pH Studies on the Chemical Mechanism of Rabbit Muscle Pyruvate Kinase. 1. Alternate Substrates Oxalacetate, Glycolate, Hydroxylamine, and Fluoride†

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ABSTRACT: The decarboxylation of oxalacetate shows equilibrium-ordered kinetics, with Mg^{2+} adding before oxalacetate. The K_i for Mg^{2+} increases below a pK of 6.9, corresponding to a ligand of the metal that is probably glutamate, and decreases above a pK of 9.2, corresponding to water coordinated to enzyme-bound Mg^{2+}. Both V and V/K_{OAA} decrease above the pK of 9.2, suggesting that the carbonyl oxygen of oxalacetate must replace water in the inner coordination sphere of Mg^{2+} prior to decarboxylation. The enzyme–Mg^{2+}–oxalacetate complex must be largely an outer sphere one, however, since the pK of 9.2 is seen in the V profile. The phosphorylation of glycolate or *N*-hydroxycarbamate (the actual substrate that results from reaction of hydroxylamine with bicarbonate) occurs only above the pK of 9.2, with V/K profiles decreasing below this pH. The alkoxides of these substrates appear to be the active species, replacing water in the coordination sphere of Mg^{2+} prior to phosphorylation by MgATP. Glycolate, but not *N*-hydroxycarbamate, can bind when not an alkoxide, since the V profile for the former decreases below a pK of 8.9, while V for the latter is pH independent. Initial velocity patterns for phosphorylation of fluoride in the presence of bicarbonate show saturation by MgATP but not by fluoride. The V/K profile for fluoride decreases above the pK of 9.0, showing that fluoride must replace water in the coordination sphere of Mg^{2+} prior to phosphorylation. None of the above reactions is sensitive to the protonation state of the acid–base catalyst that assists the enolization of pyruvate in the physiological reaction.

Pyruvate kinase catalyzes the physiological phosphorylation of MgADP by phosphoenolpyruvate and, in the reverse direction, the phosphorylation of pyruvate by MgATP:

$$\text{PEP} + \text{MgADP} \rightleftharpoons \text{MgATP} + \text{pyruvate} \quad (1)$$

The enzyme also catalyzes the phosphorylation of a number of other molecules such as fluoride [in the presence of bicarbonate (Tietz & Ochoa, 1958)], hydroxylamine [as its CO_2 adduct, *N*-hydroxycarbamate (Kupiecki & Coon, 1959; Weiss et al., 1984)], and glycolate (Kayne, 1974), as well as the decarboxylation of oxalacetate (Creighton & Rose, 1976a,b) and the detritiation of pyruvate (Robinson & Rose, 1972). It is clear that a base on the enzyme is needed to remove the proton from pyruvate during its phosphorylation (or during the detritiation reaction), but what is not clear is whether this same group can remove a proton from the hydroxyl group of glycolate or *N*-hydroxycarbamate during their phosphorylations or whether a different group or no group is involved. Do such bases play a role in the fluoride reaction or the decarboxylation of oxalacetate? To answer these questions and to try to delineate the role of the second divalent metal ion bound directly to the enzyme [the first divalent metal ion is coordinated to the nucleotide substrate (Gupta et al., 1976)], we have carried out a study of the pH dependence of the kinetic parameters of the various reactions carried out by pyruvate kinase. This paper will deal with the decarboxylation of oxalacetate and the phosphorylations of glycolate, *N*-hydroxycarbamate, and fluoride, and the following paper (Dougherty & Cleland, 1985) will describe the reactions involving phosphoenolpyruvate and the alternate substrate phosphoenol-α-ketobutyrate.

Materials and Methods

Chemicals. Pyruvate kinase and lactate dehydrogenase were obtained from Boehringer as ammonium sulfate suspensions. Each solution was centrifuged, redissolved in 25 mM Tes,[1] pH 7.0, and dialyzed twice for 12 h against 500 volumes of the same buffer. The tris(cyclohexylammonium) salt of phosphoenolpyruvate from Sigma was converted to the potassium salt by passing a solution through a column of Dowex 50-K^+. NADH, glycolic acid, oxalacetic acid were from Sigma, and solutions of the latter two were titrated to alkaline pH with KOH. The potassium salt of ATP was from Calbiochem.

Assay Procedure. Kinetic studies were run in 3-mL total volume in 1-cm cuvettes by measuring absorbance changes at 340 nm with a Beckman monochromator equipped with a Gilford optical density converter and a 10-mV recorder with adjustable zero and multispeed drive. Full-scale sensitivity of 0.05 absorbance was used, and the temperature was maintained at 25 °C with thermospacers and a circulating water bath.

The decarboxylation of oxalacetate was measured by coupling the pyruvate produced with NADH and lactate dehydrogenase and monitoring the decrease of absorbance at 340 nm. The reaction mixture contained 100 mM buffer, 0.2 mM NADH, 1.0 mg of pyruvate kinase, 0.5 mg of lactate dehydrogenase, and various amounts of oxalacetate and $MgCl_2$. Since oxalacetate spontaneously decarboxylates in the presence of divalent metals at a significant rate, a blank containing all reaction components except pyruvate kinase was run at every level of oxalacetate and Mg^{2+} used. This rate was subtracted from the rate in the presence of pyruvate kinase to yield the reported net rate. The rate of the blank was in no case more than 25% of the rate with pyruvate kinase.

The phosphorylations of glycolate, hydroxylamine, and fluoride were measured with the internal ADP assay of Dougherty (1982). In this assay, a moderate level of phos-

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[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.

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phoenolpyruvate is added to convert MgADP to MgATP, so that pyruvate kinase and lactate dehydrogenase are the coupling enzymes. In such an assay, the rate equation for pyruvate production (as measured with the lactate dehydrogenase couple) is

$$\frac{1}{v} = \frac{K_b}{V_1}\left(1 + \frac{K_{ia}}{A} + \frac{K_{ia}P}{K_{ip}A}\right)\frac{1}{B} + \frac{1}{V_1}\left(1 + \frac{K_a}{A} + \frac{V_1K_p}{V_2P}\right) \quad (2)$$

where A, B, and P are the concentrations of MgATP, glycolate, and phosphoenolpyruvate, respectively. If MgATP is held at a fixed level above K_a and K_{ia} and the concentration of phosphoenolpyruvate is not high enough to cause competitive inhibition [from the $P/(AB)$ term in eq 2], then correct values will be seen for V_1/K_b and V_1, as long as phosphoenolpyruvate is high enough for the $V_1K_p/(V_2P)$ term to be negligible. This is helped by the fact that V for the slow alternate substrates (V_1) is much less than V for reaction of MgADP and phosphoenolpyruvate (V_2).

For the glycolate or hydroxylamine reaction, the assay mixture contained 100 mM buffer, 0.2 mM phosphoenolpyruvate, 0.2 mM NADH, 0.01–0.25 mg of pyruvate kinase, and 0.1–0.2 mg of lactate dehydrogenase. Glycolate or hydroxylamine were varied from 10–50 mM at MgATP levels of 0.25, 0.50, and 1.0 mM. The concentration of free Mg^{2+} was 1.0 mM, and KCl was added so the total concentration of K^+ was 100 mM above the level introduced with the buffer. For the hydroxylamine reaction, the assays contained 10 mM $KHCO_3$.

For the fluoride reaction, the assay mixtures contained 100 mM buffer, 90 mM KCl, 10 mM $KHCO_3$, 0.2 mM phosphoenolpyruvate, 0.2 mM NADH, 5 mg of pyruvate kinase, and 0.2 mg of lactate dehydrogenase. Fluoride was varied from 0.4 to 2.0 mM at MgATP levels of 0.50, 1.0, and 2.0 mM. The concentration of free Mg^{2+} was 1.0 mM. A background rate was detected with the fluoride reaction that depended only on the levels of MgATP and pyruvate kinase used. The net rates after subtracting the rates in the absence of fluoride are the ones reported. The background rate varied from 10% to 80% of the rate with fluoride present, the higher background rate occurring at high pH.

The following buffers were used over the indicated range of pH: Mes, pH 5.5–6.7; 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. For all assays, the coupling system was checked at the lowest and highest pH values being used, as well as at an intermediate pH, by obtaining a linear plot of velocity vs. the concentration of pyruvate kinase.

Data Analysis. Reciprocal initial velocities were plotted against reciprocal substrate concentrations, and the experimental data were fitted to eq 3–8 with the Fortran programs

$$v = VAB/(K_{ia}K_b + K_bA + AB) \quad (3)$$

$$v = VAB/(K_{ia}K_b + K_aB + K_bA + AB) \quad (4)$$

$$v = VAB/(K_{ia}K_b + K_bA) \quad (5)$$

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

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

$$\log y = \log [C(1 + K_2/H)/(1 + K_1/H)] \quad (8)$$

of Cleland (1979). The points in the figures are experimental values, while the curves are calculated from fits of the data to the appropriate equation.

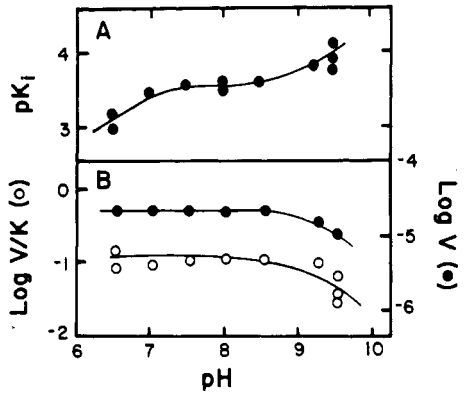


FIGURE 1: pH profiles for the pyruvate kinase catalyzed decarboxylation of oxalacetate. (A) A plot of pK_i for Mg^{2+} vs. pH. The data were fitted to eq 8. (B) V and V/K_{OAA} profiles with units of M min^{-1} and min^{-1}, respectively (1 mg/mL enzyme used). The data were fitted to eq 6.

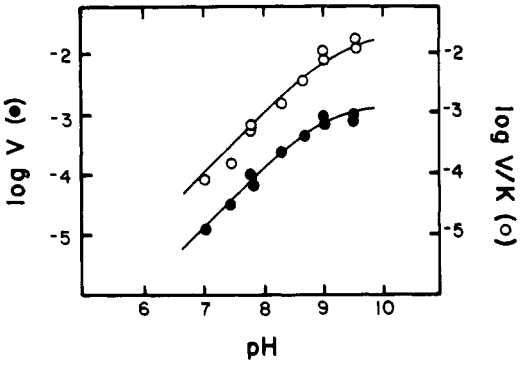


FIGURE 2: pH profiles for the phosphorylation of glycolate. The units are M min^{-1} and min^{-1} for V and V/K (0.17 mg/mL enzyme used). The data were fitted to eq 7.

RESULTS

The initial velocity pattern for oxalacetate at different concentrations of Mg^{2+} was an equilibrium-ordered one at each pH value (Mg^{2+} adding before oxalacetate), with reciprocal plots intersecting on the vertical axis when oxalacetate was the variable substrate. The data at each pH value were fitted to eq 3, and V/K_{OAA}, V, and the dissociation constant (K_i) for Mg^{2+} were tabulated as a function of pH (these are V/K_b, V, and K_{ia} in eq 3). The pK_i profile for Mg^{2+} (Figure 1) drops on the acid side with a pK of 6.95 ± 0.07 and appears to rise on the basic side with a pK of 9.15 ± 0.13. The value of K_i on the plateau at neutral pH was 0.28 ± 0.03 mM. Both V/K_{OAA} and V decrease on the basic side with pK's of 9.2 ± 0.1 and 9.17 ± 0.05, respectively. The plateau values were (1.7 ± 0.1) × 10^{-5} M min^{-1} mg^{-1} for V and (3.5 ± 0.1) × 10^{-2} min^{-1} mg^{-1} for V/K_{OAA}, and the average K_{OAA} was 0.5 ± 0.2 mM.

Initial velocity patterns for glycolate at different levels of MgATP were intersecting, and the data were fitted to eq 4. The V/K values for glycolate obtained in this manner are the true values, as is the value of V, since the concentration of phosphoenolpyruvate used in the internal assay was sufficiently high to convert MgADP to MgATP but not high enough to inhibit the reaction of glycolate competitively. The K_{ia} value for MgATP is only an apparent value, however, and must be divided by $(1 + P/K_{ip})$, where P is phosphoenolpyruvate concentration and K_{ip} its dissociation constant, to yield the true K_{ia} (see eq 2). The corrected K_{ia} values were relatively constant over the pH range studied, averaging 0.7 mM. The $V/K_{glycolate}$ and V profiles both decrease on the acid side with pK's of 9.32 ± 0.07 and 8.89 ± 0.05, respectively (Figure 2). The plateau

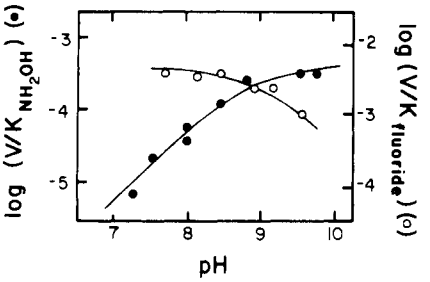


FIGURE 3: V/K profiles for the phosphorylation of fluoride and hydroxylamine (units are min^{-1} for V/K, with 1.67 and 0.53 mg/mL enzyme used, respectively). The data for fluoride are fitted to eq 7 and those for hydroxylamine to eq 6.

values were $(2.8 \pm 0.7) \times 10^{-2}$ min^{-1} mg^{-1} and $(1.6 \pm 0.2) \times 10^{-3}$ M min^{-1} mg^{-1} for V/K and V, respectively, corresponding to a value of 58 ± 23 mM for $K_{glycolate}$, while the value of the latter changed to ~160 mM at neutral pH.

Initial velocity patterns for hydroxylamine at different levels of MgATP and at a fixed bicarbonate level of 10 mM were intersecting, and the data were fitted to eq 4. The true substrate for this reaction is *N*-hydroxycarbamate (Weiss et al., 1984), but at a fixed bicarbonate level the level of *N*-hydroxycarbamate is proportional to the level of added hydroxylamine, since K_{eq} for carbamate formation from bicarbonate and hydroxylamine is 1.33 M^{-1}. The corrected K_{ia} values for MgATP were relatively constant over the pH range studied, averaging 0.2 mM. $V/K_{hydroxylamine}$ decreased on the acid side with a pK of 8.90 ± 0.06 (Figure 3), while the value of V was pH independent with a value of $(2.3 \pm 0.4) \times 10^{-5}$ M min^{-1} mg^{-1} (~0.01% that for the reaction of phosphoenolpyruvate and MgADP at optimal pH). $K_{hydroxylamine}$ had a value of 77 ± 36 mM at high pH, corresponding to 1 mM for the carbamate.

Initial velocity patterns for fluoride at different levels of MgATP and a fixed bicarbonate level of 10 mM appeared to pass through the origin (Figure 4), and the data were fitted to eq 5. The corrected K_{ia} value for MgATP was relatively constant over the pH range studied, averaging 1.7 mM. $V/K_{fluoride}$ decreased on the basic side with a pK of 8.98 ± 0.08 and had a plateau value of $(8.0 \pm 0.7) \times 10^{-4}$ min^{-1} mg^{-1} at neutral pH (Figure 3). The background rate observed in the absence of fluoride was highest above pH 9.2 and decreased below this pH, leveling off at pH 7.75 with a rate one-fifth that at pH 9.2. This background rate was not stimulated by bicarbonate but depended only on the concentrations of MgATP and pyruvate kinase. The background rate was 2×10^6 times slower than the maximum velocity of the physiological reaction of MgADP with phosphoenolpyruvate and presumably represents an ATPase or PEPase reaction catalyzed by pyruvate kinase or an enzyme present as an impurity.

Discussion

Decarboxylation of Oxalacetate. The initial velocity patterns for this reaction were equilibrium ordered, with Mg^{2+} adding before oxalacetate. Since the concentration of oxalacetate is extrapolated to zero in order to determine K_i for Mg^{2+}, the K_i value represents the equilibrium dissociation constant of Mg^{2+} from the enzyme, and the pK's seen in the pK_i profile should have their correct values. Our value of 0.29 mM for the K_i of Mg^{2+} is in good agreement with the value of 0.38 mM obtained by Mildvan & Cohn (1965) from EPR and PRR measurements of Mg^{2+} competition with Mn^{2+}. The drop in the pK_i profile below a pK of 6.95 indicates that there is a group on the enzyme that when protonated prevents binding of Mg^{2+}. Mildvan & Cohn (1965) similarly found

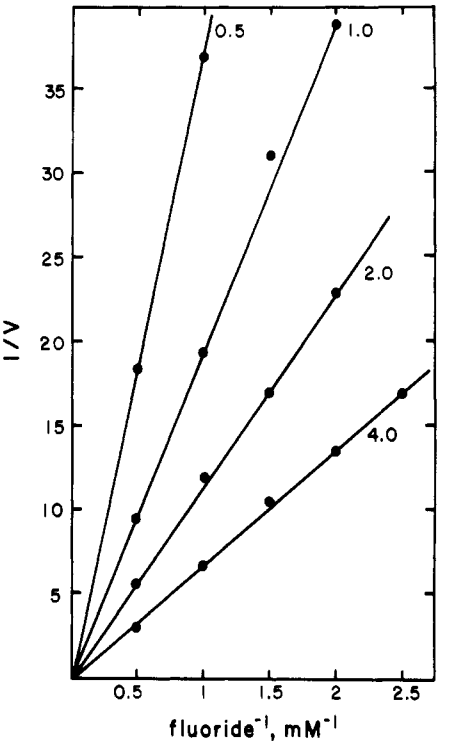


FIGURE 4: Initial velocity pattern for fluoride at the indicated millimolar levels of MgATP at pH 7.0. Free Mg^{2+} was 1 mM, and bicarbonate was 10 mM. The corrected K_{ia} for MgATP was 1.6 mM at this pH. The data were fitted to eq 5. The units of $1/v$ are min $M^{-1} \times 10^{-5}$ (1.67 mg/mL enzyme used).

from EPR and PRR measurements that the pK_i profile for Mn^{2+} with pyruvate kinase dropped below a pK of 6.8 and assigned this pK to a ligand of Mn^{2+}.

Recent proton NMR work of Meshitsuka et al. (1981) resolved resonance lines of six of the 14 histidines in each subunit of pyruvate kinase and showed that the chemical shift of one of the histidines changed dramatically upon binding of phosphoenolpyruvate, which caused a decrease of the pK by 0.4 unit. These authors concluded that since a direct interaction of phosphoenolpyruvate with the ligand would raise its pK, the decrease most likely resulted from binding Mg^{2+}. However, in the X-ray structure of the cat enzyme no histidines are close to the metal binding site, and it appears that Glu-271 is the metal ligand (Hilary Muirhead, personal communication).

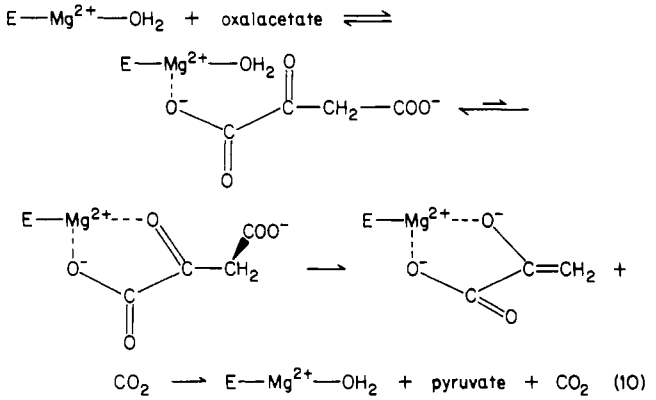
What is most unusual about the pK_i profile in Figure 1 is that it appears to rise above a pK of 9.15. Mildvan & Cohn (1965) also saw this with the pK_i profile for Mn^{2+}, but the pK was 7.8 in that case. We believe that this pK results from the ionization of water coordinated to the metal ion. Since the pK of this water coordinated to enzyme-bound Mg^{2+} is 9.2, while the pK of water coordinated to Mg^{2+} in solution is 12.8, the equilibrium between free Mg^{2+} and enzyme-bound Mg^{2+} will be displaced in favor of the latter as the pH is raised above 9. We can diagram this situation as follows, where we consider only one water molecule in the inner coordination sphere of Mg^{2+}:

$$\begin{array}{ccccc} E + Mg(OH_2) & \rightleftharpoons & E\text{-}Mg(OH_2) & \overset{pK=9.2}{\rightleftharpoons} & E\text{-}MgOH + H^+ \quad (9) \\ & & \Big\| pK=12.3 & & \\ & & MgOH + H^+ & & \end{array}$$

Since the pK of water coordinated to Mn^{2+} in solution is 10.6, it is reasonable that the pK of water coordinated to

enzyme-bound Mn^{2+} would be 7.8, as found by Mildvan & Cohn (1965). The pK of Co^{2+} bound to pyruvate kinase is even lower, as shown by the pH dependence of the detritiation of pyruvate in the presence of MgATP (Robinson & Rose, 1972). This activity is expected to have a similar pH dependence to the rate of phosphoenolpyruvate formation (the detritiation experiments were carried out at equilibrium), which is shown in the following paper to be at a maximum between the pK values of the acid–base catalyst for enolization (~8.3) and the pK of water in the coordination sphere of the enzyme-bound metal ion. The detritiation of pyruvate is at a maximum with Mg^{2+} at pH 8.6 or above, but with Mn^{2+} at pH 8 and with Co^{2+} at pH 7.3 (Robinson & Rose, 1972). The profile with Mn^{2+} peaks between the pK's of 7.8 and 8.3 of the enzyme-bound $Mn(OH_2)$ and the acid–base catalyst, and the Co^{2+} profile suggests that the pK of $Co(OH_2)$ on the enzyme is ~6.3 (the pK of water bound to Co^{2+} in solution is 8.9). Further evidence for the pK of 9.2 resulting from water coordinated to enzyme-bound Mg^{2+} comes from the temperature and solvent dependence of this pK reported in the following paper.

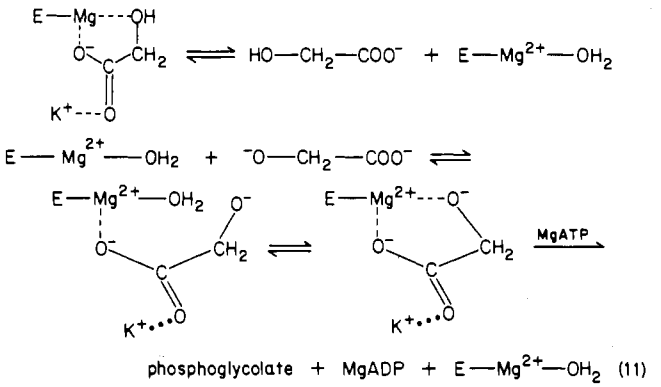
The V/K and V profiles in Figure 1 both show what appears to be the pK of water bound to Mg^{2+}. These data are most easily rationalized by the following mechanism, in which only a small proportion of the bidentate inner sphere complex is present in the steady state:



If the outer sphere complex, which can still lose a proton to give a coordinated hydroxide ion that cannot be displaced by the carbonyl oxygen, were not the major form present, the V profile would not show the pK of metal-bound water. The postulated bidentate inner sphere complex is similar to the oxalate complex, which is known to be bidentate (Ash, 1982). Protonation of enolpyruvate and its release from the enzyme are presumably fast enough not to limit V for this reaction, and thus we cannot tell whether the group that protonates enolpyruvate during the physiological reaction (see the following paper) plays any role in this reaction.

Phosphorylation of Glycolate. The V/K profile for glycolate in Figure 2 shows a pK similar to that of metal-bound water. This could mean either that the active species are glycolate and E–Mg^{2+}–OH^- or that they are the alkoxide of glycolate plus E–Mg^{2+}–OH_2. In the former case, one might expect that the metal-bound hydroxide would be the base that accepted the proton from the hydroxyl of glycolate when it was phosphorylated. However, Ash (1982) has shown with the use of ^{17}O-labeled glycolate that the hydroxyl group enters the inner coordination sphere of Mn^{2+}, and thus, this mechanism is not viable. While reaction of the alkoxide of glycolate and E–Mg^{2+}–OH_2 at first glance seems implausible, there will be 1 part in 10^6 of glycolate in the alkoxide form at pH 9 if the pK of the hydroxyl is 15.[2] We estimate the V/K for glycolate above pH 9 to be $\sim 2 \times 10^3$ M^{-1} s^{-1}, and allowing for the proportion of alkoxide in glycolate, the corrected V/K value is only 2×10^9 M^{-1} s^{-1}, which does not exceed the diffusion-controlled limit. The alkoxide will form at a rate of over 10^5 s^{-1}, so its level will always be at equilibrium in solution.

Although the above calculation shows that it is plausible for the alkoxide of glycolate to displace water from enzyme-bound Mg^{2+} and thus to generate a Mg^{2+}–alkoxide that would be very rapidly phosphorylated, the V profile also decreases as the pH is lowered, and thus, it is is clear that glycolate will bind to the enzyme with its hydroxyl still protonated. The experiments of Ash (1982) were carried out in fact at a pH where glycolate was not present as an alkoxide. We can diagram the probable mechanism as



We presume that the inner sphere alkoxide complex is phosphorylated faster than it isomerizes back to the outer sphere one or (above pH 9) is protonated, so that the critical step is formation of the inner sphere complex (a step that requires the glycolate hydroxyl to be ionized and the bound water to be protonated). There should of course be a second path for formation of the inner sphere alkoxide complex, namely, direct loss of a proton from the inner sphere complex where glycolate is not an alkoxide. This is thermodynamically an equivalent mechanism, but whether there are kinetic barriers to this path cannot be told by steady-state kinetic studies.

The phosphorylation of glycolate, like that of pyruvate, requires K^+ or NH_4^+ as an activator, and the monovalent ion is thought to bind to the carboxyl of the substrate as well as the enzyme (Nowak & Mildvan, 1972). We have shown it in a position consistent with NMR measurements [NH_4^+ is 4.4 Å from Mn^{2+} when phosphoenolpyruvate is present (Raushel & Villafranca, 1980)], but its exact location and mode of interaction with the substrate are not yet known.

Phosphorylation of Hydroxylamine. This reaction requires bicarbonate, and Weiss et al. (1984) have shown that the actual substrate is *N*-hydroxycarbamate, which is formed by the following reactions:

$$HCO_3^- \rightleftharpoons CO_2 + OH^- \qquad (12)$$

$$CO_2 + NH_2OH \rightleftharpoons HO\text{–}NH\text{–}CO_2^- + H^+$$

The overall equilibrium constant for carbamate formation from bicarbonate and hydroxylamine is 1.33 M^{-1} (Weiss et al., 1984), so that the level of carbamate is low (1.33% of the

[2] Since thioglycolate and ethanethiol have nearly identical pK values (the negative charge balancing the inductive effect of the carboxyl), the pK of glycolate might be expected to be similar to that of ethanol (~16). What is critical, however, is the pK of the glycolate hydroxyl in the active site where the carboxyl is coordinated to Mg^{2+}. This value is probably at least 1 pH unit less than 16.

hydroxylamine level at the 10 mM bicarbonate level used in the present study). *N*-Hydroxycarbamate is isosteric with glycolate, and thus it is not surprising to see a pH dependence similar to that for glycolate. Presumably, the alkoxide (^-O–NH–CO_2^-) is the active form of the substrate. The pK for alkoxide formation is probably somewhat lower than that for glycolate, since hydroxylamine has a lower pK (13.7) than methanol (15.5). One difference from the glycolate reaction is that V was pH independent, showing that *N*-hydroxcarbamate does not bind appreciably to the enzyme when the hydroxyl is not ionized.

Our data can help explain the unusual preference for the divalent metal activator for the hydroxylamine reaction and why the reaction has been reported not to occur with Mg^{2+}. Previous studies (Kupiecki & Coon, 1959, 1960; Cottam et al., 1968)) have been conducted at pH 7.5, well over the edge of the pH profile with Mg^{2+}. Since one-fifth the amount of enzyme we have employed was used in these studies, it is easy to see how the activity with Mg^{2+} could have escaped detection at this pH.

The higher activity with Zn^{2+} probably results from the low pK of water coordinated to enzyme-bound Zn^{2+}, as opposed to the value of 9 for Mg^{2+}. [We estimate ~6, since water coordinated to enzyme-bound Mn^{2+} has a pK of 7.8 (Mildvan & Cohn, 1965), and the value with Co^{2+} was estimated above from the detritiation studies of Robinson & Rose (1972) to be ~6.3, while the pK's of metal-bound water in solution are 12.8 for Mg^{2+}, 10.6 for Mn^{2+}, 8.9 for Co^{2+}, and 8.7 for Zn^{2+}.] Zn^{2+} is a good activator at low pH for the CrATP-induced enolization of pyruvate, and for the phosphorylation of glycolate by CrATP (Dunaway-Mariano et al., 1979). Its low activity for the physiological reaction (3% that with Mg^{2+} at pH 6.7; not detected at pH 7.5; Dunaway-Mariano et al., (1979) may result partly from a nonproductive mode of binding to ADP or ATP when the natural substrates are present but certainly stems also from the fact that activity drops above the pK of coordinated water (~6 in this case).

Phosphorylation of Fluoride. The initial velocity patterns for fluoride at different fixed levels of MgATP all appear to go through the origin, although a finite dissociation constant was observed for MgATP. This suggests a direct chemical reaction between fluoride and enzyme-bound MgATP. Conceivably, the K_m for fluoride could be very high, but to avoid forming a precipitate of MgF_2 (solubility product 6.4 mM^3), concentrations of fluoride above 2 mM could not be used.

The V/K profile for fluoride decreases on the basic side with a pK of 9.0, which appears to be the pK of water coordinated to enzyme-bound Mg^{2+}. Presumably, fluoride can displace water but not hydroxide from the coordination sphere of Mg^{2+} and is then phosphorylated. The dissociation constant of MgF^+ in solution is 50 mM at 0.5 M ionic strength (Connick & Tsao, 1954), so it is not surprising not to see a finite K_m for fluoride. Transfer of fluoride from Mg^{2+} to phosphate should be a facile process, since nonhydrated fluoride should be an excellent nucleophile. Thus, the role of Mg^{2+} coordination may be largely to dehydrate the fluoride ion.

Bicarbonate is an obligate activator for the phosphorylation of fluoride (Tietz & Ochoa, 1958). We presume that its function is to combine in that portion of the active site normally occupied by the carboxyl group of the substrate and induce the proper conformation change to activate phosphate transfer from MgATP.

Glyoxylate-Induced ATPase. We should mention this activity of pyruvate kinase, which has been studied by Rendina & Cleland (1984). The rate is optimal at high pH (V is 18% and $V/K_{glyoxylate}$ is 31% the values for the phosphorylation of pyruvate by MgATP), with V/K for glyoxylate decreasing below a pK of 9.3, but leveling out below pH 7 at 1% the rate at high pH. No ^{18}O transfer takes place from glyoxylate to phosphate during the reaction, and there is no deuterium isotope effect on V/K with [2-^{2}H]glyoxylate, so it is the predominant hydrate that is the activator. It appears that the presence of glyoxylate in the active site induces the enzyme to transfer phosphate from MgATP to hydroxide coordinated to enzyme-bound Mg^{2+}, although transfer to water also occurs with a rate that is 2 orders of magnitude slower.

Conclusions. The data presented in this paper show evidence of only two groups in the pH profiles for the reactions studied. First, a group with a pK near 7 (probably glutamate) appears to be a ligand for the divalent metal ion that combines directly with the enzyme. The pK of this group is seen in the pK_i profile for Mg^{2+} but not in any pH profile where Mg^{2+} was kept at a saturating level. Second, a pK of near 9 is seen in the pH profiles for all of the reactions studied. Since loss of this proton tightens the binding of Mg^{2+}, we believe this group is water coordinated to the enzyme-bound Mg^{2+}. This pK is nearly 4 pH units lower than the value for free Mg^{2+} in solution, presumably because the active site has a net positive charge prior to adsorption of the anionic substrate ($MgATP^{2-}$ and Mg^{2+} effectively neutralize each other, so the active site plus the monovalent cation activator must provide the positive charge). Activity occurs only when this water is not ionized. Formation of hydroxide in the inner coordination sphere of Mg^{2+} prevents all reactions (presumably by not permitting ligand substitutions), although for phosphorylation of glycolate or *N*-hydroxycarbamate where the alkoxide is the active species the pH range where both alkoxide and E–Mg^{2+}–OH_2 exist lies above the pK of this coordinated water.

In none of the pH profiles reported here do we see any indication of an acid–base catalyst for the enolization of pyruvate. We will demonstrate in the following paper that such a group exists, but its state of protonation clearly has no influence on the reactions studied here.

In all of the phosphorylation reactions described here, pyruvate kinase is unique in transferring phosphate to a metal-bound nucleophile. Thus, Mg^{2+}–alkoxides or Mg^{2+}–F^- appear to be the species phosphorylated, and the transfer is between the metal ion and phosphorus. For most kinases either no acid–base catalyst is needed (acetate, 3-phosphoglycerate, and adenylate kinases), or a carboxyl group or histidine on the enzyme accepts the proton from the alcohol or other group being phosphorylated [hexokinase, Viola & Cleland (1978); fructokinase, Raushel & Cleland (1977); creatine kinase, Cook et al. (1981)]. We shall see in the following paper that a Mg^{2+}–enolate of pyruvate appears to be an intermediate in the pyruvate reaction and that an acid–base catalytic group on the enzyme is required to assist in the enolization or ketonization of pyruvate.

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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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