Mechanisms of Aldehyde-Induced Adenosinetriphosphatase Activities of Kinases†

Alan R. Rendina‡ and W. W. Cleland*

ABSTRACT: Aldehyde analogues of the normal alcohol substrates induce ATPase activities by glycerokinase (D-glyceraldehyde), fructose-6-phosphate kinase (2,5-anhydromannose 6-phosphate), fructokinase (2,5-anhydromannose or 2,5-anhydrotalose), hexokinase (D-*gluco*-hexodialdose), choline kinase (betaine aldehyde), and pyruvate kinase (glyoxylate). Since purified deuterated aldehydes give V and V/K isotope effects near 1.0 for glycerokinase, fructokinase with 2,5-anhydro[1-^{2}H]talose, hexokinase, choline kinase, and pyruvate kinase, the hydrates of these almost fully hydrated aldehydes are the activators of the ATPase reactions. Fructose-6-phosphate kinase and fructokinase with 2,5-anhydro[1-^{2}H]-mannose show V/K deuterium isotope effects of 1.10 and 1.22, respectively, suggesting either that both hydrate and free aldehyde may be activators (predicted values are 1.37 if only the free aldehyde activates the ATPase) or, more likely, that the phosphorylated hydrate breaks down in a rate-limiting step on the enzyme while MgADP is still present and the back-reaction to yield free hydrate in solution is still possible. ^{18}O was transferred from the aldehyde hydrate to phosphate during the ATPase reactions of glycerokinase, fructose-6-phosphate kinase, fructokinase, and hexokinase but not with choline kinase or pyruvate kinase. Thus, direct phosphorylation of the hydrates by the first four enzymes gives the phosphate adduct of the aldehyde, which decomposes nonenzymatically, while with choline kinase and pyruvate kinase the hydrates induce transfer to water (metal-bound hydroxide or water with pyruvate kinase on the basis of pH profiles). Observation of a lag in the release of phosphate from the glycerokinase ATPase reaction at 15 °C supports the existence of a phosphorylated hydrate intermediate with a rate constant for breakdown of 0.035–0.043 s^{-1} at this temperature. Kinases that phosphorylate creatine, 3-phosphoglycerate, and acetate did not exhibit ATPase activities in the presence of keto or aldehyde analogues (*N*-methylhydantoic acid, D-glyceraldehyde 3-phosphate, and acetaldehyde, respectively), possibly because of the absence of an acid–base catalytic group in the latter two cases. These analogues were competitive inhibitors vs. the normal substrates, and in the latter case, the hydrate of acetaldehyde was shown to be the inhibitory species on the basis of the deuterium isotope effect on the inhibition constant.

The ATPase activities of kinases induced by aldehyde analogues of the normal substrates were first reported for *Escherichia coli* (Hayishi & Lin, 1967) and *Candida* glycerokinase (Gancedo et al., 1968; Janson & Cleland, 1974) in the presence of D-glyceraldehyde. Recently, both fructose-6-phosphate kinase (fructose-6-P kinase) and fructokinase were shown to exhibit ATPase activity in the presence of aldehyde analogues of their substrates (Viola & Cleland, 1980). We have now found that hexokinase, pyruvate kinase, and choline kinase also show ATPase activity with the appropriate aldehydes.

While much is known about the stereochemistry (Knowles, 1980) and the coordination of Mg^{2+} to the phosphate of ATP and the products during reactions catalyzed by kinases (Cleland & Mildvan, 1979), the chemical mechanism is still not well understood. pH studies on fructokinase (Raushel & Cleland, 1977b), hexokinase (Viola & Cleland, 1978), and creatine kinase (Cook et al., 1981) have shown that there is an acid–base catalyst (carboxyl for the first two; histidine for the latter) that accepts a proton from the substrate to allow nucleophilic attack on the activated γ-phosphate of MgATP. For hexokinase, X-ray crystallographic studies show an aspartate hydrogen bonded to the 6-hydroxyl of glucose (Anderson et al., 1978; Bennett & Steitz, 1980). In this paper, we compare pH profiles for the ATPase and kinase activities of several kinases in order to establish whether both activities require the same protonation state of the acid–base catalytic group.

Since the hydration equilibrium constant increases by a factor of 1.37 when aldehydes are deuterated (Hill & Milosevich, 1976; Lewis & Wolfenden, 1977), the deuterium isotope effects on V/K for the aldehyde can be used to deduce whether the hydrate or free aldehyde is the activator of the ATPase reaction. For heavily hydrated aldehydes, the value should be 1.37 if the aldehyde is the activator and 1.00 if the hydrate were responsible. Since fructose-6-P kinase showed a normal deuterium isotope of 1.23 on V/K for the ATPase reaction, Viola & Cleland (1980) suggested that the aldehyde was the active species. Their conclusion was based on an incorrectly interpreted NMR spectrum [2,5-anhydromannose is actually almost fully hydrated, not 52% hydrated as reported by Viola & Cleland (1980)], and thus, the isotope effect is too low for the aldehyde to be the only active species. We will report isotope effects for all of the aldehyde-induced ATPase activities we have studied and show that in all cases it appears that the hydrates are the activators, being directly phosphorylated in cases where ^{18}O is transfered from aldehyde to phosphate (Reindina et al., 1984) or inducing phosphate transfer to water (or metal-bound water) when it is not. Normal isotope effects when seen are kinetic ones on breakdown of the phosphorylated hydrate on the enzyme.

Experimental Procedures

Materials. D_2O was from Bio-Rad (99.8 atom % D) or Aldrich (100.00 atom % D), and DCl (>99 atom % D) was from Sigma. [1-^{2}H]Glucose (98 atom % D) was from Stohler. [1,1,2,2-$^2H_4$]Choline bromide (98 atom % D) was from MSD

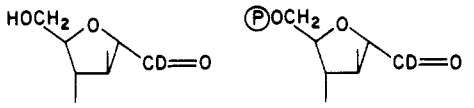
† From the Department of Biochemistry, University of Wisconsin, Madison, Wisconsin 53706. *Received February 22, 1984.* This work was supported by a grant from the National Institutes of Health (GM-18938). A.R.R. was a postdoctoral trainee on Grant AM-07049 from the National Institutes of Health. A preliminary report of this work has been presented (Rendina & Cleland, 1983).

‡ Present address: Chevron Chemical Co., Richmond, CA 94804.

0006-2960/84/0423-5157$01.50/0

Isotopes (Merck). Sodium borodeuteride (97.6 atom % D) was from Bio-Rad, and D_2 gas (>99.5 atom % D) was from Matheson. [^{14}C]Methanol and [γ-^{32}P]ATP were from New England Nuclear. Pd/$BaSO_4$ catalyst and 2,3-butanedione were from Aldrich. In addition to the materials listed in the previous paper (Rendina et al., 1984), the following reagents and enzymes were from Sigma: Bicine,[1] Caps, Pipes, Tes, choline, pyruvate, creatine, D-3-phosphoglycerate, glucose 1,6-bisphosphate, creatinine, D-lyxose, D-glyceraldehyde 3-phosphate, bovine liver glycogen, D-arabinose, oxalate, rabbit muscle aldolase, creatine kinase, myokinase, α-glycerophosphate dehydrogenase, phosphorylase *a*, phosphoglucomutase, *Escherichia coli* acetate kinase, *Candida mycoderma* glycerokinase, *Leuconostoc mesenteroides* glucose-6-phosphate dehydrogenase, *Pseudomonas fluorescens* galactose dehydrogenase, *Propionibacterium freudenreichii* fructose-6-phosphate kinase (pyrophosphate dependent), bovine intestinal alkaline phosphatase, yeast triosephosphate isomerase, glyceraldehyde-3-phosphate dehydrogenase, 3-phosphoglycerate kinase, and phosphoglucose isomerase. *N*-Methylhydantoic acid was synthesized from creatinine by the method of Gaebler (1926). Bovine liver fructokinase was purified by the method of Raushel & Cleland (1977a). All enzymes used were tested for ATPase activity in the absence of aldehydes; in no case was such an activity as much as 5% of the rate in the presence of aldehyde. For kinetic measurements, these background rates were subtracted from the observed ones in the presence of the aldehyde.

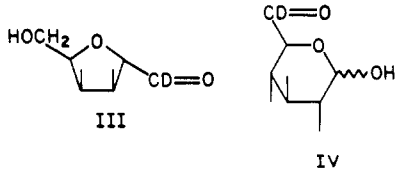
Synthesis and Purification of Deuterated Aldehydes. 2,5-Anhydro[1-^{2}H]mannose (I) and 2,5-anhydro[1-^{2}H]-



I II

mannose 6-phosphate (II) were synthesized from D-arabinose (Viola & Cleland, 1980) and purified on a column of Dowex 50 in the ethylenediamine form (Rendina & Cleland, 1981). 2,5-Anhydromannose solutions were calibrated with hexokinase by using a coupled assay for ADP (Rendina & Cleland, 1981), while concentrations of 2,5-anhydromannose 6-phosphate were determined from the ATPase activity induced by this substrate with fructose-6-phosphate kinase compared to a standard curve, or else by semicarbazone formation (Rendina & Cleland, 1981).

2,5-Anhydro[1-^{2}H]talose (III) was synthesized by nitrous



III IV

acid deamination of D-[1-^{2}H]galactosamine (Defaye, 1964) and purified by Dowex 50–ethylenediamine chromatography (Rendina & Cleland, 1981). The synthesis of D-galactosamine involved catalytic reduction by D_2 in D_2O containing 0.5 M DCl of the 2-aminonitrile made by addition of anhydrous hydrogen cyanide to the Schiff's base of α-D-lyxose and ammonia (Brossmer, 1962). Galactosamine solutions were calibrated with galactose dehydrogenase and showed isotope effects of 5.33 ± 0.11 on V/K and 1.0 on V with that enzyme (data fitted to eq 5). Since 2,5-anhydrotalose was not a substrate for aldehyde or alcohol dehydrogenase and was a poor substrate for hexokinase, solutions were calibrated with semicarbazide and checked by periodate cleavage (Rendina et al., 1984). The isotope effects for the ATPase activity of fructokinase induced by 2,5-anhydrotalose were determined immediately after purification because solutions of this aldehyde at room temperature rapidly eliminate water to form 5-(hydroxymethyl)furfural (Defaye, 1964), which is a substrate for aldehyde dehydrogenase and could be detected in aged solutions of 2,5-anhydrotalose [solutions of 5-(hydroxymethyl)[1-^{2}H]furfural calibrated with aldehyde dehydrogenase gave $^DV/K$ = 1.26 ± 0.02 and ^DV = 1.0 with this enzyme (data fitted to eq 5)].

D-*gluco*-[6-^{2}H]Hexodialdose (IV) was prepared and purified by the procedure of Fischer & Schmidt (1960) except that reduction of D-glucuronic 3,6-lactone with Na/Hg amalgam was conducted in 99.8 atom % D_2O. Solutions of deuterated and unlabeled dialdose were calibrated by measuring A_{230} due to semicarbazone formation (Rendina et al., 1984). Since the proton NMR showed more than 10 distinct resonances in the anomeric proton region and the ^{13}C NMR produced more than 60 lines, these calibrations represented the total concentration of sugar species in equilibrium with the active aldehydo form. However, deuteration at C-6 was confirmed by proton NMR, since half of the signals in the anomeric proton region were missing in the deuterated compound.

D-[1-^{2}H]Glyceraldehyde was isolated from a 20-mL reaction mixture in which 500 μmol of B-side NADD and 60 units of D-glyceraldehyde-3-phosphate dehydrogenase were used to reduce 500 μmol of 1,3-bisphosphoglycerate (generated from 500 μmol of 3-phosphoglycerate, MgATP, and 50 units of 3-phosphoglycerate kinase with 500 μmol of phosphoenolpyruvate and 20 units of pyruvate kinase to recycle ADP). The NADD was generated from oxidation with 25 units of glucose-6-phosphate dehydrogenase of 500 μmol of glucose 6-phosphate made from 500 μmol of [1-^{2}H]glucose with 50 units of hexokinase (MgATP was regenerated with phosphoenolpyruvate and pyruvate kinase). After induction for sufficient time to form D-glyceraldehyde 3-phosphate, the pH was adjusted to 9 with KOH, and 1000 units of alkaline phosphatase was added to liberate D-glyceraldehyde, which was then purified on a Dowex 50–ethylenediamine column (Rendina & Cleland, 1981). Unlabeled D-glyceraldehyde (Sigma) was purified in a similar manner, and solutions of both were calibrated with aldehyde dehydrogenase (Rendina & Cleland, 1981). Specific deuteration at C-1 of D-glyceraldehyde was confirmed by determination of a primary isotope effect with aldehyde dehydrogenase: $^DV/K$ = 2.50 ± 0.17 and ^DV = 1.0 (data fitted to eq 5).

To prepare [1-^{2}H]acetaldehyde, 36.5 mmol of 2,3-butanedione was reduced with 35.9 mmol of $NaBD_4$ (97.6 atom % D from Bio-Rad) in 4 mL of water at 25 °C. After 15 min, the mixture was cooled to 0 °C and Dowex 50, H^+, added to lower the pH to 3 and remove unreacted borodeuteride. The ion-exchange resin was removed by filtration and washed with water, and the filtrate and washings were cooled to 0 °C for 30 min. Excess sodium periodate (50 mmol) was added, and the acetaldehyde was distilled through glass helices with a stream of N_2 gas and collected in a trap cooled with liquid N_2. Solutions of deuterated and unlabeled acetaldehyde (syn-

[1] Abbreviations: Bicine, *N*,*N*-bis(2-hydroxyethyl)glycine; Mes, 2-(*N*-morpholino)ethanesulfonate; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*'-2-ethanesulfonate; Taps, 3-[[tris(hydroxymethyl)methyl]amino]-propanesulfonate; Ches, 2-(*N*-cyclohexylamino)ethanesulfonate; Caps, 3-(*N*-cyclohexylamino)propanesulfonate; Tes, 2-[[tris(hydroxymethyl)-methyl]amino]ethanesulfonate; TLC, thin-layer chromatography; HPLC, high-pressure liquid chromatography.

thesized in the same manner) were calibrated with yeast aldehyde dehydrogenase (Rendina & Cleland, 1981).

[2-^{2}H]Glyoxylate was synthesized by reduction of oxalic acid with magnesium metal in 99.8 atom % D_2O (Lewis & Weinhouse, 1957) followed by purification on a Sephadex A-25 anion-exchange column (eluted with a linear gradient from 0 to 0.8 M $KHCO_3$). Solutions were calibrated with lactate dehydrogenase (Rendina et al., 1984), and specific deuteration was established by observation of a primary isotope effect on V/K of 1.36 ± 0.01 with this enzyme (data fitted to eq 5).

[1,2,2-$^2H_3$]Betaine aldehyde was synthesized by oxidation of 0.49 mmol of [1,1,2,2-$^2H_4$]choline in 5 mL of dimethyl sulfoxide containing 2 mmol of *N,N'*-dicyclohexylcarbodiimide, 0.5 mmol of pyridine, and 0.25 mmol of pyridinium trifluoroacetate (Jones & Moffatt, 1972) and purified by Dowex 50, H^+, column chromatography (washed with water then eluted with a linear gradient of 0–1 M HCl). Choline and betaine aldehyde solutions were calibrated with choline oxidase by using a coupled assay for H_2O_2 (Rendina et al., 1984). Specific deuteration was confirmed by determining the primary isotope effects with choline oxidase. [$^2H_4$]Choline gave $^DV/K$ = 5.09 ± 0.09 and ^DV = 2.51 ± 0.05, and [$^2H_3$]betaine aldehyde gave $^DV/K$ = 2.06 ± 0.07 and ^DV = 2.72 ± 0.08 (data fitted to eq 4).

Whenever possible, both unlabeled and deuterated aldehydes were purified by the same technique, such as Dowex 50–ethylenediamine chromatography. The absence of primary isotope effects on V_{max} with several dehydrogenases indicates that no contaminating inhibitors were present, since the presence of a competitive inhibitor affects V and not V/K. In addition, no bursts in ADP formation were detected with the appropriate kinase and a coupled assay for ADP, suggesting that the aldehyde solutions did not contain the normal substrates [since the normal substrates generally have much higher V/K values than the aldehydes (see Table I), a burst of ADP would be expected if any were present]. The purity of the aldehydes was also checked by TLC, HPLC (Hendrix et al., 1981), and proton NMR (instrumentation described in the preceding paper). Specific deuteration of the aldehyde group was confirmed in each case by proton NMR since the hydrate resonances at 4.7–5.0 ppm were missing in spectra of the deuterated aldehydes. Since the secondary isotope effects on the ATPase activities are predicted to be very small (37% at most), concentrations of the unlabeled and deuterated aldehydes were carefully matched to give the same end points enzymatically or with semicarbazide.

Initial Velocity Studies. Initial velocities were obtained by monitoring absorbance changes at 340 nm in 1-cm cuvettes by using either a Cary 118 spectrophotometer or a Beckman DU monochromator equipped with a Gilford OD converter and a 10-mV strip chart recorder. Assay temperatures were maintained at 25.0 ± 0.1 °C by using thermospacers and a circulating water bath. pH values were measured with a Radiometer 26 pH meter equipped with a combined microelectrode standardized to ±0.01 pH unit. Except for pyruvate kinase, the ATPase and kinase reactions were followed by measuring the production of ADP with a pyruvate kinase/lactate dehydrogenase couple.

Assay mixtures for following the kinase or ATPase activity of *E. coli* glycerokinase contained 0.1 M K-Hepes, pH 8.0, 2 mM $MgCl_2$, 0.5 mM phosphoenolpyruvate, 0.3 mM NADH, 1 mM DTT, 10 units of pyruvate kinase, 14 units of lactate dehydrogenase, 0.125–0.25 unit of *E. coli* glycerokinase, and varying levels of D-glyceraldehyde or glycerol and ATP (up to 0.3 mM) in a total volume of 2 mL. The assays for the ATPase activity of hexokinase were the same as above except that 12.5 units of hexokinase and varying levels of D-*gluco*-hexodialdose and ATP were added with $MgCl_2$ maintained at 1 mM excess over ATP. Assays for the ATPase activity of fructokinase induced by 2,5-anhdrotalose contained 0.05 M Pipes, pH 7.0, 0.1 M KCl, 1 mM PEP, the remaining components above, 0.34 unit of fructokinase, and varying levels of 2,5-anhydrotalose and ATP, with $MgCl_2$ present at 2 mM excess over ATP. Choline kinase ATPase activity was assayed in the same manner with 0.1 M Hepes, pH 8.3, 0.1 unit of choline kinase, 15 mM $MgCl_2$, and varying amounts of betaine aldehyde and ATP (up to 1.5 mM). In each case, the reactions were started by the addition of substrate. For each aldehyde-induced ATPase reaction, the release of inorganic phosphate was confirmed by a phosphomolybdate assay (Lanzetta et al., 1979).

ATP solutions were calibrated by summing the ATP and ADP contents when coupled assays for ADP were used. The end-point assay for ATP contained 0.1 M Hepes, pH 8, 5 mM $MgCl_2$, 0.5 mM NAD, 5 mM glucose, and 50 units each of hexokinase and glucose-6-phosphate dehydrogenase in 2 mL. The ADP content of ATP solutions was determined in 2-mL solutions containing the components of the coupled assay for ADP described above. Glycerol solutions were calibrated by using glycerokinase and the coupled assay for ADP.

The glyoxylate-induced ATPase activity of pyruvate kinase was monitored by fixed time assay of the production of inorganic phosphate. At 5- or 10-min intervals, 5-μL aliquots of the assay mixtures were quenched with 45 μL of 0.1 M acetic acid, and the inorganic phosphate concentration was determined by the method of Lanzetta et al. (1979), which can detect 1 nmol of phosphate. The components of the reaction mixture (in particular ATP) are stable under the conditions of this assay. The absorbance was calibrated with standard curves run on known phosphate solutions. Assay mixtures for following the ATPase activity of pyruvate kinase contained 0.1 M buffer (either Mes, pH 6.5, Taps, pH 8.47, or Ches, pH 9.9), 0.1 M KCl, 10–50 units of pyruvate kinase (Boehringer-Mannheim), and varying amounts of glyoxylate and ATP, with $MgCl_2$ present at 5 mM excess over ATP. At least four time points were sampled at four different levels of glyoxylate and four different levels of ATP at each pH. Glyoxylate did not interfere with the determination of phosphate at any level used at any of the pHs.

Inhibition Studies. Aldehydes that did not induce ATPase activities with particular kinases were examined as competitive inhibitors of those enzymes. For acetate kinase, 3-phosphoglycerate kinase, and creatine kinase, the normal substrates were varied at different fixed levels of the inhibitors acetaldehyde, D-glyceraldehyde 3-phosphate, and *N*-methylhydantoic acid, respectively, and the initial velocities were obtained by the coupled assay for ADP at pH 8.0, 25 °C. Specific conditions used are listed in the footnotes to Table II. Assay mixtures for the inhibition of the pyrophosphate-dependent fructose-6-phosphate kinase by 2,5-anhydromannose 6-phosphate contained 0.1 M Taps, pH 8.0, 2 mM pyrophosphate, 5 mM $MgCl_2$, 0.25 mM NADH, 8 units of aldolase, 7 units of triosephosphate isomerase, 4 units of α-glycerophosphate dehydrogenase, 0.05 unit of fructose-6-phosphate kinase (pyrophosphate-dependent), and varying amounts of fructose 6-phosphate at fixed levels of 2,5-anhydromannose 6-phosphate. Solutions of the normal substrates of these kinases were calibrated by allowing the kinase reactions to go to completion and by recording the net change in absorbance

with the coupled assays described above (D-glyceraldehyde-3-phosphate dehydrogenase was added to the assay mixture for 3-phosphoglycerate determinations).

pH Studies. For fructokinase and glycerokinase in the direction of ATP hydrolysis, the coupled assay for ADP was used as described above at 25 °C. The following buffers were used at 0.1 M concentrations at the stated pH values: Mes (5.5–6.5), Pipes (7.0), Hepes (8.0), Bicine (8.44), and Caps (9.3–9.45). Fructokinase acts on 2,5-anhydromannose in two ways, phosphorylating the hydroxymethyl group at C-6 or giving an ATPase activity when C-1 of the hydrate is adjacent to MgATP, while hexokinase only phosphorylates 2,5-anhydromannose at C-6 (Viola & Cleland, 1980). Therefore, the ratio of the excess ADP formed during the fructokinase reaction compared to the hexokinase reaction is a measure of the ratio of the V/K values for the ATPase reaction and the kinase reaction with 2,5-anhydromannose.

Isotope Effects. Except where noted, isotope effects on intial velocities were obtained at ATP concentrations at least $5K_m$ in the presence of excess Mg^{2+} by comparing deuterated and unlabeled substrates at 25 °C (no substrate inhibition was observed by these ATP levels). Conditions for the determination of isotope effects on the aldehyde-induced ATPase reactions were very similar to those described for the initial velocity, inhibition, and pH studies (that is, 0.1 M Hepes, pH 8.0, 25 °C, and the components of the coupled assay for ADP). Specific conditions are listed in the tables.

Conditions for the primary deuterium isotope effects used to check for specific deuteration of the aldehydes are listed below. For the galactose dehydrogenase isotope effect with [1-^{2}H]galactosamine, conditions were 0.1 M Hepes, pH 8.5, 1 mM NAD, 5 mM dithiothreitol, and 2.1 units of galactose dehydrogenase. For the aldehyde dehydrogenase isotope effect determinations with 5-(hydroxymethyl)[1-^{2}H]furfural or D-[1-^{2}H]glyceraldehyde, reaction mixtures contained 0.1 M Hepes, pH 8.0. 0.1 M KCl, 1 mM NAD, 5 mM dithiothreitol, and 1.2 units of aldehyde dehydrogenase. The coupled assay for hydrogen peroxide described in the preceding paper (Rendina et al., 1984) was used to determine the isotope effects for choline oxidase with [1,1,2,2-^{3}H$_4$]choline and [1,2,2-^{2}H$_3$]betaine aldehyde in 0.1 M Hepes, pH 8.0, 25 °C. Choline solutions were calibrated with choline kinase by allowing coupled assays for ADP (see above) to reach completion in the presence of aliquots of labeled or unlabeled choline. Isotope effects for lactate dehydrogenase with [2-^{2}H]glyoxylate were determined by assays described by Rendina et al. (1984). The dehydrogenases were used to calibrate solutions of their respective substrates by allowing assays to reach completion and measuring the net change in absorbance.

Data Analysis. Reciprocal initial velocities were plotted vs. reciprocal substrate concentrations, and all plots were linear. All data were fitted to the appropriate equations with the Fortran programs of Cleland (1979a). Individual saturation curves were fitted to eq 1. Data obtained by varying the levels

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

of two substrates were fitted to eq 2. Competitive inhibition

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

data were fitted to eq 3, where I represents the inhibitor

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

concentration. Data for the direct comparison of rates with unlabeled and deuterated substrates were fitted to eq 4, which

$$v = VA/[K(1 + F_iE_{V/K}) + A(1 + F_iE_V)] \quad (4)$$

assumes different isotope effects on V and V/K, or to eq 5 and

$$v = VA/[K(1 + F_iE_{V/K}) + A] \quad (5)$$

6, which assume isotope effects on V/K and V only, respec-

$$v = VA/[K + A(1 + F_iE_V)] \quad (6)$$

tively. Where appropriate, the data were also fitted to eq 7,

$$v = VA/[(K + A)(1 + F_iE_V)] \quad (7)$$

which assumes equal isotope effects on V and V/K. In these equations, F_i is the fraction of deuterium label in the substrate, and E_V and $E_{V/K}$ are the isotope effects minus 1 on V and V/K, respectively. Data were also fitted to forms of eq 1 to 7 where the logarithm was taken of both sides (this changes the assumed error distribution from one with constant errors in initial velocities to one with proportional errors). Data for pH profiles showing a drop in log V or log (V/K) with a slope of 1 as the pH is lowered were fitted to eq 8, while when log V or log

$$\log y = \log [C/(1 + H/K_1)] \quad (8)$$

(V/K) decreased at both high and low pH, the data were fitted to eq 9. In these equations, K_1 and K_2 are the dissociation

$$\log y = \log [C/(1 + H/K_1 + K_2/H)] \quad (9)$$

constants for groups on the enzyme, y is V or V/K, and C is the pH-independent value of y. The data for log V and log (V/K) as a function of pH with the glyoxylate-induced ATPase activity of pyruvate kinase were fitted to eq 10, where y is the

$$\log y = \log ([YL + YH(K_1/H)]/[1 + K_1/H]) \quad (10)$$

observed value of the parameter, YL is the value of y at low pH, YH is the value of y at high pH, and K_1 represents the dissociation constant of the group whose protonation decreases y. Data for the lag in phosphate released during the ATPase reaction of glycerokinase were fitted to eq 11, where y is the

$$y = Ae^{-kt} + Bt + C \quad (11)$$

absorbance at 340 nm, t is elapsed time, A and k describe the apparent first-order region of the curve, B is the final steady-state rate, and C is the absorbance at $t = 0$.

^{18}O Transfer Experiments. Procedures and instrumentation for monitoring the transfer of ^{18}O from the aldehydes to inorganic phosphate during the ATPase reactions are described in detail in the preceding paper (Rendina et al., 1984).

Methanol Trapping Experiments. We attempted to trap possible phosphorylated oxycarbonium ion intermediates with methanol present in relatively high concentrations in aqueous solution. If [^{14}C]methanol and [γ-^{32}P]ATP are used, the phosphorylated methyl hemiacetal formed in this way would be stable and doubly labeled. Conditions for trapping intermediates generated during the glycerokinase ATPase reaction were 40% methanol (v/v) containing 1 mCi of [^{14}C]methanol (20 μCi/mmol), 30 mM ATP containing 1.5 μCi of [γ-^{32}P]ATP (10 μCi/mmol), 10 mM magnesium diacetate, 5 mM D-glyceraldehyde, and 3 mg of *E. coli* glycerokinase in 5.05-mL total volume. [^{14}C]Methanol was distilled and passed through an anion-exchange column prior to use. [γ-^{32}P]ATP was purified by ion-exchange chromatography on Sephadex A-25 prior to use. The control was identical with the sample except that no glyceraldehyde was added. The pH was maintained at 8 with 0.1 M KOH and monitored continuously for 8 h until the ATP was consumed in the sample. Excess [^{14}C]methanol was removed by repeated rotary evaporation with unlabeled methanol, and the samples were diluted to 50 mL with water and loaded onto identical Dowex 1, Cl, columns

Table I: Comparison of Kinetic Constants for Normal and Aldehyde Substrates[a]

enzyme	substrate	activity	rel V_{max}	$K_{substrate}$ (mM)	$K_{i\ substrate}$ (mM)	K_{ATP} (mM)	$K_{i\ ATP}$ (mM)	rel V/K_{sub}
E. coli glycerokinase	glycerol[b]	K	100	0.008 ± 0.003	0.041 ± 0.009	0.042 ± 0.009	0.212 ± 0.088	100
	D-glyceraldehyde[b]	A	20	0.54 ± 0.07	3.94 ± 0.57	0.022 ± 0.004	0.162 ± 0.019	0.294
fructose-6-P kinase	fructose-6-P[c]	K	100	0.07 ± 0.009	0.002 ± 0.008	0.027 ± 0.003	0.001 ± 0.003	100
	2,5-anhydromannose-6-P[d]	A	3	0.30 ± 0.05	0.65 ± 0.32	0.003 ± 0.001	0.007 ± 0.002	0.70
fructokinase	fructose[e]	K	100	0.10 ± 0.01	0.29 ± 0.04	0.17 ± 0.02	0.51 ± 0.08	100
	2,5-anhydromannose[e]	K + A	59	1.0 ± 0.1	5.8 ± 0.6	0.30 ± 0.02	1.8 ± 0.2	5.9
	2,5-anhydrotalose[f]	A	21	0.67 ± 0.14	3.15 ± 0.74	0.24 ± 0.07	1.12 ± 0.16	3.1
hexokinase	glucose[g]	K	100	0.10	0.1	0.1	5	100
	D-*gluco*-hexodialdose[h]	A	0.09	~0.01	>5	<0.008	5	0.18
	lyxose[i]	A	0.07	17.2 ± 0.1		0.1[i]	5	0.0004
choline kinase	choline[j]	K	100	0.15	0.15	0.14	0.14	100
	betaine aldehyde[k]	A	7.0	0.15 ± 0.01	0.34 ± 0.08	0.034 ± 0.005	0.074 ± 0.011	0.7
pyruvate kinase	pyruvate[l]	K	100	6.0	6.0	0.66	0.66	100
	glyoxylate[m]	A	18.0	3.5 ± 0.8	3.24 ± 0.98	0.9 ± 0.32	0.84 ± 0.14	31

[a] The rel V and rel V/K_{sub} are expressed as a percentage of that with the normal substrate and were determined separately for each enzyme by using a coupled assay for ADP in each case except with pyruvate kinase where fixed-time assays for P_i were used. Values reported here for each pair of substrates were determined under the same pH, temperature, and assay conditions and at saturating MgATP. The remaining kinetic constants are from the literature as noted or else from the present work. Under the column headed activity, K means kinase and A means ATPase. [b] Kinetic parameters were determined at pH 8, and the data were fitted to the log form of eq 2. [c] Kinetic parameters were determined at pH 7.4 by Bar-Tana & Cleland (1974). [d] Determined at pH 7.9 (Viola & Cleland, 1980). [e] Determined at pH 7.0 (Raushel & Cleland, 1977a). The values for 2,5-anhydromannose represent the sum of ATPase and kinase activities of the substrate, although the kinase activity dominates: relative V/K for ATPase to kinase = 0.15 (this work). [f] Kinetic parameters determined at pH 7.0; data were fitted to the log form of eq 2. [g] Determined at pH 8 by Danenberg & Cleland (1975). $K_{i\ MgATP}$ appears to be 0.1 mM from the initial velocity pattern but is actually ~5 mM. [h] Parameters determined at pH 8. The binding of MgATP and the dialdose is so synergistic that it is difficult to determine either Michaelis constant because the K_aB and K_bA terms in eq 2 are so small. The constant term is 0.04 mM^2, however; so if $K_{i\ MgATP}$ is taken as 5 mM, the K_m of the dialdose is 8 μM. The K_i for the dialdose is at least 5 mM, which makes K_{MgATP} less than 8 μM, the lowest value known for any hexokinase substrate. [i] DelaFuenta et al. (1970) at pH 7.0, 30 °C. This is an ATPase activity. [j] Brostrom & Browning (1973) at pH 8.5. [k] Data were obtained at pH 8.3 and fitted to eq 2. [l] Dougherty (1982) at pH 8.0. [m] Kinetic parameters determined at pH 8.5; data were fitted to the log form of eq 2.

previously equilibrated with water. After being washed with 300 mL of water to remove methanol and glyceraldehyde, the columns were eluted with 1-L linear gradients from 0 to 0.5 M NH_4Cl in 50 mM NH_4BO_3 at pH 8.5 (7-mL fractions). ^{32}P and ^{14}C were counted simultaneously in 1-mL aliquots of each fraction in a scintillation counter.

For the trapping experiments with the fructose-6-phosphate kinase ATPase, the sample contained in 2.22 mL of 40.5% methanol (v/v), 0.1 M NH_4-Hepes, pH 8.0, 25 °C, 30.8 mM ATP containing 2.2 μCi of [γ-^{32}P]ATP, 20 mM magnesium diacetate, 1 mM citrate, 9 mM 2,5-anhydromannose 6-phosphate, and 420 units of fructose-6-phosphate kinase. The control was identical except that no 2,5-anhydromannose 6-phosphate was added. After 8 h, both sample and control were treated in the same manner as described for the glycerokinase experiments except that components of the reaction mixture were separated at 4 °C on identical 2 × 30 cm anion-exchange columns of Sephadex A-25, eluted with a 0.6-L linear gradient from 0.05 to 0.8 M in triethylammonium bicarbonate, pH 7.6 at 25 °C. The entire fraction was counted without fluor by using the Cerenkov radiation of ^{32}P. A separate experiment was conducted for this ATPase activity where the reactions were quenched after 8 h with 20 mM glucose, 50 units of hexokinase, and 100 units of myokinase, which converted all ADP and ATP to AMP and glucose 6-phosphate. This was done in order to detect any ^{32}P-labeled components that might elute in the same place as ADP or ATP. Conditions and workup of this experiment were the same as the other trapping experiment with fructose-6-phosphate kinase.

Lag in Phosphate Released during Glycerokinase ATPase Reaction. The rate of inorganic phosphate formation was determined from the increase in absorbance at 340 nm resulting from the formation of NADH in the following coupling system (Knight et al., 1981): phosphorylase *a*, 60–100 units/mL; glycogen, 25 mM in glucose units; AMP, 0.1 mM; phosphoglucomutase, 100 units/mL; glucose 1,6-bisphosphate, 12 μM; phosphoglucose isomerase, 100 units/mL; glucose-6-phosphate dehydrogenase, 80 units/mL; NAD, 1 mM. The reactions were initiated by the addition of *E. coli* glycerokinase to 1-mL reaction mixtures containing 50 mM Pipes, pH 7.0, 1 mM ATP, 2 mM $MgCl_2$, 9 mM D-glyceraldehyde, and 1 mM dithiothreitol, at 15 ± 0.1 °C. Mixing times were <10 s. Inorganic phosphate was removed from the coupling enzymes and glycerokinase by centrifugal desalting (Neal & Florini, 1973). Residual phosphate in the reagents was consumed by the coupling system prior to initiation of the reaction with glycerokinase (the base line was flat). The amount of glycerokinase was adjusted so that the final steady-state rate nearly matched the initial rate observed upon addition of 5 μM phosphate.

Phosphoglucose isomerase was added to the coupling system to catalyze the conversion of α-glucose 6-phosphate (the product of phosphoglucomutase) to β-glucose 6-phosphate (the substrate for glucose-6-phosphate dehydrogenase; Wurster & Hess, 1975). The mutarotation lag is about 6.3 s at 25 °C and would be expected to be larger at lower temperatures (Cleland, 1979b). When this coupling system was used to study the pyruvate kinase ATPase reaction, phosphoglucose isomerase was left out of the reaction mixture since the mixing times were longer than 10 s.

Results and Discussion

Initial Velocity Studies. In addition to the ATPase activities induced by D-glyceraldehyde with glycerokinase from *E. coli* (Hayishi & Lin, 1967) or from *Candida* (Gancedo et al., 1968) and those reported for fructose and fructose-6-phosphate kinases with 2,5-anhydromannose and 2,5-anhydromannose 6-phosphate, respectively (Viola & Cleland, 1980), we have found four additional aldehyde-induced ATPase activities. The kinetic constants for the normal substrates are compared to those for the aldehyde analogues in Table I. Coupled enzymatic assays for ADP were used in each case except pyruvate kinase, but the release of inorganic phosphate was confirmed

Table II: Comparison of Kinetic Constants for Substrates and Aldehyde Inhibitors

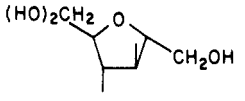
enzyme	substrate	K_m (mM)	inhibitor	K_i (mM)
acetate kinase[a]	acetate	98 ± 9	acetaldehyde	57 ± 2
3-P-glycerate kinase[b]	3-P-glycerate	0.25 ± 0.05	D-glyceraldehyde-3-P	0.68 ± 0.15
creatine kinase[c]	creatine	18 ± 3	*N*-methylhydantoic acid	109 ± 14
fructose-6-P-kinase (pyrophosphate-dependent)[d]	fructose-6-P	0.37 ± 0.01	2,5-anhydromannose-6-P	4.73 ± 0.19

[a] Conditions were 0.1 M Hepes, pH 8.0, 25 °C, 1 mM ATP, and 2 mM $MgCl_2$; data were fitted to eq 3. [b] Data were obtained at 0.1 M Hepes, pH 8, 25 °C, 3 mM ATP, and 5 mM $MgCl_2$ and fitted to the log form of eq 3. [c] Conditions were the same as in footnote *b* except the data were fitted to eq 3. [d] Kinetic parameters were determined in 0.1 M Taps, pH 8, with 2 mM pyrophosphate and 5 mM $MgCl_2$. The data were fitted to eq 3.

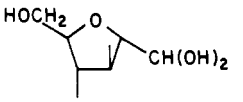
for all the ATPase activities with either fixed-time assays or coupled enzymatic assays for inorganic phosphate. The coupled assay for P_i could not be used when 2,5-anhydromannose 6-phosphate was present, since this aldehyde strongly inhibited phosphoglucomutase, one of the enzymes in the coupled assay.

The kinetic parameters we have observed for *E. coli* glycerokinase are similar to those reported by Thorner & Paulus (1973) for the same enzyme and comparable to those reported for the *Candida* enzyme by Janson & Cleland (1974). Previously reported data for fructose and fructose-6-phosphate kinases with 2,5-anhydromannose and its 6-phosphate are included for comparison with the other ATPase reactions.

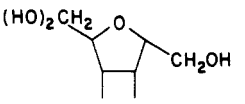
With fructokinase, 2,5-anhydromannose is phosphorylated at C-6 (in binding mode V) as well as inducing an ATPase



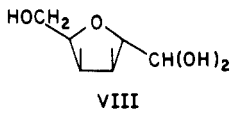
V



VI



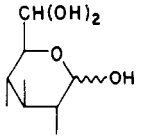
VII



VIII

activity when C-1 binds to the active site in binding mode VI (Viola & Cleland, 1980). 2,5-Anhydrotalose, which has the opposite configuration at C-4 from 2,5-anhydromannose, is not phosphorylated but only induces ATPase activity, since it only assumes binding mode VIII and not VII. Greater than 10 times the amount of ADP and phosphate are released than the amount of 2,5-anhydrotalose added. Because of the inverted configuration at C-4, 2,5-anhydrotalose is also a very poor substrate for hexokinase (V = 0.07% that of glucose; K_m = 0.14 M).

Although glucopyranose, gulopyranose, and fused-ring furanose–furanose and furanose–pyranose structures are possible for D-*gluco*-hexodialdose and on the basis of the proton and ^{13}C NMR spectra all probably exist, it is presumably the hydrate (IX) that is the activator of the ATPase activity



IX

observed with hexokinase. The relative V_{max} for this aldehyde-induced ATPase activity is quite low compared to that for glucose but is comparable to that observed with the ATPase induced by D-lyxose or D-xylose (DelaFuente et al., 1970). However, D-*gluco*-hexodialdose is much more tightly bound than D-lyxose, and product release may be the slow step limiting V_{max} in this case. The low V_{max} and K_m might also result from nonproductive binding of one or more of the other anomeric forms present in solution, especially those with a glucopyranose ring and a second hemiacetal ring formed by reaction of the β-hydroxyl at C-1 or the hydroxyl at C-3 with the 6-aldehyde group. Such structures ought to fit the active site well but would probably not induce ATPase activity. Both the lyxose- and aldehyde-induced ATPase activities are about 20 times faster than the native ATPase activity of hexokinase in the absence of sugar substrate under optimal conditions (DelaFuente et al., 1970). As we will show later, the aldehyde-induced ATPase of hexokinase has a different mechanism than the ATPase induced by lyxose, which has no phosphorylatable functional group in the 6-position.

Betaine aldehyde is structurally similar to choline, except that again an aldehyde group replaces the hydroxymethyl group that is normally phosphorylated by choline kinase and an ATPase activity is observed. With pyruvate kinase, glyoxylate, which is an aldehyde analogue of glycolate, a reasonably good substrate for pyruvate kinase ($V_{rel\ Pyr} \simeq 1$; K_m = 2.3 mM; Kayne, 1974), induced a relatively rapid ATPase activity. The aldehyde group or hydroxyl group of these substrates replaces the enolate form of pyruvate, which is thought to be formed prior to phosphorylation (Kuo et al., 1979).

The relative maximal velocities for the ATPase activities reported in Table I are 3–20% of the V_{max} observed with the normal substrates with the exception of hexokinase, where nonproductive binding of other anomers may lower the observed V_{max}. The aldehydes have K_m's and dissociation constants comparable to those observed with the normal alcohol substrates. They also produce similar K_m's for MgATP as with the normal substrates, suggesting that these analogues induce the same conformation changes (presumably necessary to activate the γ-phosphate of MgATP for transfer) in the kinases as binding of the normal substrates.

Inhibition Studies. Not all of the aldehyde analogues that we tried with the appropriate kinases induced an ATPase activity. Several were competitive inhibitors vs. the normal substrates, and the apparent K_m's for the substrates are compared to the inhibition constants in Table II. These kinases were tested for ATPase activity at saturating MgATP levels in the presence of aldehyde concentrations from 5 to 10 times larger than their respective inhibition constants and at 100–1000 times more enzyme than was required for kinase activity, but no ADP or phosphate was detected. Possible explanations for the failure to observe an aldehyde-induced ATPase activity with these kinases are discussed later.

pH Studies. Detailed pH studies on fructokinase (Raushel & Cleland, 1977b) and hexokinase (Viola & Cleland, 1978) indicate that the chemical mechanisms for these two kinases are similar, with what was deduced from temperature variation and solvent perturbation studies to be a carboxylate group on the enzyme accepting a proton from the sugar hydroxyl in the forward direction an donating a proton in the reverse reaction. X-ray work (Anderson et al., 1978) confirms the presence of a group (probably aspartate) on hexokinase that is interacting with the 6-hydroxyl group of glucose in the enzyme–glucose

Table III: Kinetic Parameters as a Function of pH for Glyoxylate-Induced ATPase Activity of Pyruvate Kinase[a]

pH	V_{max}	K_{ATP} (mM)	$K_{i\,ATP}$ (mM)	V/K_{ATP}	$K_{glyoxylate}$ (mM)	$K_{i\,glyoxylate}$ (mM)	$V/K_{glyoxylate}$
6.5	4.88 ± 2.06	1.52 ± 1.39	33.3 ± 29.5	3.2 ± 1.7	1.49 ± 1.8	32.5 ± 18.8	3.28 ± 2.77
8.46	26.5 ± 2.6	0.90 ± 0.32	0.84 ± 0.14	29.3 ± 6.3	3.5 ± 0.8	3.24 ± 0.98	7.57 ± 0.57
9.99	169.0 ± 79	8.6 ± 5.4	1.65 ± 1.05	19.8 ± 3.5	2.5 ± 1.7	0.48 ± 0.29	67.9 ± 15.4

[a] Data were fitted to eq 2 or its log form. Velocities are expressed as μmol min^{-1} (mL of enzyme)$^{-1}$. Conditions were 0.1 M buffer (Mes, pH 6.5; Taps, pH 8.46; Ches, pH 9.99), 0.1 M KCl, and $MgCl_2$ in 5 mM excess over ATP. Fixed-time assays for P_i released were used. At pH 6.5, the initial velocity pattern is nearly equilibrium ordered with MgATP adding first. At both pH 6.5 and 9.99, glyoxylate gives substrate inhibition at high concentrations above 6 mM at pH 6.5 and 2 mM at pH 10, so the initial velocity patterns were obtained at low, noninhibitory levels of glyoxylate.

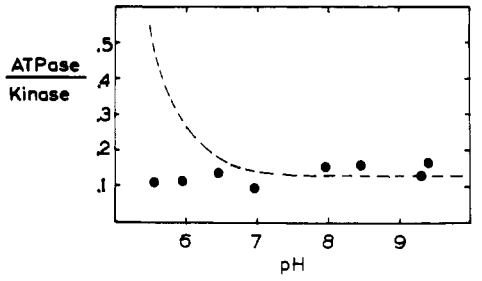


FIGURE 1: pH profile for fructokinase ATPase and kinase activities induced by 2,5-anhydromannose. At each pH, the same amount of 2,5-anhydromannose was added to end-point assay mixtures containing 3 mM ATP, 10 mM $MgCl_2$, and either 350 units of hexokinase or 2 units of fructokinase at 25 °C. We assume that negligible ATPase activity occurs with hexokinase (Viola & Cleland, 1980). The dashed line shows the predicted behavior if the ATPase reaction did not decrease below a pK of 6, as the kinase reaction does (Raushel & Cleland, 1977b).

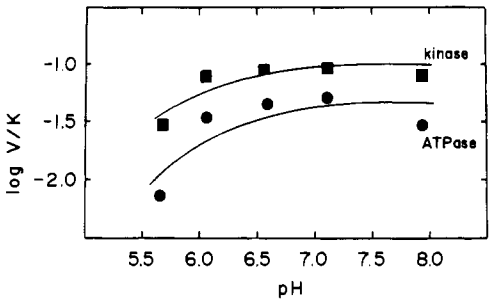


FIGURE 2: Glycerokinase pH profile with variable MgATP. The concentrations of MgATP were corrected for a pK of 5.5 for these complexes. The kinase profile (■) was obtained with 330 μM glycerol and 3 mM $MgCl_2$ at 25 °C. ATPase activity (●) was obtained with 7.5 mM D-glyceraldehyde and 3 mM $MgCl_2$ at 25 °C. Lines shown are from fits of these data to eq 8.

complex. The pH dependence of three kinases and their ATPase activities were studied to see whether the same protonation state of the acid–base catalyst was required for both kinase and ATPase activities.

Figure 1 shows the pH dependence of the ratio of V/K values for the ATPase and kinase activities of fructokinase with 2,5-anhydromannose. The ratio of the V/K's is constant from pH 5.5 to 9.5, with a value of 0.15. The ATPase reaction must have the same pH dependence as the kinase activity, since V/K for the kinase reaction with fructose decreases below a pK of 6 (Raushel & Cleland, 1977b). Both ATPase and kinase reactions thus require the same protonation state of the carboxyl acid–base catalytic group.

The pH profiles of V/K_{MgATP} for the phosphorylation of glycerol catalyzed by *E. coli* glycerokinase and the ATPase activity induced by D-glyceraldehyde are shown in Figure 2. These profiles decreased below pK's of 5.9 ± 0.1 for the kinase activity and 6.14 ± 0.15 for the ATPase. V_{max} for the ATPase decreased below a pK of 5.67 ± 0.07, while V for the kinase reaction decreased below a pK of 7.01 ± 0.06. For the reverse reaction of glycerokinase, V decreased below a pK of 6.9 ± 0.15 and above a pK of 7.6 ± 0.16, while V/K_{MgADP} decreased below a pK of 6.54 ± 0.11 and above a pK of 7.56 ± 0.12 (data, which are not shown, were fitted to eq 9).

The V/K's of both kinase and ATPase activities decrease with a pK around 6, and if this pK is due to an acid–base catalyst similar to the ones proposed for fructokinase and hexokinase, the same protonation state of that group is required for both activities. Further work with the temperature and solvent perturbation of the pH profiles is needed to determine whether this enzyme group is a carboxylic acid residue as proposed for fructokinase and hexokinase. The pK of the catalytic group is perturbed to 7.6 in the V/K profile of ADP for the reverse reaction, presumably due to the stickiness of glycerol phosphate (when a substrate is sticky, it reacts to give products faster than it dissociates from the enzyme), although more experiments are needed to demonstrate this. A similar

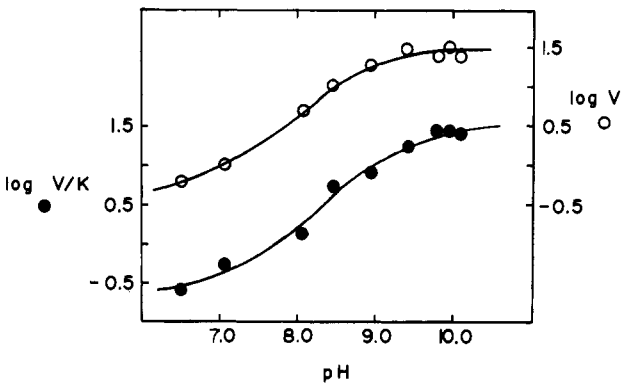


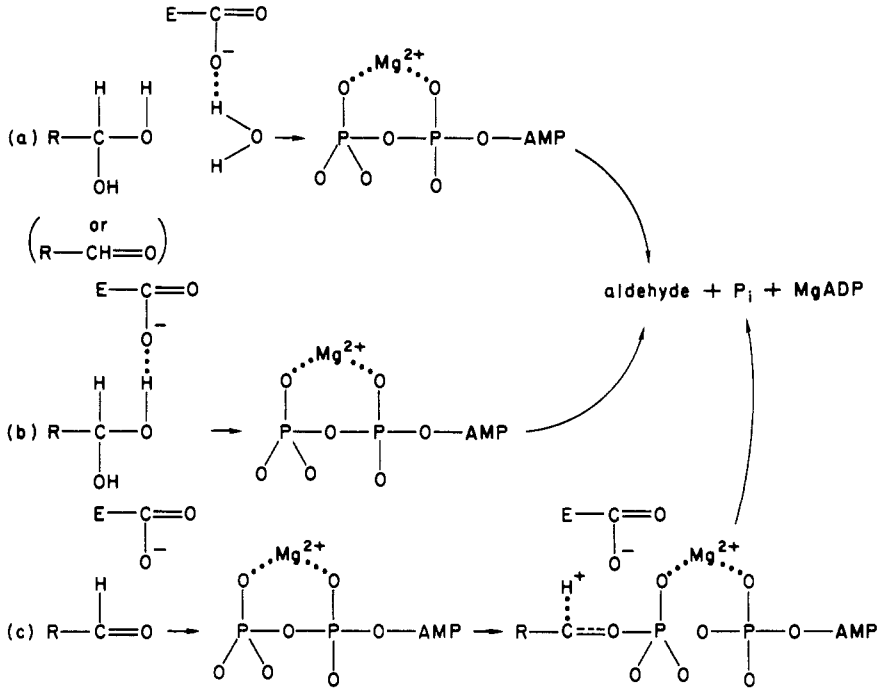
FIGURE 3: pH dependence of V and V/K for the glyoxylate-induced ATPase activity of pyruvate kinase obtained by using fixed-time assays for P_i at 2 mM ATP, 15 mM $MgCl_2$, and variable levels of glyoxylate at 25 °C. The solid lines represent fits of the data to eq 10.

displacement was observed for V/K_{MgADP} for the reverse reaction of hexokinase (Viola & Cleland, 1978), where glucose has been shown to be a sticky substrate (Rose et al., 1974).

The pH profiles for V and V/K for the glyoxylate-induced ATPase activity of pyruvate kinase determined at saturating MgATP are presented in Figure 3. Both parameters reach a constant value at high and low pH. V decreases below a pK of 8.80 ± 0.07 and reaches a plateau on the acid side at a pK of 7.0 ± 0.1, while V/K drops below a pK of 9.3 ± 0.1 and levels out at low pH with a pK of 7.25 ± 0.15 (data fitted to eq 10). Table III shows the kinetic parameters from initial velocity patterns for glyoxylate and MgATP at three pH values.

The V/K profile with glycolate as variable substrate also decreases below a pK near 9 (Dougherty, 1982), showing that the ATPase reaction prefers the same protonation state of the catalytic group that has this pH dependence. This pK is probably that of water coordinated to a second divalent Mg^{2+} in the active site of pyruvate kinase. Since no isotope effect is observed with the ATPase activity (Table V) and no ^{18}O is transferred from glyoxylate to inorganic phosphate [Table

Scheme I: Possible Mechanisms for Aldehyde-Induced ATPase Reaction[a]

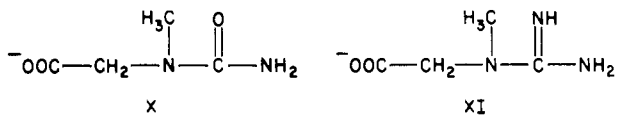


[a] (a) Transfer to water induced by the hydrate or free aldehyde. (b) Direct phosphorylation of the hydrate to give an adduct decomposing on or off of the enzyme. (c) Phosphorylation of free aldehyde to give an oxycarbonium ion stabilized by a carboxyl group on the enzyme and reacting subsequently with water on or off of the enzyme to give an unstable phosphorylated hydrate.

I of Rendina et al. (1984)], the hydrate of glyoxylate probably induces the proper conformation of pyruvate kinase to allow attack of Mg···OH^- on the γ-phosphate of ATP. The form of the pH profile implies that Mg-bound water can also attack MgATP, but at a 100-fold slower rate.

On the basis of these pH profiles, it appears that when aldehyde-induced ATPase activities are observed, both kinase and ATPase activities require the same protonation state of the acid–base catalyst on the enzyme. On the other hand, when no ATPase activity is observed with suitable aldehyde analogues, as with acetate and 3-phosphoglycerate kinases, the substrates are anions at neutral pH, and these kinases do not require an acid–base catalyst to activate the substrates. The lack of an acid–base catalyst may thus explain why no ATPase activity is observed with suitable aldehyde analogues in these cases.

With creatine kinase, *N*-methylhydantoic acid (X) is a keto



X XI

rather than an aldehyde analogue of creatine (XI). Its failure to induce an ATPase activity or to be phosphorylated probably reflects its chemical inertness (it is a substituted urea) and also possibly the lack of a positive charge on the ureido group.

While not much is known about the mechanism of the pyrophosphate-dependent fructose-6-phosphate kinase, it presumably requires an acid–base catalyst to remove a proton from fructose 6-phosphate. It is reported to use 2,5-anhydromannitol bisphosphate as a slow substrate (Bertagnolli & Cook, 1984), and thus it is not clear why it fails to show a PPase activity with 2,5-anhydromannose 6-phosphate.

^{18}O Transfer from Aldehydes to Phosphate. Several mechanisms are possible for an aldehyde-induced ATPase (Scheme I). First, binding of the hydrate or free aldehyde induces a nucleophilic attack by a bound water molecule on the activated γ-phosphate of MgATP. Second, direct phosphorylation of one of the hydroxyl groups of the hydrate with proton removal by the acid–base catalyst gives a phosphorylated hydrate that subsequently decomposes either on or off the enzyme to phosphate and free aldehyde. Third, phosphorylation of a free aldehyde could produce an oxycarbonium ion stabilized by the negative charge on the acid–base catalyst but decomposing to phosphate and free aldehyde upon reaction with water. The latter two mechanisms will involve transfer of ^{18}O from labeled aldehydes (in equilibrium with their hydrates) to inorganic phosphate during the ATPase reactions, but no ^{18}O will be incorporated into P_i when an induced transfer to water takes place. The data in Table I of the preceding paper (Rendina et al., 1984) show direct phosphorylation mechanisms with ^{18}O transfer to phosphate for the ATPase reactions of glycerokinase with D-glyceraldehyde, fructose-6-phosphate kinase with 2,5-anhydromannose 6-phosphate, fructokinase with either 2,5-anhydromannose or 2,5-anhydrotalose, and hexokinase with D-*gluco*-hexodialdose.

The remaining two ATPase reactions catalyzed by pyruvate kinase with glyoxylate and choline kinase with betaine aldehyde proceed by induced transfer of phosphate to metal-bound water or hydroxide as discussed above for pyruvate kinase or else by induced transfer to a bound water molecule. Further studies on choline kinase are needed to distinguish between these two possibilities. The latter mechanism applies to the lyxose-induced ATPase activity of hexokinase where the binding of lyxose promotes the necessary conformation change to activate the γ-phosphate of ATP toward nucleophilic attack by a water molecule that binds in the pocket left by the missing 6-(hydroxymethyl) group of the substrate (Dela-Fuente et al., 1970; Danenberg & Cleland, 1975).

^{18}O transfer studies do not usually distinguish whether the aldehyde or the hydrate is the activator of the ATPase activity, since ^{18}O will be transferred to phosphate in either case if the reaction proceeds by direct phosphorylation. However, when the washout of label via the hydration–dehydration equilibrium

Table IV: Observed Isotope Effects for Aldehyde-Induced ATPase Activities of Kinases[a]

enzyme	aldehyde	$^{D}V/K$	conditions
fructose-6-P kinase	2,5-anhydro[1-^{2}H]mannose-6-P	1.23 ± 0.03[b]	0.1 M Hepes, pH 8, 1 mM ATP, 2 mM $MgCl_2$
		1.10 ± 0.014[c]	same buffer, 1.5 mM ATP, 5 mM $MgCl_2$
fructokinase	2,5-anhydro[1-^{2}H]mannose	1.23 ± 0.14[d]	same buffer, 1 mM ATP, 2 mM $MgCl_2$
		1.04 ± 0.02[e]	same buffer, 5 mM ATP, 6 mM $MgCl_2$
	2,5-anhydro[1-^{2}H]talose	1.011 ± 0.011	50 mM Pipes, pH 7, 3 mM ATP, 6 mM $MgCl_2$
		1.002 ± 0.025	0.1 M Hepes, pH 8, 2.5 mM ATP, 6 mM $MgCl_2$
		0.95 ± 0.02	same buffer, 0.125 mM ATP
hexokinase	D-*gluco*-[6-^{2}H]hexodialdose	1.07 ± 0.05[f]	same buffer, 2 mM ATP, 5 mM $MgCl_2$
		1.0[g]	same except HPLC-purified aldehydes
E. coli glycerokinase	D-[1-^{2}H]glyceraldehyde	0.96 ± 0.01	same buffer, 1 mM ATP, 2 mM $MgCl_2$
		0.97 ± 0.02[h]	same except 9.5 μM ATP, 3 mM $MgCl_2$
Candida glycerokinase		0.92 ± 0.03	same
choline kinase	betaine aldehyde	1.02 ± 0.03[h]	Hepes, pH 8.2, 1.5 mM ATP, 15 mM $MgCl_2$

[a] Data are reported from fits to equations that give the lowest standard deviation in the isotope effect. Unless otherwise noted, this was eq 7, which assumes equal isotope effects on V/K and V. [b] Viola & Cleland (1980). Data were fitted to eq 5. [c] Value from the present work; data were fitted to the log form of eq 5. [d] Average of four determinations by end-point assay with fructokinase vs. hexokinase (that is, the isotope effect is on the ATPase to kinase ratio). [e] The isotope effect is on the total initial velocity of ADP formation, which includes both kinase and ATPase activities; data were fitted to eq 5. [f] Fit to eq 5. A fit to eq 6 gave 1.02 ± 0.02 for ^{D}V. [g] Fit to eq 6; ^{D}V = 1.04 ± 0.01. A fit to eq 4 gave $^{D}V/K$ = 0.95 ± 0.04 and ^{D}V = 1.05 ± 0.02. [h] Fit to eq 5.

process is slowed down relative to the enzymatic rate at low temperatures and the hydrate is the predominant form in solution, the observation of large amounts of label in the phosphate can only be accounted for by a mechanism in which it is the hydrate that is phosphorylated.[2] With D-glyceraldehyde and glycerokinase at 2 °C, 91% of the ^{18}O label was incorporated into P_i, which is greater than the maximum possible incorporation under the conditions of the experiment (82%) if the free aldehyde were the activator. Thus, the hydrate is the activator of this ATPase. Due to the prohibitively large amounts of enzymes required for such studies, for the remaining aldehydes we used isotope effects on the kinetic parameters of the ATPase reactions to distinguish whether the aldehyde or the hydrate was the activator.

Isotope Effects on ATPase Reactions. Direct comparison of the kinetic parameters of the ATPase activities induced by labeled and unlabeled aldehydes can be used to determine which species is the activator, since the equilibrium between hydrate and aldehyde is shifted toward the former by a factor of 1.37 upon deuteration at C-1 (Hill & Milosevich, 1976; Lewis & Wolfenden, 1977). Normal isotope effects are predicted if the free aldehyde is the activator (near 1.37 for the heavily hydrated aldehydes used in the present study, but 1.22 for acetaldehyde[3]). When the hydrated aldehyde is the active form, there should be an inverse isotope effect, but when the hydrate is the dominant species present, the isotope effect will be near unity. For acetaldehyde the value would be 0.89.[3]

The observed deuterium isotope effects on V and V/K for the ATPase activities are listed in Table IV. The pH dependence of the isotope effects on the kinetic parameters of the glyoxylate-induced ATPase activity of pyruvate kinase are tabulated in Table V. For five of the kinases, the isotope effects either are inverse or else are not significantly different from 1.0, suggesting that the ATPase activities are hydrate activated. With glycerokinase and fructokinase with 2,5-anhydrotalose, varying the level of ATP to below saturating did not change the isotope effect from 1.0. Also, the isotope effect for the pyruvate kinase ATPase activity did not change significantly from unity as a function of pH. When ADP

Table V: Deuterium Isotope Effects for Glyoxylate-Dependent Pyruvate Kinase ATPase vs. pH[a]

pH	isotope effect[b]	assay
6.02	1.064 ± 0.054	*c*
8.47	0.97 ± 0.02	*c*
9.95	0.97 ± 0.04	*c*
6.0	1.054 ± 0.068	*d*
6.92	0.954 ± 0.025	*d*
8.35	1.068 ± 0.027	*d*

[a] 2 mM ATP, 5 mM $MgCl_2$, 0.1 M buffer (Mes at pH 6, Tes at pH 6.9, Taps at pH 8.4, Ches at pH 10). [b] Assumed equal on V/K and V (data were fitted to eq 7). The values were not significantly different when the data were fitted to eq 5 or 6. [c] Fixed-time assays for P_i were used. [d] Coupled enzymatic assays for P_i were used.

production was monitored with fructokinase, no isotope effect on V or V/K was observed with 2,5-anhydromannose, since this molecule induces both kinase and ATPase activities and the V/K of the kinase is 7 times larger than the V/K of the ATPase reaction.

With fructose-6-phosphate kinase and fructokinase in the presence of 2,5-anhydromannose, small normal isotope effects on V/K of the ATPase activities were observed that are significantly different from 1.0. However, these effects are also significantly smaller than the predicted isotope effects near 1.37 for such heavily hydrated aldehydes if the aldehyde is the activator. One explanation is that both the free aldehyde and the hydrate of these particular aldehydes can activate the ATPase reactions. More likely is that a different kinetic mechanism with bound intermediates accounts for normal isotope effects when only the hydrate is the activator; this mechanism will be discussed later.

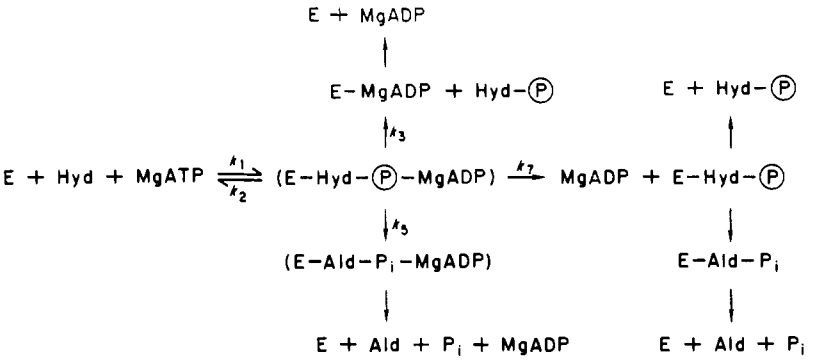
This technique was also applied to the inhibition of acetate kinase by acetaldehyde. Deuterated acetaldehyde gave a slope inhibition constant of 49.4 ± 2.9 mM while the K_I of unlabeled acetaldehyde was 56.5 ± 2.1 mM. The corresponding inverse isotope effect of 0.87 ± 0.06 on the inhibition constant (the deuterated compound is a better inhibitor) agrees well with the predicted isotope effect of 0.89 if the hydrate of acetaldehyde is the inhibitory species. It seems reasonable that the hydrate would be adsorbed on the enzyme, since the substrate binding pocket is designed for acetate, which also has two oxygens on C-1. The lack of aldehyde-induced ATPase activity is probably because of the absence of an acid–base catalyst to remove the proton from the hydroxyl group of the hydrate.

Attempted Trapping of Phosphorylated Intermediates with Methanol. If the ATPase reaction were activated by the free

[2] If the small amount of free aldehyde were the substrate, the rapid ATPase activity would deplete it of ^{18}O faster than fresh ^{18}O-labeled aldehyde could be generated by dehydration of the hydrate, and the phosphate formed would contain a considerable level of ^{16}O derived from the solvent.

[3] These numbers are the ratios of the fraction of free aldehyde in deuterated and unlabeled acetaldehyde or of hydrate for the two species.

Scheme II: Mechanism for Decomposition of Phosphorylated Hydrate on the Enzyme That Can Account for a Normal α-Secondary Deuterium Isotope Effect (See Text)[a]



[a] Hyd is hydrate; Ald is free aldehyde.

aldehyde and ^{18}O is transferred to inorganic phosphate, as the isotope effects suggest might be the case with fructose-6-phosphate kinase and fructokinase with 2,5-anhydromannose, a likely chemical mechanism would be direct phosphorylation of the aldehyde to give an unstable oxycarbonium ion, as shown in Scheme I. The requirement for the proper protonation state of the acid–base catalyst as shown by parallel V/K pH profiles for kinase and ATPase activity is consistent with this mechanism.

To test the third mechanism shown in Scheme I, we attempted to trap the proposed oxycarbonium ion intermediate by running the ATPase reaction in the presence of methanol, which should react with the oxycarbonium ion to give a phorphorylated methyl hemiacetal. When 40% [^{14}C]methanol and [γ-^{32}P]ATP were used with the glycerokinase ATPase reaction induced by D-glyceraldehyde, no doubly labeled fractions were seen on anion-exchange chromatography. The control experiment under identical conditions in the absence of D-glyceraldehyde also showed no doubly labeled peak. In retrospect, no oxycarbonium ion intermediate would be predicted for the ATPase activity of this kinase since the hydrate is the activator on the basis of the deuterium isotope effects. Therefore, these results are consistent with the mechanism involving direct phosphorylation of the hydrate.

Similar experiments were conducted with fructose-6-phosphate kinase in the presence of 2,5-anhydromannose 6-phosphate, 40% methanol, and [γ-^{32}P]ATP. No labeled fraction was observed that was not also present in the control conducted in the absence of 2,5-anhydromannose 6-phosphate. We also found no additional labeled fractions when control and sample were not quenched with glucose and hexokinase. The results suggest either that no oxycarbonium ion is generated or else that a bound water molecule reacts with it before release from the enzyme. Since metaphosphate cleavage of MgATP in the presence of methanol might also be expected to give methyl phosphate (doubly labeled in the glycerokinase experiment), these results apparently rule out this mechanism as well. Failure to trap an oxycarbonium ion intermediate is consistent with the idea that even the ATPase reactions where normal isotope effects are observed (fructose-6-phosphate kinase and fructokinase with 2,5-anhydromannose) are probably not activated by the free aldehyde, especially since the isotope effects can be explained by the kinetic model described below.

Evidence for a Phosphorylated Hydrate Intermediate. Direct phosphorylation of the hydrate followed by nonenzymatic breakdown of the hydrate adduct is a likely chemical mechanism for hydrate-activated ATPase reactions of kinases where ^{18}O is transferred to phosphate. Breakdown of the phosphorylated hydrate adduct may occur on the enzyme or after release into solution. In either case, there should be a lag in the release of phosphate at low temperatures where the adduct is expected to be more stable. Figure 4 shows the lag in phosphate released during the D-glyceraldehyde-induced ATPase reaction of glycerokinase at 15 °C, pH 7, by a coupled assay for phosphate. Lag times of 23–29 s slower than the time required to build up a corresponding steady-state level of phosphate were observed in duplicate experiments. The rate constant for breakdown of phosphorylated hydrate is estimated to be 0.035–0.043 s^{-1}, compared to the dehydration rate constant of 0.01 s^{-1} calculated for D-glyceraldehyde at 15 °C. These results indicate that a phosphorylated adduct is formed during the ATPase reaction of glycerokinase as was initially proposed when these reactions were first observed with the enzymes from *E. coli* (Hayishi & Lin, 1967) and *Candida* (Gancedo et al., 1968).

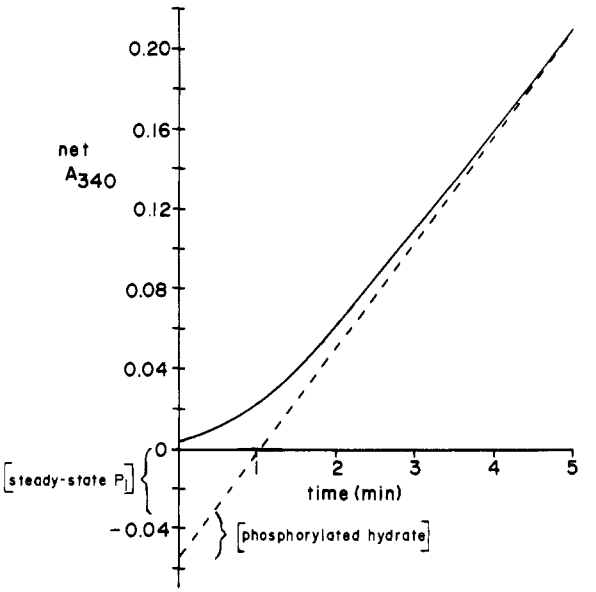


FIGURE 4: Lag in phosphate released determined by coupled enzymatic assay during the ATPase reaction catalyzed by *E. coli* glycerokinase in the presence of D-glyceraldehyde at 15 °C. The experimental data (solid line) were fitted to eq 11, and the dashed line represents the final steady-state rate of the ATPase reaction given by this fit. The intersection of this line with the vertical axis gives the total level of intermediates in the coupled system. Subtraction of the level of phosphate required to give the same steady-state rate with the same levels of coupling enzymes gives the level of phosphorylated hydrate present in the steady state.

Kinetic Mechanism for Hydrate-Activated ATPases. In order to explain normal isotope effects observed with activation by the hydrate where ^{18}O is transferred to phosphate, we

propose the kinetic mechanism shown in Scheme II. In this scheme, k_1 and k_2 are net rate constants for the steps leading to formation of the phosphorylated hydrate (binding of hydrate and MgATP may be ordered or random), k_7 is the rate constant for ADP release, and k_3 is that for release of phosphorylated hydrate, and k_5 is that for breakdown of the phosphorylated hydrate on the enzyme. k_5 should show a normal α-secondary deuterium isotope effect, the size of which would depend on the nature of the transition state for the cleavage step and on the relative rates of the other steps. The intrinsic isotope effect on k_5 is unlikely, however, to exceed 1.2 unless the transition state is quite late. Since k_3, k_5, and k_7 are irreversible steps, the subsequent product release steps shown do not affect V/K or the isotope effect on it, which are given by eq 12 and 13 From these equations, we predict no isotope

$$V/K = \frac{k_1(k_3 + k_5 + k_7)}{k_2 + k_3 + k_5 + k_7} \quad (12)$$

$$^{D}V/K = \frac{(k_2 + k_3 + k_{5D} + k_7)(k_3 + k_{5H} + k_7)}{(k_2 + k_3 + k_{5H} + k_7)(k_3 + k_{5D} + k_7)} \quad (13)$$

effect will be observed if k_3 or $k_7 > k_5$ or if $k_2 < k_5$, while the full isotope effect will be observed when $k_5 > (k_3 + k_7)$ and $k_2 > k_5$. Thus, normal isotope effects of any size between 1 and perhaps 1.2 can be accounted for when the hydrate is the activator, depending on the relative rates of each step in the kinetic mechanism.

It is interesting to note that fructose-6-phosphate kinase, an enzyme where significant normal isotope effects were observed, is the only kinase where there is evidence that the nucleotides are sticky (Uyeda, 1970). Thus, k_7 may be slow relative to the rates of the other steps, one of the criteria needed in order to observe an isotope effect. We attempted to look for an isotope effect in the presence of inhibitory levels of ADP (the net value of k_7 is in effect reduced by the high MgADP concentration) where no isotope effects were observed in the absence of ADP. Glycerokinase was chosen, and the deuterium isotope effect for the ATPase reaction in the presence of 18 mM MgADP with a coupled assay for P_i was 1.024 ± 0.033 (assuming equal isotope effects on V and V/K). The failure to observe a significant normal isotope effect on the kinetic parameters does not rule out the proposed kinetic mechanism but suggests that release of the phosphorylated hydrate (k_3) is fast relative to its breakdown (k_5) or else its formation is slow relative to its decomposition on the enzyme. However, the apparent K_i for MgADP at the 0.5 mM level of MgATP used in the experiment is about 4.5 mM, so that MgADP levels of 4 times K_i may not be sufficient to slow down the step represented by k_7 sufficiently to see an isotope effect.

Conclusions

In summary, all the aldehyde-induced ATPase activities studied appear to be activated by the hydrated forms of the aldehydes. Different deuterium isotope effects for the ATPase reactions can be explained by differences in the relative rates of steps in the kinetic mechanism for hydrate-activated ATPases. Whether direct phosphorylation of the hydrate occurs or not depends on the nature of the kinase. To get induced transfer to water, the geometry of the active site must accomodate water plus the hydrate or else involve a second metal with bound water, as with pyruvate kinase. Kinases that are most likely to exhibit aldehyde-induced ATPase activities are those that normally require an acid–base catalyst to remove a proton from the substrate. For kinases where an acid–base catalyst is not needed for the normal reaction, the hydrates by themselves are not good enough nucleophiles to attack the γ-phosphate of MgATP, and they act as inhibitors but are not phosphorylated.

Acknowledgments

We thank Professor Laurens B. Anderson for discussions on the synthesis of aldehydes, Dr. John Schloss for the concept of the enzymatic synthesis of deuterated D-glyceraldehyde, and Drs. Vernon A. Anderson, Jeffrey D. Hermes, and Vincent L. Pecoraro and Charles B. Grissom for many helpful discussions.

References

Anderson, C. M., Stenkamp, R. E., McDonald, R. C., & Steitz, T. A. (1978) *J. Mol. Biol. 123*, 207–219.
Bar-Tana, J., & Cleland, W. W. (1974) *J. Biol. Chem. 249*, 1263–1270.
Bennett, W. S., & Steitz, T. A. (1980) *J. Mol. Biol. 140*, 211–230.
Bertagnolli, B. L., & Cook, P. F. (1984) *Biochemistry 23*, 4101–4108.
Brossmer, R. (1962) *Methods Carbohydr. Chem. 1*, 216–221.
Brostrom, M. A., & Browning, E. T. (1973) *J. Biol. Chem. 248*, 2364–2371.
Cleland, W. W. (1979a) *Methods Enzymol. 63*, 103–138.
Cleland, W. W. (1979b) *Anal. Biochem. 99*, 142–145.
Cleland, W. W., & Mildvan, A. J. (1979) *Adv. Inorg. Biochem. 1*, 163–191.
Cook, P. F., Kenyon, G. L., & Cleland, W. W. (1981) *Biochemistry 20*, 1204–1210.
Danenberg, K. D., & Cleland, W. W. (1975) *Biochemistry 14*, 28–39.
Defaye, J. (1964) *Bull. Soc. Chim. Fr.*, 999–1002.
DelaFuente, G., Lagunao, R., & Sols, A. (1970) *Eur. J. Biochem. 16*, 226–233.
Dougherty, T. M. (1982) Ph.D. Thesis, University of Wisconsin, Madison, WI.
Fischer, F. G., & Schmidt, H. (1960) *Chem. Ber. 93*, 658–662.
Gaebler, O. H. (1926) *J. Biol. Chem. 69*, 613–624.
Gancedo, C., Gancedo, J. M., & Sols, A. (1968) *Eur. J. Biochem. 5*, 165–172.
Hayishi, S.-I., & Lin, E. C. C. (1967) *J. Biol. Chem. 242*, 1030–1035.
Hendrix, D. L., Lee, R. E., Baust, J. G., & James, H. (1981) *J. Chromatogr. 210*, 45–53.
Hill, E. A., & Milosevich, S. A. (1976) *Tetrahedron Lett. 50*, 4553–4554.
Janson, C. A., & Cleland, W. W. (1974) *J. Biol. Chem. 249*, 2562–2566.
Jones, G. H., & Moffatt, J. G. (1972) *Methods Carbohydr. Chem. 6*, 315–322.
Kayne, F. J. (1974) *Biochem. Biophys. Res. Commun. 59*, 8–13.
Knight, W. B., Fitts, S. W., & Dunaway-Mariano, D. (1981) *Biochemistry 20*, 4079–4086.
Knowles, J. R. (1980) *Ann. Rev. Biochem. 49*, 877–919.
Kuo, D. J., O'Connell, E. L., & Rose, I. A. (1979) *J. Am. Chem. Soc. 101*, 5025–5030.
Lanzetta, P. A., Alvarez, L. J., Reinach, P. S., & Candic, O. A. (1979) *Anal. Biochem. 100*, 95–97.
Lewis, C. A., & Wolfenden, R. (1977) *Biochemistry 16*, 4886–4890.
Lewis, K. F., & Weinhouse, S. (1957) *Methods Enzymol. 3*, 269–276.
Neal, M. W., & Florini, J. R. (1973) *Anal. Biochem. 55*, 328–330.

Raushel, F. M., & Cleland, W. W. (1977a) *Biochemistry 16*, 2169–2175.
Raushel, F. M., & Cleland, W. W. (1977b) *Biochemistry 16*, 2176–2181.
Rendina, A. R., & Cleland, W. W. (1981) *Anal. Biochem. 117*, 213–218.
Rendina, A. R., & Cleland, W. W. (1983) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 42*, 1921.
Rendina, A. R., Hermes, J. D., & Cleland, W. W. (1984) *Biochemistry* (preceding paper in this issue).
Rose, I. A., O'Connell, E. L., Litwin, S., & Bar-Tana, J. (1974) *J. Biol. Chem. 249*, 5163–5168.
Shoham, M., & Steitz, T. A. (1980) *J. Mol. Biol. 140*, 1–14.
Thorner, J. W., & Paulus, H. (1973) *J. Biol. Chem. 248*, 3922–3932.
Uyeda, K. (1970) *J. Biol. Chem. 245*, 2268–2275.
Viola, R. E., & Cleland, W. W. (1978) *Biochemistry 17*, 4111–4117.
Viola, R. E., & Cleland, W. W. (1980) *Biochemistry 19*, 1861–1866.
Wurster, B., & Hess, B. (1975) *Methods Enzymol. 41*, 488–493.

Kinetic Mechanism and Location of Rate-Determining Steps for Aspartase from *Hafnia alvei*†

Ileana I. Nuiry,‡ Jeffrey D. Hermes, Paul M. Weiss, Cheau-Yun Chen, and Paul F. Cook*

ABSTRACT: Coupled spectrophotometric assays that monitor the formation of fumarate and ammonia in the direction of aspartate deamination and aspartate in the direction of fumarate amination were used to collect initial velocity data for the aspartase reaction. Data are consistent with rapid equilibrium ordered addition of Mg^{2+} prior to aspartate but completely random release of Mg^{2+}, NH_4^+, or fumarate. In addition to Mg^{2+}, Mn^{2+} can also be used as a divalent metal with V_{max} 80% and a $K_{aspartate}$ 3.5-fold lower than when Mg^{2+} is used. Monovalent cations such as Li^+, K^+, Cs^+, and Rb^+ are competitive vs. either aspartate or NH_4^+ but noncompetitive vs. fumarate. A primary deuterium isotope effect of about 1 on both V and $V/K_{aspartate}$ is obtained with (3*R*)-L-aspartate-*3-d*, while a primary ^{15}N isotope effect on $V/K_{aspartate}$ of 1.0239 ± 0.0014 is obtained in the direction of aspartate deamination. A secondary isotope effect on V of 1.13 ± 0.04 is obtained with L-aspartate-*2-d*. In addition, a secondary isotope effect of 0.81 ± 0.05 on V is obtained with fumarate-d_2, while a value of 1.18 ± 0.05 on V is obtained by using (2*S*,3*S*)-L-aspartate-*2,3*-d_2. These data are interpreted in terms of a two-step mechanism with an intermediate carbanion in which C–N bond cleavage limits the overall rate and the rate-limiting transition state is intermediate between the carbanion and fumarate.

Aspartase (EC 4.3.1.1) catalyzes the divalent metal dependent deamination of L-aspartate to yield fumarate and ammonia (Quastel & Woolf, 1926). The aspartase reaction was first demonstrated in bacteria by Harden (1901). Aspartase has been regarded as a catabolic enzyme in both bacteria and plants, but the reaction is reversible and favors aspartate formation with $\Delta G° = 3.2$ kcal/mol for aspartate deamination. The equilibrium constant for the aspartase reaction, measured directly at 25 °C by Bada & Miller (1968), is 5×10^{-3} M.

Nucleotides such as IMP, AMP, and GDP (to a lesser extent) and adenosine activate aspartase, while GTP and UTP inhibit (Williams & Scott, 1968). The nucleotide triphosphates increase $K_{aspartate}$ while the activators decrease $K_{aspartate}$; V_{max} is unaffected.

Gawron & Fondy (1959), using the data of Englard (1958) and Krasna (1958), showed that ammonia is added trans across the double bond of fumarate. The enzyme is specific for the amino acid substrate and fumarate, but NH_2OH can substitute for ammonia as a substrate (Emery, 1963). A variety of divalent metal ions will activate the reaction including Mg^{2+} and Mn^{2+} (Williams & Lartigue, 1967). The most complete study of the kinetic mechanism for aspartase was carried out by Dougherty et al. (1972), who suggested a uni-bi rapid equilibrium random mechanism. However, these studies neglected any role of the metal ion.

In this study, we present data on the overall kinetic mechanism for aspartase from *Hafnia alvei*, which includes the divalent metal ion as a pseudoreactant. In addition, through the use of primary and secondary kinetic isotope effects, it is suggested that the deamination of aspartate proceeds via a carbanion intermediate and that C–N bond cleavage limits the overall rate.

Materials and Methods

Chemicals and Enzymes. H. alvei aspartase (1 unit/mg), chicken liver malic enzyme, pig heart fumarase, bovine liver glutamate dehydrogenase, pig heart aspartate aminotransferase, and pig heart malate dehydrogenase were obtained

† From the Department of Biochemistry, University of Wisconsin, Madison, Wisconsin 53706 (J.D.H. and P.M.W.), and the Department of Biochemistry, North Texas State University/Texas College of Osteopathic Medicine, Denton, Texas 76203 (C.-Y.C. and P.F.C.). *Received February 29, 1984.* Portions of this work were completed at Louisiana State University Medical Center, New Orleans, LA 70112. I.I.N. was a M.S. student in the lab of P.F.C. at Louisiana State University Medical Center when this work was completed. This work was supported in part by a Research Corporation grant and an NIH grant (GM 31686) to P.F.C. and also by an NIH grant (GM 18936) to Dr. W. W. Cleland at the University of Wisconsin, Madison (J.D.H. and P.M.W. are presently in Dr. Cleland's laboratory). P.F.C. is the recipient of a Research Career Development Award from NIH (AM 01155).
‡ Present address: Department of Biochemistry, Louisiana State University Medical Center, New Orleans, LA 70112.

0006-2960/84/0423-5168$01.50/0