D-isomer concentration of DL-GAP. Reactions were studied in 0.1 M pH 8.6 HEPPS buffer with 1.0 mM DTT, 0.5 mM EDTA, and 1.6 μg/mL GAPDH at 25.0 °C. Error bars mark least-squares estimates of standard deviations for V and V/K. The line drawn through the data of the upper panel is the least-squares line. The curve in the lower panel is the least-squares fit to $V = A[NAD^+]/(B + [NAD^+])$; $A = 1.680 \pm 0.062$ μM/s, $B = 47.4 \pm 8.5$ μM.

Dependence of Steady-State Parameters on Substrate Concentration. Figures 1–3 show steady-state parameters V and V/K for GAP or NAD^+ as functions of other substrate concentrations. These parameters were determined by varying the measuring initial velocity as one substrate concentration was varied while the remaining concentrations were fixed, followed by least-squares fitting to eq 1. Solid lines are least-squares fits of the data to the equations described in the individual figure legends.

DISCUSSION

Observed Primary Hydrogen Isotope Effects. Our observations of hydrogen isotope effects on the GAPDH reaction at pH 8.6 are very different from the values reported by Canellas and Cleland (1991). These authors found $^{D}V_G = ^{D}(V/K)_G = 2.55$ (taking the aldehyde hydrate as the reactant state).

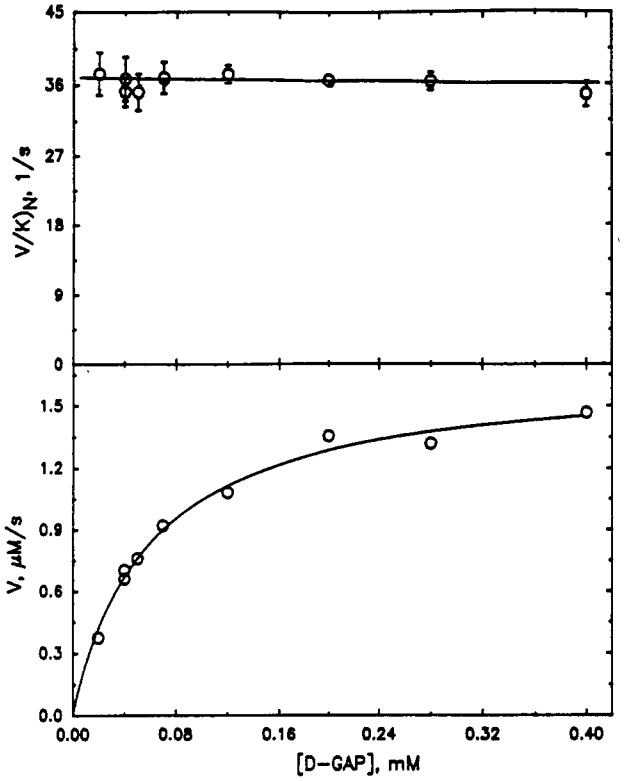

FIGURE 2: Apparent V and V/K (determined by varying [NAD^+]) as functions of [D-GAP] (used DL-GAP) in 3.00 mM total arsenate. See the legend of Figure 1 for reaction conditions. The line through the $(V/K)_N$ data is the least-squares line. The curve drawn through the V data is the least-squares fit to $V = A$[D-GAP]$/(B +$ [D-GAP]); $A = 1.665 \pm 0.042$ μM/s, $B = 59.7 \pm 4.4$ μM.

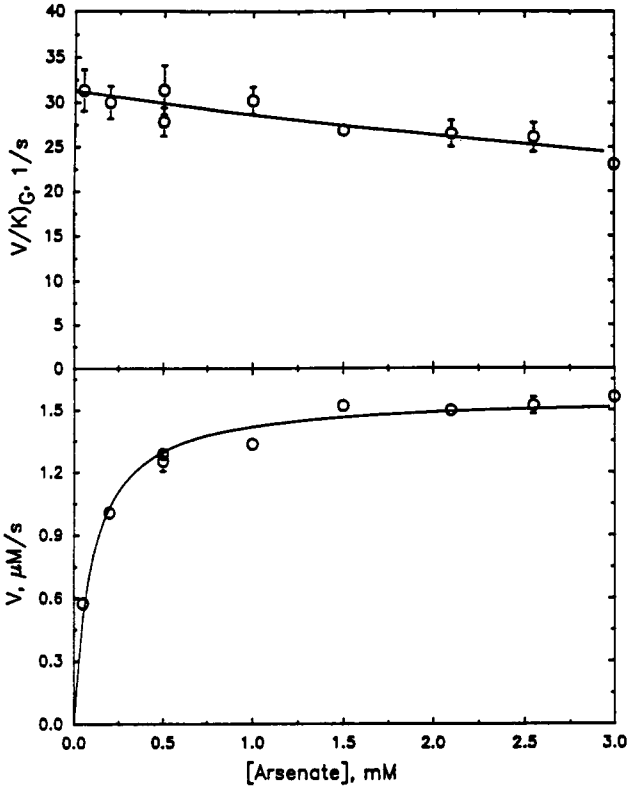

FIGURE 3: Apparent V and V/K (determined by varying [DL-GAP]) as functions of [arsenate] in 3.00 mM total arsenate. V/K was calculated based on the D-isomer concentration of DL-GAP. See the legend of Figure 1 for reaction conditions. The curve drawn through the $(V/K)_G$ data (accounts for competitive inhibition by arsenate) is the least-squares fit to $V/K = A/(B +$ [arsenate]); $A = 0.332 \pm 0.067$ s^{-1}, $B = 10.6 \pm 2.3$ mM. The curve drawn through the V data is the least-squares fit to $V = A$[D-GAP]$/(B +$ [D-GAP]); $A = 1.568 \pm 0.030$ μM/s, $B = 104 \pm 13$ μM.

We find ${}^DV_G = 1$ and ${}^D(V/K)_G = 1.4$–1.5 using two different preparations of our deuterated substrate (one chemical and one enzymatic synthesis) and two different assays (the direct arsenate assay and the coupled assay). We have been unable to explain the differences between our observations and those of Canellas and Cleland. Our results with the rabbit muscle enzyme do fit in well with the isotope effect measurements on reactions catalyzed by GAPDH from other sources, however. Trentham (1971) studied the reverse reaction catalyzed by the lobster muscle and sturgeon muscle enzymes using half-deuterated NADH and observed no kinetic isotope effect, and Cobier et al. (1990) found ${}^DV_G = 1.32 \pm 0.04$ for the forward-reaction primary isotope effect on a reaction catalyzed by the *Bacillus stearothermophilus* enzyme.

In contrast to these several instances of direct evidence for a modest primary hydrogen isotope effect on V/K, indirect evidence that there is a normal primary hydrogen isotope effect on V/K comes from the H/D isotope effect on carbon isotope effects reported by Canellas and Cleland (1991). These authors found ${}^{13}(V/K)_H = 1.0125 \pm 0.0003$ using protiated substrates and ${}^{13}(V/K)_D = 1.0283 \pm 0.0005$ for the same carbon isotope effect determined using GAP deuterated in the aldehydic site. The apparent isotope effect on the carbon isotope effect is consistent with a multiple-rate-limiting-step model in which the carbon-isotope-sensitive step becomes more important when a deuterated substate is used. Because we were concened that this class of models would require large primary hydrogen isotope effects to reproduce the carbon isotope effects, we sought to demonstrate in the next section that our modest primary hydrogen isotope effects are in fact compatible with the Canellas and Cleland carbon isotope effects in a multiple-rate-limiting-step model.

Analysis of Isotope Effects on Isotope Effects. Using our values for primary hydrogen isotope effects we can reanalyze the carbon isotope effects of Canellas and Cleland (1991). These authors chose to interpret their isotope effects using a model showing multiple-rate-limiting steps that required a detailed description of the reaction mechanism. We opted to evaluate the isotope effects using an approach that is largely independent of assumptions about the reaction mechanism.

Isotope effects on V/K were considered to reflect two rate-limiting steps designated by the rate constants k_x and k_y for a particular conversion from an initial state to a transition state (Alvarez et al. 1991). In this treatment,[2] any isotope effect on V/K [designated by ${}^a(V/K)$] will be a simple weighted average of component isotope effects ${}^ak_x$ and ${}^ak_y$ (eq 2). The weighting factor w_x (eq 3) will be unity in the limit of complete rate control by k_x and zero in the limit of complete rate control by k_y. Relationships like eqs 2 and 3 were written for our

$$ {}^a(V/K) = w_x {}^ak_x + (1 - w_x) {}^ak_y \quad (2) $$

$$ w_x = k_y/(k_y + k_x) \quad (3) $$

primary hydrogen isotope effects along with the carbon and secondary hydrogen isotope effects of Canellas and Cleland (1991). Isotope effects on k_x were found by solving the equations (see the Appendix) using two assumptions about k_y.

[2] The formulas of Northrup (1977) can be recast (Ray, 1983) as weighted-average expressions and represent specific cases of the more general weighted-average approach.

Table II: Isotope Effects Calculated Using a Model with Two Serially Related Rate-Limiting Steps[a]

Isotope Effects Assumed for One Component Rate Constant		
$^{13}k_{y,H}$	1	0.995
$^{13}k_{y,D}$	1	0.995
$^{D}k_{y}$	1	1.145
$^{\alpha D}k_{y}$	1	0.890
Calculated Results		
$^{13}k_{x,H}$	1.085 ± 0.036	1.0353 ± 0.0033
$^{13}k_{x,D}$	1.0693 ± 0.0082	1.0556 ± 0.0055
$^{D}k_{x}$	3.98 ± 1.3	1.83 ± 0.14
$^{\alpha D}k_{x}$	1.345 ± 0.074	1.261 ± 0.055
$w_{x,H}$	0.147 ± 0.063	0.434 ± 0.063
$w_{x,D}$	0.408 ± 0.048	0.550 ± 0.052
$w_{x,\alpha D}$	0.189 ± 0.077	0.520 ± 0.059

[a] Refer to the Appendix for the values of experimental isotope effects used in the analysis and the full details of the calculations.

Our analysis gives the results shown in Table II for the case where unit isotope effects are assumed for one component (k_y), and a second analysis was conducted using isotope effects assumed if NADH release is rate-limiting (based on equilibrium isotope effects described in Canellas and Cleland). Most of the calculated isotope effects (Table II) on k_x are sensitive to small changes in assumed values for isotope effects on the second component, but the differences are not large given the estimated error limits for the calculated values, and the magnitudes of the effects are reasonable. We conclude, therefore, that our primary hydrogen isotope effects are compatible with the carbon isotope effects of Canellas and Cleland (1991) and multiple-rate-limiting-step models.

Kinetic Mechanism of GAPDH at pH 8.6. Although numerous steady-state rate studies have been conducted on the GAPDH reaction from a variety of sources (Furfine & Velick, 1965; Orsi & Cleland, 1972; Duggleby & Dennis, 1974; Meunier & Dalziel, 1978; Crow & Wittenberger, 1979), we conducted our own investigation under the conditions of our isotope effect experiments. Our results, displayed as plots of apparent V/K or V in Figures 1–3, are consistent with the mechanism proposed by Segal and Boyer (1953) in which NADH is released before NAD^+ and the acyl acceptor binds to the enzyme. In each of the V/K plots, the data are either independent of the substrate concentration shown or seem to extrapolate to nonzero intercepts [$(V/K)_G$ shows inhibition by arsenate but extrapolates to a nonzero intercept at zero arsenate concentration]. The V/K data from each of the three plots thus indicated that the binding event for GAP is isolated by apparent irreversible steps in the catalytic cycle from the binding events for NAD^+ and the acyl acceptor (arsenate in our experiments). In the Segal and Boyer mechanism, NADH release serves as the key irreversible step separating GAP binding from the binding steps for the remaining substrates.

In the sequential mechanism favored by Orsi and Cleland (1972) and Canellas and Cleland (1991), the apparent irreversible steps needed to bring the scheme into consistency with our results could be assigned to a collection of substrate-binding steps (with very low binding constants) and other reactions in the catalytic cycle. We cannot, therefore, strictly eliminate the Orsi and Cleland (1972) mechanism for the GAPDH reaction at pH 8.6 solely on the basis of our results. However, as Meunier and Dalziel (1978) have noted, the product-inhibition results that support the Orsi and Cleland mechanism were obtained using an unnatural aldehyde substrate, and it seems that the kinetic mechanism is dependent on the identity of this substrate. Meunier and Dalziel (1978) also used results from product-inhibition studies with the natural substrate to argue against the Orsi and Cleland (1972) scheme. Additionally, Scheek et al. (1979) have used coenzyme-binding studies with rabbit muscle GAPDH and the thioacyl form of the enzyme as evidence against the Orsi and Cleland mechanism.

A possible rationale for the dependence of the timing of NADH release on the structure of the aldehyde substrate may lie in a need for the enzyme to protect the thioacyl–enzyme intermediate from water. In reactions of poor aldehyde substrates, water or buffer might serve as the acyl acceptor through a kinetic mechanism that differs from that of the natural substrate. In preliminary studies, we find that when glyceraldehyde is used as a substrate, for example, a substantial catalytic reaction is observed in the absence of an added acyl acceptor such as phosphate or arsenate.

Our primary hydrogen isotope effects are easily accommodated by the Segal and Boyer mechanism. The modest isotope effects seen on V/K_G are independent of both acyl acceptor concentration and NAD^+ concentration (Table I), suggesting that hydride transfer is only partially rate-limiting under these V/K conditions and that the binding of NAD^+ and arsenate is separated by an apparent irreversible step (such as NADH release) from the binding of GAP. Unit values of $^{D}V_G$ are most easily explained by assigning both initial and final states of this parameter to states after NADH release and during the acyl-transfer phase of the reaction. If this is the case, the label is always in the reduced coenzyme for all kinetically significant conversions of V_G so isotope effects must be unity.

CONCLUSIONS

Our steady-state studies and isotope effects for the rabbit muscle GAPDH reaction at pH 8.6 are fully consistent with the Segal and Boyer (1953) mechanism for GAPDH action involving NADH release prior to NAD^+ and acyl-acceptor binding. In conjunction with the carbon isotope effects of Canellas and Cleland, our results show that hydride transfer is a partial contributor to rate determination at low GAP concentrations [$(V/K)_G$ conditions] but makes no contribution to rate control at saturating concentrations of GAP.

APPENDIX

Details of the Analysis of Isotope Effects on Isotope Effects. The following equations describe several kinetic isotope effects on V/K for a general model in which two steps in series control a reaction rate. Following Alvarez et al. (1991), k_x and k_y are rate constants that reflect distinct conversions from an initial state (for V/K, it is the same initial state) to a transition state.

$$^{13}(V/K)_H = w_{x,H}(^{13}k_{x,H}) + (1 - w_{x,H})(^{13}k_y) \quad (4)$$

$$^{13}(V/K)_D = w_{x,D}(^{13}k_{x,D}) + (1 - w_{x,D})(^{13}k_y) \quad (5)$$

$$^{13}(V/K)_{\alpha D} = w_{x,\alpha D}(^{13}k_{x,H}) + (1 - w_{x,\alpha D})(^{13}k_y) \quad (6)$$

$$^{D}(V/K) = w_{x,H}(^{D}k_x) + (1 - w_{x,H})(^{D}k_y) \quad (7)$$

$$(w_{x,H}^{-1} - 1)/(w_{x,D}^{-1} - 1) = {}^{D}k_x/{}^{D}k_y \quad (8)$$

$$(w_{x,H}^{-1} - 1)/(w_{x,\alpha D}^{-1} - 1) = {}^{\alpha D}k_x/{}^{\alpha D}k_y \quad (9)$$

$$^{\alpha D}(V/K) = w_{x,H}(^{\alpha D}k_x) + (1 - w_{x,H})(^{\alpha D}k_y) \quad (10)$$

Conventional nomenclature is used to identify isotope effects in these equations ($^{heavy}k = k_{light}/k_{heavy}$). $^{13}(V/K)_H$ (1.0125 ± 0.0003) is the carbon isotope effect (C-1) determined with protiated substrates, $^{13}(V/K)_D$ (1.0283 ± 0.0005) is the same

carbon isotope effect determined using GAP deuterated in the aldehydic site, and $^{13}(V/K)_{\alpha D}$ (1.0160 ± 0.0007) is the same effect determined using protiated GAD but NAD^+ deuterated at C-4 of the nicotinamide ring. $^{\alpha D}(V/K)$ (1.051 ± 0.023) is the secondary hydrogen isotope effect arising from isotopic substitution at C-4 of the nicotinamide ring of NAD^+. Values in parentheses are from Canellas and Cleland (1991), referenced to the aldehyde hydrate reactant state. For $^{D}(V/K)$ we now use the average value found in Table I (1.440 ± 0.045).

The seven equations (eqs 4–10) can be solved to obtain w_H, w_D, $w_{\alpha D}$, $^{D}k_x$, $^{13}k_{x,H}$, $^{13}k_{x,D}$, and $^{\alpha D}k_x$ provided that values for $^{D}k_y$, $^{13}k_y$, and $^{\alpha D}k_y$ are assumed (for convenience, we have also assumed that $^{13}k_y = {}^{13}k_{y,H} = {}^{13}k_{y,D}$). Solutions (Table II) were obtained numerically using standard methods [see Press et al. (1986) or any modern numerical methods text], and errors for the unknowns were estimated by propagating error estimates of the observed isotope effects using standard methods employing numerical estimates of the necessary derivatives (Clifford, 1973).

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