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pH Studies on the Chemical Mechanism of Rabbit Muscle Pyruvate Kinase. 2. Physiological Substrates and Phosphoenol-α-ketobutyrate†

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ABSTRACT: pH profiles have been determined for the reactions catalyzed by pyruvate kinase between pyruvate and MgATP and between phosphoenolpyruvate and MgADP. V, V/K_{MgATP}, and $V/K_{pyruvate}$ all decrease below a pK of 8.3 and above one of 9.2. The group with pK = 8.3 is probably a lysine that removes the proton from pyruvate during enolization, while the pK of 9.2 is that of water coordinated to enzyme-bound Mg^{2+}. The fact that this pK shows in all three pH profiles shows that pyruvate forms a predominantly second sphere complex and cannot replace hydroxide to form the inner sphere complex that results in enolization and subsequent phosphorylation. On the basis of the displacement of the pK of the acid–base catalytic group in its V/K profile, phosphoenolpyruvate is a sticky substrate, reacting to give pyruvate ~5 times faster than it dissociates. The V/K profile for the slow substrate phosphoenol-α-ketobutyrate shows the pK of 8.3 for the acid–base catalytic group in its correct position, but this group must be protonated so that it can donate a proton to the intermediate enolate following phosphoryl transfer. The secondary phosphate pK of the substrate is seen in this V/K profile as well as in the pK_i profile for phosphoglycolate (but not in those for glycolate O-sulfate or oxalate), showing a preference for the trianion for binding. The chemical mechanism with the natural substrates thus appears to involve phosphoryl transfer between MgADP and a Mg^{2+}-bound enolate with metal coordination of the enolate serving to make it a good leaving group.

Pyruvate kinase catalyzes the transfer of the phosphate group of phosphoenolpyruvate to MgADP and stereospecifically protonates the intermediate enolate to form pyruvate. As indicated by NMR distance measurements that suggest molecular contact between the carbonyl oxygen of pyruvate and the γ-phosphate of ATP (Mildvan et al., 1976) and the stereochemical studies of Blättler & Knowles (1979) with chiral [γ-^{16}O,γ-^{17}O,γ-^{18}O]-ATP, which unambiguously demonstrated inversion of configuration around phosphorus during the reaction, the phosphoryl-transfer step appears to occur by a direct, in-line displacement.

Unlike other kinases, however, pyruvate kinase requires an enzyme-bound divalent metal ion for catalysis in addition to the metal ion required to complex the nucleotide substrate. Gupta et al. (1976) showed that substitution-inert CrATP would activate the detritiation of pyruvate only in the presence of an added divalent cation. Further evidence for the dual divalent metal ion requirement has come from NMR chemical shift titrations of enzyme-bound ATP with Mg^{2+}, which indicated that at least 2 equiv of Mg^{2+} are required for saturation (Nageswara Rao et al., 1979), and from synergistic activation of pyruvate kinase by mixed divalent metal ions (Baek & Nowak, 1982).

In the previous paper (Dougherty & Cleland, 1985) we reported pH profiles for several alternate reactions catalyzed by pyruvate kinase. These profiles showed pK's of only two groups, one interpreted as a ligand for the enzyme-bound Mg^{2+} and the other as water coordinated to this Mg^{2+}. In this paper we will report the pH profiles for the natural substrates phosphoenolpyruvate and pyruvate and for the slow alternate substrate phosphoenol-α-ketobutyrate, as well as for the

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binding of a number of competitive inhibitors. pH profiles for the substrates in which enolate–keto conversion are involved show a third pK corresponding to the acid–base catalyst for the enolization process, which we believe may be a lysine. We will show that phosphoryl transfer to MgADP gives a Mg^{2+}-bound enolate, which is protonated to a Mg^{2+}-coordinated keto acid that leaves the coordination sphere of Mg^{2+} prior to dissociation from the enzyme.

Materials and Methods

Chemicals. Pyruvate kinase, lactate dehydrogenase, phosphoenolpyruvate carboxylase, acetate kinase, and malate dehydrogenase were purchased from Boehringer as ammonium sulfate suspensions. Each enzyme solution was centrifuged, redissolved in 25 mM Tes,[1] pH 7.0, and dialyzed 2 times for 12 h against 500 volumes of the same buffer. The tris(cyclohexylammonium) salts of phosphoenolpyruvate and phosphoglycolate and the sodium salt of pyruvate from Sigma were converted to potassium salts by passing a solution of each through a column of Dowex 50-K^+. NADH and acetylpyridine–NADH were from Sigma, while the potassium salts of ADP and ATP were from Boehringer and Calbiochem, respectively.

Synthesis of Phosphoenol-α-ketobutyrate. 3-Bromo-2-ketobutyric acid from Adams Chemical Co. was reacted with trimethyl phosphite in anhydrous ether according to the procedure of Stubbe & Kenyon (1972). Cyclohexylammonium phosphoenol-α-ketobutyrate was converted to the potassium salt by passing a solution through a column of Dowex 50-K^+. The resulting product after crystallization had a proton NMR spectrum in D_2O identical with that reported by Duffy et al. (1982) for pure (*Z*)-tripotassium phosphoenol-α-ketobutyrate.

Synthesis of Glycolate O-Sulfate. Five grams of glycolic acid (from Sigma; lyophized before use) was combined with a small amount of sodium [^{14}C]glycolate from Amersham and dissolved in 250 mL of anhydrous ethanol. Dry HCl gas was bubbled through the solution until the pH was below 1, and the solution was heated to boiling and the azeotrope of ethanol and water removed. The remaining ethanol was removed with a rotary evaporator, and 3 g of the ester was mixed with 7 g of sulfur trioxide–trimethylamine complex in 200 mL of anhydrous dimethylformamide and refluxed for 24 h. After cooling, the dimethylformamide was removed with a rotary evaporator, and the remaining solid was dissolved in water and the pH raised to 12 with barium hydroxide. Barium sulfate was filtered off and the solution heated for 1 h, which was sufficient time to hydrolyze most of the ethyl ester. Carbon dioxide was then bubbled into the solution until the pH was 8. The precipitate was filtered off and the filtrate diluted and applied to a column of Dowex 1, bicarbonate form. A void volume of water was passed through the column, followed by a linear 0.1–1 M gradient of ammonium bicarbonate. Fractions from the second major peak containing radioactivity were pooled and flashed to dryness, and the resulting solid was lyophilized for 24 h. It was then dissolved and passed through a column of Dowex 50-K^+. The resulting compound gave a negative test for sulfate at 25 °C and neutral pH. However, a solution in 1 N HCl boiled for 15 min gave a heavy precipitate upon addition of $BaCl_2$ to the cooled solution. A proton NMR spectrum of the glycolate *O*-sulfate preparation revealed a singlet at 4.76 ppm (this peak was 0.1 ppm downfield from the glycolate peak in a mixture of the two compounds).

Assay Conditions. Kinetic studies were run in 3-mL total volume in 1-cm cuvettes by measuring absorbance changes with a Beckman monochromator, a Gilford optical density converter, and a 10-mV recorder with adjustable zero and multispeed drive. Full-scale sensitivity of 0.05A was used.

Reaction rates with phosphoenolpyruvate as the variable substrate were measured by coupling the pyruvate produced with lactate dehydrogenase and monitoring the disappearance of absorbance at 340 nm. Reaction mixtures contained 100 mM buffer, 100 mM KCl, 0.2 mM NADH, ADP (5 mM at pH 6.5 or below, or 2.5 mM at higher pHs), 0.6–1.2 μg of pyruvate kinase, 0.1 mg of lactate dehydrogenase, and variable amounts of phosphoenolpyruvate and $MgCl_2$. Reaction rates with phosphoenolketobutyrate as a substrate were measured with the same coupling system as with phosphoenolpyruvate. Reaction mixtures were the same except that ADP was 15 mM and 25 μg of pyruvate kinase and 0.2 mg of lactate dehydrogenase were used.

Reaction rates with pyruvate as the variable substrate were measured by coupling the phosphoenolpyruvate produced with phosphoenolpyruvate carboxylase and reducing the oxalacetate formed with malate dehydrogenase. Acetylpyridine–NADH (monitored at 363 nm) was used as the cofactor, since it was a poorer substrate than NADH for the lactate dehydrogenase, which was present in low levels in the commercial preparations of phosphoenolpyruvate carboxylase that we used. Acetyl phosphate and acetate kinase were also included to recycle the ADP produced to ATP, thus keeping its concentration constant. The reaction mixtures contained 100 mM buffer, 90 mM KCl, 10 mM $KHCO_3$, 0.125 mM acetylpyridine–NADH, 10 mM acetyl phosphate, 15–30 μg of pyruvate kinase, 0.2 mg of phosphoenolpyruvate carboxylase, 0.02 mg of malate dehydrogenase, 0.02 mg of acetate kinase, and variable levels of pyruvate, ATP, and $MgCl_2$.

For all coupled assays, the levels of coupling enzymes were varied to verify that the rates being measured were totally limited by the levels of pyruvate kinase and its substrates and were independent of the levels of coupling enzymes present in the assay. This was done at each pH; and at the highest, lowest, and an intermediate pH being studied the coupling system was checked by obtaining a linear plot of velocity vs. the concentration of pyruvate kinase.

The following buffers were used over the indicated range of pH: Mes, pH 5.0–6.7; Tes, pH 6.6–7.8; Taps, pH 7.7–8.8; Ches, pH 8.7–9.6. All buffers were titrated to pH with KOH. Sufficient overlaps were obtained between buffers to rule out any buffer effects.

Data Analysis. Reciprocal initial velocities were plotted vs. reciprocal substrate concentrations, and the data were fitted to eq 1–8 with the Fortran programs of Cleland (1979). The

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

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

$$v = VA/[K(1 + I/K_{is}) + A] \quad (3)$$

$$v = VA/[K(1 + I/K_{is}) + A(1 + I/K_{ii})] \quad (4)$$

$$\log y = \log [C/(1 + K_1/H)] \quad (5)$$

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

$$\log y = \log [C/(1 + H/K_1 + K_2/H)] \quad (7)$$

$$pK = \Delta H_{ion}/(2.303RT) + B \quad (8)$$

points in the figures are the experimentally determined values, while the curves are calculated from fits of these data to the

[1] Abbreviations: Mes, 2-(*N*-morpholino)ethanesulfonic acid; Tes, 2-[[tris(hydroxymethyl)methyl]amino]ethanesulfonic acid; Taps, 3-[[tris(hydroxymethyl)methyl]amino]propanesulfonic acid; Ches, 2-(*N*-cyclohexylamino)ethanesulfonic acid.

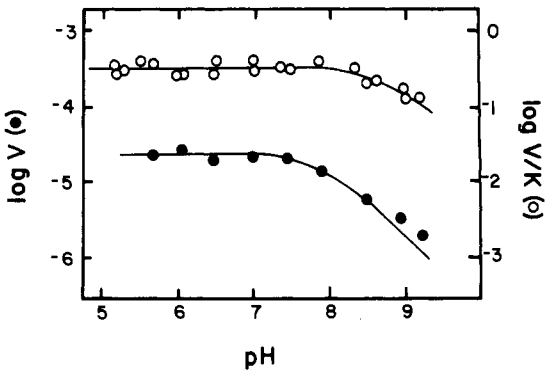

FIGURE 1: pH profiles for the reaction of phosphoenolpyruvate and MgADP. MgADP was at least $10K_m$ at all pH values, and the level of free Mg^{2+} was 10 mM. The values of V, V/K, and K_{PEP} on the plateaus were 0.4 M min^{-1} mg^{-1}, 5×10^3 min^{-1} mg^{-1}, and 80 μM. The units of V/K and V are min^{-1} and M min^{-1}, respectively (1.67×10^{-5} mg/mL enzyme used).

appropriate equation. Linear reciprocal plots were fitted to eq 1, intersecting initial velocity patterns to eq 2, and linear competitive and linear noncompetitive inhibition patterns to eq 3 and 4, respectively. Data for pH profiles that showed a decrease with a slope of −1 as the pH was increased were fitted to eq 5. Data for pK_i profiles that showed a decrease in pK_i with a slope of 1 as the pH was lowered were fitted to eq 6. When the profile decreased at both low and high pH, the data were fitted to eq 7. Apparent pK values as a function of temperature were fitted to eq 8. In eq 5–7 y is V/K, V, or $1/K_i$, C is the pH-independent value of y, and K_1 and K_2 are acid dissociation constants.

Results

The log (V/K) profile for phosphoenolpyruvate drops on the basic side with an apparent pK of 9.0, while the log V profile shows a pK of 8.2 (Figure 1). pK's are not seen at low pH. The log (V/K) profile for phosphoenolketobutyrate decreases at both low and high pH with pK's of 6.3 and 8.2, respectively, while log V was pH independent over the pH range studied (Figure 2). However, if one uses the concentration of the trianion of phosphoenolketobutyrate, rather than the total concentration, when plotting reciprocal plots, the log (V/K) profile shows only the pK of 8.2. These profiles were all run with the level of free Mg^{2+} at 10 mM, which is high enough to saturate the enzyme binding site for Mg^{2+}, so that the pK of 7 seen in the previous paper for a ligand for this metal ion was not observed. When the V/K profile for phosphoenolketobutyrate was run at 1 mM free Mg^{2+}, the low-side pK was 6.94 ± 0.07, while the one at high pH was not significantly different (8.17 ± 0.07). The low-side pK observed at low Mg^{2+} is presumably that of a ligand for the enzyme-bound Mg^{2+}, and the value agrees with that determined in the previous paper.

The V/K profile for phosphoenolketobutyrate was run at 20, 25, and 35 °C with the level of free Mg^{2+} held at 10 mM and also under conditions where there should be little free Mg^{2+}. At 10 mM Mg^{2+}, the low-side pK of 6.3 varied by only 0.1 pH unit from 20 to 35 °C, which is consistent with this pK being that of the phosphate group of the substrate. At low free Mg^{2+}, however, this pK showed a ΔH_{ion} value of 6 kcal/mol, which is consistent with this group being Glu-271 as suggested by the X-ray evidence (Hilary Muirhead, personal communication), with a conformation change in the protein partly coupled to the protonation of this group so as to induce the temperature variation. The group with a pK of 8.2 showed a ΔH_{ion} value of 12–14 kcal/mol, and the pK was not perturbed

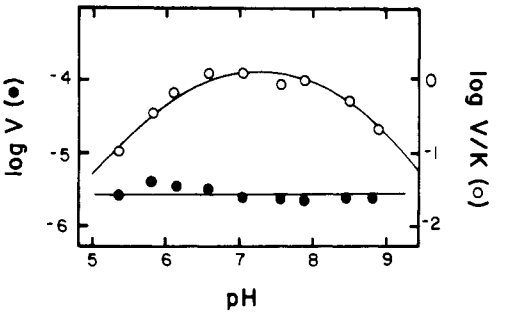

FIGURE 2: pH profiles for reaction of phosphoenolketobutyrate. MgADP was at least $10K_m$ at all pH values, and free Mg^{2+} was 10 mM. The plateau values of V, V/K, and K_{PEKB} were 4.5×10^{-5} M min^{-1} mg^{-1}, 22 min^{-1} mg^{-1}, and 2 μM. Units of V and V/K are min^{-1} and M min^{-1} respectively (0.017 mg/mL enzyme used).

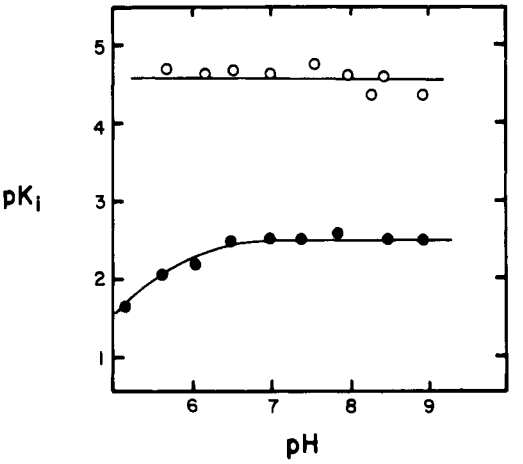

FIGURE 3: pK_i profiles for phosphoglycolate (●) and oxalate (○) as competitive inhibitors vs. phosphoenolpyruvate. MgADP was at least $10K_m$ at each pH value, and free Mg^{2+} was 10 mM. The values of K_i on the plateau were 3.5 ± 0.6 mM for phosphoglycolate and 28 ± 7 μM for oxalate. The pK in the profile for phosphoglycolate was 6.00.

by the presence of 20% propylene glycol.

Oxalate and phosphoglycolate are good competitive inhibitors of pyruvate kinase, with K_i values of 20 μM and 3 mM, respectively, when tested against phosphoenolpyruvate in the forward reaction at pH 7.0. Glycolate O-sulfate was a poorer inhibitor with a K_i of 16 mM at the pH optimum.[2] The pK_i profile for oxalate as a competitive inhibitor vs. phosphoenolpyruvate was pH independent over the pH region studied, while the pK_i profile for phosphoglycolate showed a decrease at low pH with a pK of 6.0 (Figure 3). These profiles were run with the level of free Mg^{2+} at 10 mM. The pK_i profile for glycolate O-sulfate (not shown) decreased on the acid side with a pK of 6.9 when this profile was run with 1 mM free Mg^{2+}.

With the assay procedure originally devised for the back-reaction by Dyson et al. (1975), pyruvate was varied from 1 to 5 mM at MgATP levels of 0.25, 0.5, and 1.0 mM with 1 mM free Mg^{2+}. V/K profiles for pyruvate and MgATP and the log V profile (Figure 4) all decrease on the acid side with a pK of 8.3 and on the basic side with a pK of 9.2. The pyruvate profile was also run at 2 mM MgATP and 3 mM free Mg^{2+} at 25 and 35 °C, and the ΔH_{ion} was 11–12 kcal/mol for both pK's.

[2] There was a small intercept effect seen in the inhibition patterns for glycolate O-sulfate, but we believe this to be due to residual barium resulting from overloading of the Dowex 50 column. The K_i we have reported is the K_{is} value.

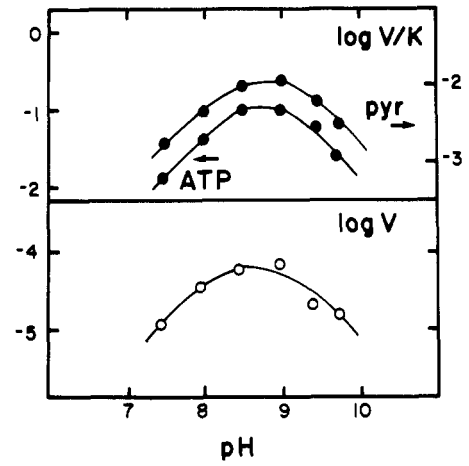

FIGURE 4: pH profiles for the reaction of pyruvate and MgATP. All three profiles showed pK values of 8.3 and 9.2. The data are from initial velocity patterns fitted to eq 2. Free Mg^{2+} was 1 mM. The pH-independent values were $V = (1.3 \pm 0.2) \times 10^{-2}$ M min^{-1} mg^{-1}, $V/K_{pyruvate} = 2.2 \pm 0.3$ min^{-1} mg^{-1}, and $V/K_{MgATP} = 20 \pm 6$ min^{-1} mg^{-1}. Units of V and V/K are M min^{-1} and min^{-1}, respectively (1.6 μg/mL enzyme used).

Analysis of Haldane Relationship. The kinetic constants were determined in both directions at pH 8.0, 25 °C, with Taps buffer as follows: $K_{i\,MgATP} = 0.99$ mM; $K_{pyruvate} = 3.9$ mM; $V_2 = 6.3 \times 10^{-3}$ M min^{-1} mg^{-1}; $K_{i\,PEP} = 72$ μM; $K_{MgADP} = 0.50$ mM; $V_1 = 0.27$ M min^{-1} mg^{-1}. The internal consistency of these kinetic parameters was checked with the kinetic Haldane relationship for a Bi Bi mechanism (Cleland, 1963):

$$\text{apparent } K_{eq} = \frac{V_1 K_{i\,MgATP} K_{pyruvate}}{V_2 K_{i\,PEP} K_{MgADP}} \qquad (9)$$

where V_1 is the maximum velocity with phosphoenolpyruvate and MgADP as substrates and V_2 is that in the reverse reaction. These kinetic constants give a value of 4.6×10^3 for this expression, which agrees with the experimentally observed value of 3.3×10^3 (McQuate & Utter, 1959).

Discussion

Evidence for the Stickiness of Phosphoenolpyruvate. Most enzymes have evolved to the point where product release rather than the chemical reaction is rate limiting in the thermodynamically favorable direction. The kinetic consequence of this is that the substrate undergoes reaction faster than it dissociates from the enzyme, and such substrates are called sticky. The stickiness of a substrate can be determined by the isotope partition method of Rose et al. (1974). This has been done with pyruvate kinase by Dann & Britton (1978), who found that over 95% of [^{32}P]phosphoenolpyruvate that was bound as a binary complex with the enzyme could be trapped as ATP when MgADP and excess unlabeled phosphoenolpyruvate were added. Phosphoenolpyruvate is thus very sticky. If properly performed, the Rose experiment allows one to determine the ratio of k_3/k_2, where k_3 is the net rate constant for the catalytic reaction and k_2 is the rate constant for dissociation of the substrate.

Cleland (1977) has shown that if a substrate is sticky and both V and V/K are pH dependent because the acid–base catalyst on the enzyme can be incorrectly protonated while the substrate is present, the pK's seen in the log (V/K) profile will not occur in the correct position but will be displaced outward (that is, to lower pH when protonation decreases activity, and to higher pH when deprotonation lowers activity) by log ($1 + k_3/k_2$). The pK's seen in the log (V/K) profile for a slow, not-sticky substrate are, however, the correct values.

The log (V/K) profile for phosphoenolpyruvate drops on the basic side with a pK of 9.0, while the corresponding pK in the V/K profile for the slow substrate phosphoenolketobutyrate is 8.23, a difference of 0.77 pH unit. Our results thus confirm the suggestion of Dann & Britton (1978) that phosphoenolpyruvate is sticky, and we calculate a ratio of 4.9 for k_3/k_2. Unfortunately, under the conditions that Dann & Britton ran their experiments only a small percentage of the labeled phosphoenolpyruvate present in the pulse solution was bound to the enzyme, preventing as accurate estimate of k_3/k_2 from their data. It would be useful to repeat the Rose experiment with pyruvate kinase to obtain an accurate independent measure of the stickiness of phosphoenolpyruvate.

Interpretation of the Phosphoenolketobutyrate and pK_i Profiles. V/K for phosphoenolketobutyrate decreases on the acid side with a pK of 6.3, which appears to correspond to the secondary phosphate pK of the substrate as determined by titration. Since one always sees the correct pK values for free enzyme and/or reactant in a pK_i profile (one extrapolates the variable substrate concentration to zero in order to determine K_i, and consequently K_i values represent equilibrium dissociation constants from the enzyme form that is present under the reaction conditions), if the pK were due to an enzyme group, it would be invariant with the nature of the inhibitor. Although this pK is seen in the pK_i profile for phosphoglycolate, it is not observed in the pK_i profile for oxalate, and both of these molecules are good competitive inhibitors vs. phosphoenolpyruvate. Consequently, the pK of 6.3 does not belong to an enzyme group but is the pK of the substrate. Further evidence for this conclusion comes from the temperature dependence of this pK (ΔH_{ion} less than 3 kcal/mol). Such a low value occurs only with carboxyl or phosphate groups.

Pyruvate kinase thus prefers the phosphate group of the substrate to be unprotonated. Although this preference for trianions may be very strong for tightly bound substrates, this is not the case for more weakly bound molecules. Thus, phosphoglycolate, which is a trianion at neutral pH, has a K_i value of 3 mM, while glycolate O-sulfate, which is a dianion, has a K_i value of 16 mM, only 5-fold higher. The pK_i profile for phosphoglycolate (Figure 3) does show that the dianion binds at least 1 order of magnitude less tightly than the trianion, however.

When the log (V/K) profile for phosphoenolketobutyrate was run at different concentrations of free Mg^{2+}, the relative V/K value at the pH optimum decreased with increasing free Mg^{2+}. This suggests that the actual substrate is phosphoenolketobutyrate and not the Mg^{2+} complex. Using the equation

$$\log (V/K)_{obsd} = \log (V/K) - \log (1 + [Mg^{2+}]/K_d) \qquad (10)$$

we calculate a dissociation constant of 25 mM for the Mg^{2+} complex of phosphoenolpyruvate under our assay conditions.

When we determined the V/K profile for phosphoenolketobutyrate or the pK_i profile for glycolate O-sulfate under conditions where there was little free Mg^{2+}, the profiles decreased on the acid side with a pK of 6.9. The temperature dependence of this pK from the V/K profile for phosphoenolketobutyrate yielded a ΔH_{ion} value of 6 kcal/mol. On the basis of X-ray studies of the cat enzyme (Hilary Muirhead, personal communication), this group is probably Glu-271, which appears to be a ligand for the Mg^{2+} that binds directly to the enzyme. The pK of this group is not seen in any of the profiles run at 10 mM free Mg^{2+}, because the dissociation constant for Mg^{2+} is 0.3 mM (see the previous paper), so that

the pK should be decreased by ~1.5 pH units and thus shifted out of the accessible pH range.

The V profile for phosphoenolpyruvate decreases at high pH with a pK of 8.15 (Figure 1). This is presumably the pK of the acid–base catalyst in the presence of the substrate (the pK in the V profile is not displaced by the stickiness of phosphoenolpyruvate). The pK appears not to be altered appreciably by the presence of the substrate, which is consistent with our belief that this group is a cationic acid and the fact that it cannot hydrogen bond to the substrate. Also, V is clearly limited by the rate of the pH-dependent step. The V profile for phosphoenolketobutyrate, however, is pH independent (Figure 2), showing either that the pK of the acid–base catalyst is perturbed upward by the presence of this substrate, even though not by phosphoenolpyruvate, or that a slow, non pH dependent step follows release of the first product. Further work is needed to elucidate the slow step for this reaction!

Evidence That the pK of 8.2 Represents the Acid–Base Catalyst. As discussed above, the pK_i profile for a competitive inhibitor detects only groups whose protonation state affects binding. In contrast, V/K profiles show the effect of ionizations on catalysis as well as on binding, and consequently, a comparison of the two types of profiles is very useful in identifying the groups responsible for catalysis, as opposed to those only involved in binding. Phosphoenolketobutyrate is a slow (0.02% the rate with phosphoenolpyruvate at neutral pH) and presumably nonsticky substrate for pyruvate kinase. A comparison of its V/K profile with the pK_i profile for phosphoglycolate suggests that the pK of 8.23 belongs to a group that acts as an acid–base catalyst or whose protonation state is important for the chemical reaction, but not for binding. This pK was not seen in any of the pH profiles reported in the previous paper for those alternate reactions catalyzed by pyruvate kinase, which do not involve enolization of the substrate.

The pK of this group also appears in the V/K profiles for pyruvate and MgATP and the V profile for the reverse reaction, but the required state of protonation of this group in the reverse reaction is opposite to that required in the forward reaction. It appears that in the direction of pyruvate phosphorylation this group is required to be ionized so it can abstract a proton from C-3 of pyruvate to form an enolate. The pH variation of the detritiation of pyruvate in the presence of MgATP (Robinson & Rose, 1972) also shows that this group must be ionized for this reaction. In the forward reaction, however, this group must be protonated so it can act as a proton donor to phosphoenolpyruvate.

The pK of 8.2 was found to remain relatively constant when the pH profiles were run in the presence or absence of 20% propylene glycol, suggesting that this enzyme group may be a cationic acid. The temperature dependence of the pK from either the phosphoenolketobutyrate or pyruvate profile gave an average ΔH_{ion} of 12 kcal/mol. One must remember that the observed ΔH_{ion} for a pK is a maximum value, since the value will be higher than expected if conformational changes in the protein are coupled to ionization of the group. The temperature and solvent perturbation experiments suggest that the acid–base catalyst is most likely a lysine with a pK lower than normal or a histidine whose pK is higher than normal. X-ray studies (Hilary Muirhead, personal communication) identify lysine-269, but no histidines, in the vicinity of the phosphoenolpyruvate binding site. Further, the fact that the pK of 9.2 for water coordinated to enzyme-bound Mg^{2+} is lower than the value for water bound to Mg^{2+} in solution argues for a net positive charge in the vicinity of the pyruvate binding site, which would lower the pK of the acid–base catalytic group. We thus conclude that this group is most likely lysine.

Evidence That the pK of 9.2 Represents Water Coordinated to Enzyme-Bound Mg^{2+}. The V/K profiles for pyruvate and MgATP and the V profile for the reverse reaction all drop at low pH with pK's of 8.2 and at high pH with pK's of 9.2. The detritiation of pyruvate was also shown by Robinson & Rose (1972) to occur between the pK of the acid–base catalyst and the pK that appears to be that of water coordinated to the enzyme-bound metal ion. Thus, detritiation was a maximum at pH 8.6 or above with Mg^{2+}, at pH 8 with Mn^{2+}, but at pH 7.3 with Co^{2+}. Since substrates are normally sticky only in the direction with the higher maximum velocity (in the reverse reaction substrates cannot be sticky because their release rates from the enzyme must exceed the velocity of the forward reaction), it is safe to assume that neither pyruvate nor MgATP is sticky and that the pK's obtained are the true ones.

When the pyruvate profile was run in the presence of 20% propylene glycol, the pK was found to shift from 9.2 to outside the accessible pH range (no evidence for a decrease at pH 9.4), which is strong evidence for this group being a neutral acid. Since the dissociation constant for Mg^{2+} decreases above this pK (see the previous paper), it can only represent water coordinated to enzyme-bound Mg^{2+}, and the ΔH_{ion} value of 12 kcal/mol is consistent with this. The decrease in this pK to 7.8 with Mn^{2+} (Mildvan & Cohn, 1965) and even lower with Co^{2+} (Robinson & Rose, 1972) also suggests this assignment.

The Chemical Mechanism. From the evidence presented in the previous paper and from the work of Ash (1982), it appears clear that the oxygen atom of glycolate enters the inner coordination sphere of the divalent metal ion bound to the enzyme prior to phosphorylation. The situation is not so clear cut with the phosphorylation of pyruvate, however. Fung et al. (1973) examined the interaction of [1-^{13}C]pyruvate and [2-^{13}C]pyruvate with pyruvate kinase plus Mn^{2+} by measurements of ^{13}C longitudinal and transverse relaxation rates of the enriched carbon atoms. In ternary complexes the Mn^{2+} to carboxyl (7.28 ± 0.08 Å) and Mn^{2+} to carbonyl (7.27 ± 0.04 Å) distances are too great by 4.1 Å for direct coordination of pyruvate, but the distances are consistent with a second sphere complex in which another ligand intervenes between the bound Mn^{2+} and the bound pyruvate. James & Cohn (1974) also calculated a distance of 7.7 Å between Mn^{2+} and the carboxyl group of pyruvate.

One of the major limitations of the NMR relaxation approach is the requirement for fast exchange between free and enzyme-bound substrate, which for ligands bound directly to the metal ion is typically realized only for very weakly bound ligands. The above results could thus be in error because of failure to achieve fast exchange, and the data could be explained if there were an equilibrium between inner and outer sphere pyruvate complexes with a preponderance of the catalytically inactive outer sphere complex.

The pH profiles for pyruvate phosphorylation support this mechanism. Both V and V/K for MgATP decrease above a pK of 9.2, showing that this group can ionize and prevent catalysis even when pyruvate is present. One would expect the water in the inner coordination sphere of Mg^{2+} still to be able to ionize when the carbonyl oxygen of pyruvate was present in the second sphere, but pyruvate should not be able to displace hydroxide to give an inner sphere complex. This model in which the carbonyl oxygen of a ketone forms a predominately second sphere complex but does occasionally enter the inner coordination sphere with resulting catalysis was

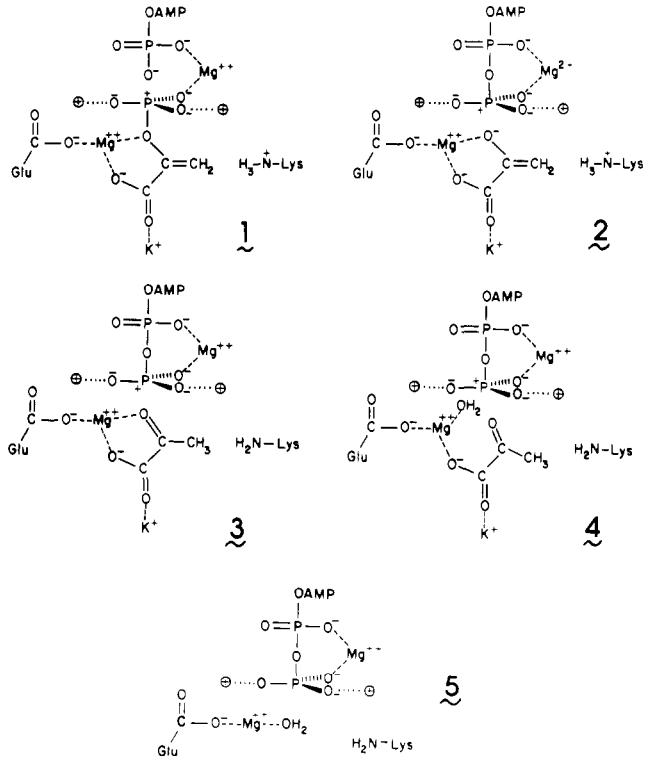

FIGURE 5: Proposed chemical mechanism for the reaction between phosphoenolpyruvate and MgADP.

also indicated by the data in the previous paper for the decarboxylation of oxalacetate catalyzed by pyruvate kinase. Such an arrangement has value for the physiological role of pyruvate kinase, since nearly irreversible conversion of the inner sphere pyruvate complex to an outer sphere one facilitates and speeds pyruvate release from the enzyme. The conversion from inner sphere to outer sphere complex is not necessarily fast, however, and in fact is probably a major rate-limiting step in the reaction, especially with metal ion activators such as Mn^{2+} or, in particular, Co^{2+} (see below).

The chemical mechanism of the pyruvate kinase catalyzed phosphoryl transfer from phosphoenolpyruvate to MgADP is shown in Figure 5. Note that the phosphate transfer is between MgADP and a Mg^{2+}-bound enolate. If Mg^{2+} were not present to bind the enolate, the latter would probably be too poor a leaving group to produce a sufficiently rapid reaction between phosphoenolpyruvate and MgADP. We thus see three types of kinase reactions in which the leaving group (1) is transfered to a metal ion, as with pyruvate kinase, (2) is transferred to a proton, as with hexokinase, or (3) has a low enough pK that such catalysis is not needed, as with adenylate or acetate kinases.

Rate-Limiting Step in the Mechanism. Robinson & Rose (1972) found that during the forward reaction 5% of tritium from phosphoenolpyruvate was found in water, rather than pyruvate at neutral pH, and they concluded that product release was therefore rate limiting. The proportion of tritium going to water increased at high pH, however, doubling at a pH corresponding to the pK of the acid–base catalyst and reaching over 50% at pH 9.3. These data and the small (2.5–4) tritium isotope effects seen on the detritiation of pyruvate in the presence of MgATP show that a step after proton transfer is slow, so that the inner sphere pyruvate complex undergoes reversal to the Mg–enolate intermediate more often than it decomposes. The increase in the amount of tritium going to water as opposed to pyruvate reflects the deprotonation of the acid–base catalyst at high pH, which prevents net conversion of the Mg–enolate to the inner sphere pyruvate complex and leads to tritium washout into water. The results of Robinson & Rose (1972) are thus fully consistent with and support the mechanism proposed above.

The percentage of tritium in phosphoenolpyruvate going to water was higher at neutral pH with Mn^{2+} (9%) and especially Co^{2+} (23%) than with Mg^{2+} (Robinson & Rose, 1972). This correlation between the partition to water and the electronegativity of the metal ion suggests that it is the conversion of the inner sphere pyruvate complex to the outer sphere one that is the slow step following proton transfer. The more electronegative ions release the carbonyl oxygen of pyruvate more slowly, thus permitting more reversal of the proton-transfer step and increased exchange of tritium into water.

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