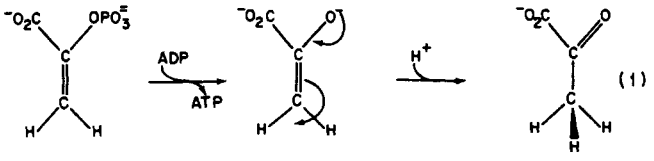
Dual Divalent Cation Requirement for Activation of Pyruvate Kinase: Essential Roles of Both Enzyme- and Nucleotide-Bound Metal Ions†

Raj K. Gupta, R. Marc Oesterling, and Albert S. Mildvan*

ABSTRACT: Rabbit muscle pyruvate kinase requires two divalent cations per active site for catalysis of the enolization of pyruvate in the presence of adenosine 5′-triphosphate (ATP). One divalent cation is bound directly to the enzyme and forms a second sphere complex with the bound ATP (site 1). The second divalent cation is directly coordinated to the phosphoryl groups of ATP and does not interact with the enzyme (site 2). The essential role of the divalent cation at site 1 is shown by the requirement for Mg^{2+} or Mn^{2+} for the enolization of pyruvate in the presence of the substitution inert Cr^{3+}-ATP complex. The rate of detritiation of pyruvate shows a hyperbolic dependence on Mn^{2+} concentration in the presence of high concentrations of enzyme and Cr^{3+}-ATP. A dissociation constant for Mn^{2+} from the pyruvate kinase-Mn^{2+}-ATP-Cr^{3+}-pyruvate complex of 1.3 ± 0.5 μM is determined by the kinetics of detritiation of pyruvate and by parallel Mn^{2+} binding studies using electron paramagnetic resonance. The essential role of the divalent cation at site 2 is shown by the sigmoidal dependence of the rate of detritiation of pyruvate on Mn^{2+} concentration in the presence of high concentrations of enzyme and ATP yielding a dissociation constant of 29 ± 9 μM for Mn^{2+} from site 2. This value is similar to the dissociation constant of the binary Mn-ATP complex (14 ± 6 μM) determined under similar conditions. The rate of detritiation of pyruvate is proportional to the concentration of the pyruvate kinase-Mn^{2+}-ATP-Mn^{2+}-pyruvate complex, as determined by parallel kinetic and binding studies. Variation of the nature of the divalent cation at site 1 in the presence of CrATP causes only a twofold change in the rate of detritiation of pyruvate which does not correlate with the pK_a of the metal-bound water. Variation of the nature of the divalent cation at both sites in the presence of ATP causes a sevenfold variation in the rate of detritiation of pyruvate that correlates with the pK_a of the metal-bound water. The greater rate of enolization observed with CrATP fits this correlation, indicating that the electrophilicity of the nucleotide bound metal (at site 2) determines the rate of enolization of pyruvate.

Rabbit muscle pyruvate kinase reversibly transfers the phosphoryl group of phosphoenolpyruvate to adenosine diphosphate and a proton from water to the enolate of pyruvate



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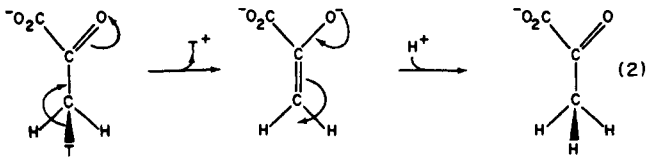
(Boyer, 1962; Kayne, 1973). The enzyme has long been known to catalyze a partial reaction, the enolization of pyruvate (Rose, 1960; Robinson and Rose, 1972; Flashner et al., 1973; Gupta



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et al., 1976). In all of these reactions the enzyme shows an absolute requirement for a divalent and a monovalent cation. The metal ion activators in vivo are Mg^{2+} and K^+, but the divalent metal ion requirement in vitro can be satisfied by Mn^{2+}, Co^{2+}, or Ni^{2+} (Boyer, 1962; Robinson and Rose, 1972; Melamud and Mildvan, 1975; Kwan et al., 1975). The enzyme is a tetramer of molecular weight 237 000, and each of its four functionally identical subunits possesses a high affinity site for the divalent cation and a site of lower affinity for the monovalent cation. Using the Mn^{2+}-pyruvate kinase complex, it has recently been demonstrated that ATP is not directly coordinated to the enzyme-bound Mn^{2+}, but that the bound nucleotide forms a second-sphere complex of the enzyme-bound Mn^{2+} (Sloan and Mildvan, 1976). Since ATP itself also has a high affinity for divalent cations and since we have previously detected the existence of a pyruvate kinase-Mn^{2+}-ATPCr complex (Gupta et al., 1976), we have now investigated the possible requirement for two functional divalent cations per active site, one bound to the protein and another one directly coordinated to bound ATP.

To examine this possibility, we chose to study the partial reaction of pyruvate enolization. This choice was based on the following considerations: (1) All of the components of the net reaction including ATP or an analogue of ATP (Rose, 1960) are essential for the enolization of pyruvate. (2) After initial equilibration, the chemical composition of the system does not change further during the course of the exchange reaction. Only the fraction of tritiated pyruvate decreases as the reaction progresses. (3) Even at saturating levels of ATP and Mn^{2+} and at the high concentrations of enzyme necessary for parallel binding studies, the rates are slow enough to be measured precisely. (4) Unlike the product inhibited overall phosphoryl transfer reaction, in the case of enolization, since no net chemical reaction takes place, no progressive inhibition by the accumulation of product can take place. (5) The observed re-

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action is unidirectional due to the large dilution factor provided by water.

Several independent approaches were used to detect the presence and to clarify the role of the two types of divalent cation sites in the functional enzyme-ATP complex. The first consisted of using CrATP, a tridentate substitution inert analogue of Mn^{2+}-ATP as an activator of the enolization reaction (Gupta et al., 1976). The second examined the rate of pyruvate enolization as a function of all forms of bound Mn^{2+} at high concentrations of the enzyme-ATP complex. The free Mn^{2+} was determined by parallel Mn^{2+}-binding studies using electron paramagnetic resonance. Third, the rates of enolization with ATP and CrATP were measured using different divalent metal activators of varying electronegativity to clarify the electronic effects at each divalent cation site.

Experimental Procedures

Materials

Rabbit muscle pyruvate kinase used in these studies was obtained from Boehringer und Soehne. The enzyme was homogeneous as judged by its specific activity (130–160 units/mg) and by acrylamide gel electrophoresis in the presence of sodium dodecyl sulfate. ATP, DPNH, and 1,4-piperazinediethanesulfonic acid[1] (Pipes) were obtained from Sigma Chemical Co. All other chemicals were reagent grade or of the highest purity commercially available. Pyruvate kinase was separated from ammonium sulfate by gel filtration using Sephadex G-25 (medium) and concentrated in collodion bags by vacuum dialysis at 4 °C.

CrATP was prepared by heating 100 ml of a solution containing 10 mM chromium perchlorate and 10 mM disodium ATP for 12 min at 80 °C and then cooling as previously described (DePamphilis and Cleland, 1973; Gupta et al., 1976). The solution was then placed on a column (1.5 × 15 cm) of Dowex 50-X2-H^+, 100–200 mesh, and eluted by extensive washing with distilled water at 4 °C. The central part of the first of two major bands eluted was used for these experiments after concentration by rotary evaporation at 35 °C or by freeze-drying. The material was stored at 4 °C or frozen at liquid nitrogen temperature. CrATP concentration was determined by measuring uv absorption at 260 nm with a millimolar extinction coefficient of 15.4 (DePamphilis and Cleland, 1973).

β-Tritiated sodium pyruvate (specific activity 10^6 counts min^{-1} μmol^{-1}) was a generous gift from Dr. Paul Meloche of this Institute. It was diluted by a factor of ten with cold pyruvate to a specific activity of 10^5 counts min^{-1} μmol^{-1} before use in exchange experiments. The concentration and purity of pyruvate solutions were checked by standard enzymatic assay procedures using lactate dehydrogenase.

Methods

Enolization of Pyruvate. All experiments were done at pH 6.7 in K^+-Pipes buffer or at pH 7.3 in Tris-Cl buffer unless otherwise specified. The following procedure was used to determine the rate of enzyme-catalyzed detritiation of pyruvate. After 10–60 min of incubation, an aliquot of 0.1 ml from the reaction mixture was transferred to a (1 × 4 cm) column of Dowex 1-Cl^- resin. The column was then washed with 3.0 ml of water and allowed to drain completely. A 0.5-ml aliquot of the effluent was then counted in an ethanol–toluene scintillation fluid. The observed counts released at varous time intervals were subjected to a first-order correction (Rose, 1960) to calculate ΔV_x the initial exchange rate, expressed as μmol of pyruvate enolized (mg of protein × min)$^{-1}$.

TABLE I: Effect of Divalent Cations on the CrATP Activated Enolization of Pyruvate.[a]

Components[a]	V_x	ΔV_x[b]
CrATP (2 mM) + Mg^{2+} (7.5 mM)	9.30	9.25
CrATP (2 mM) + Mn^{2+} (2 mM)	5.32	5.27
CrATP (2 mM)	0.39	0.34
CrATP (2 mM) + EDTA (1 mM)	0.05	—
ATP (2 mM) + Mn^{2+} (4 mM)	1.43	1.38
ATP (2 mM) + Mg^{2+} (15 mM)	0.28	0.23
ATP (2 mM)	0.05	—
Mg^{2+} (10 mM)	0.04	—

[a] Other components present were 0.1 M KCl, 50 mM K^+-Pipes, pH 6.7, and 80.6 mM sodium pyruvate, and 14.2 μM pyruvate kinase sites. $T = 23 \pm 1$ °C. [b] In the absence of the enzyme but in the presence of CrATP or ATP, 2% of the counts initially present in the substrate are found in the effluent of the anion exchange resin. This same low amount of detritiation is shown in all the controls and is taken to be a measure of the nonenzymatic exchange of the methyl hydrogens of pyruvate. This nonenzymatic exchange was subtracted from the observed rate V_x to obtain the true rate denoted by ΔV_x.

EPR Studies. In the same samples used for kinetic studies, the free Mn^{2+} concentrations were determined by measuring the average amplitude of the six lines in the first derivative EPR spectrum of Mn^{2+} at low modulation amplitudes. The EPR signal due to bound Mn^{2+} is unobservable under these conditions owing to excessive broadening of the resonances (Cohn and Townsend, 1954). All experiments were done at 23 ± 1 °C.

Calculation of Dissociation Constants. In a mixture of Mn^{2+} complexes the concentration of free Mn^{2+}, as determined by EPR, must simultaneously satisfy all equilibria involving Mn^{2+} including its reversible dissociation from the active complex. From the kinetic data, the extent of occupancy of the available active sites by Mn^{2+} is given by $\Delta V_x/(\Delta V_x)_{max}$. The dissociation constant of Mn^{2+} (K_D) from the active enzyme-Mn^{2+}-substrate complex (E*-Mn^{2+}) is therefore given by:

$$K_D = \frac{[Mn^{2+}]_f[E^*]}{[E^*\text{-}Mn^{2+}]} = \frac{[Mn^{2+}]_f([E^*]_T - [E^*\text{-}Mn^{2+}])}{[E^*\text{-}Mn^{2+}]}$$

$$= [Mn^{2+}]_f \left(\frac{(\Delta V_x)_{max}}{\Delta V_x} - 1\right) \quad (3)$$

where [E*] is the concentration of the enzyme-substrate complex. From eq 3 the concentration of free Mn^{2+} at half-maximal velocity ($(\Delta V_x)_{max}/\Delta V_x = 2$) is a direct measure of the dissociation constant K_D.

The dissociation constants of the binary enzyme-ATP and enzyme-pyruvate complexes and of the ternary enzyme-Mn^{2+}-ATP and enzyme-Mn^{2+}-pyruvate complexes were precisely determined by computer analysis (Reed et al., 1970) of previously published titration data using the proton relaxation rate (PRR) of water which had previously been analyzed by approximate graphical methods (Mildvan and Cohn, 1966).

[1] Abbreviations used are: ATP, adenosine 5′-triphosphate; DPNH, reduced diphosphopyridine nucleotide; Pipes, 1,4-piperazinediethanesulfonic acid; EPR, electron paramagnetic resonance; PRR, protein relaxation rate; EDTA, (ethylenedinitrilo)tetraacetic acid.

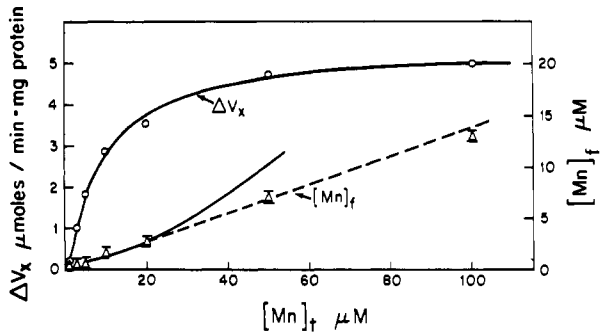

FIGURE 1: Rate of enolization of pyruvate and concentration of free Mn^{2+} as a function of total Mn^{2+} concentration in the presence of a saturating level of CrATP and a low level of pyruvate kinase. The rate of enolization was determined by the appearance of tritium counts in water from [3-^{3}H]pyruvate and the concentration of free Mn^{2+} was determined by EPR as described in the text. Components present were sodium pyruvate (91.5 mM), CrATP (2.35 mM), K^+-Pipes buffer (56 mM, pH 6.7), KCl (110 mM), and pyruvate kinase (0.208 mg/ml or 3.51 μM sites) in a total volume of 0.15 ml. $T = 23 \pm 1$°C. The solid curve through the $[Mn]_f$ points was constructed by assuming a dissociation constant of 0.9 ± 0.4 μM as calculated using eq 3.

TABLE II: Dissociation Constants of Mn^{2+} from the Quaternary Enzyme-Nucleotide-Metal Complexes in the Presence of Saturating Pyruvate.[a]

Complex	pH	K_D (μM) Site 1	K_D (μM) Site 2	Method of Determination
E-[Mn^{2+}]-ATPCr^{3+}	6.65	0.9 ± 0.4[b]	—	Kinetics + EPR[c]
E-[Mn^{2+}]-ATPCr^{3+}	6.75	1.3 ± 0.5[d]	—	Kinetics + EPR[c]
E-Mn^{2+}-ATP-[Mn^{2+}]	6.75	—	21 ± 7[e]	Kinetics + EPR[c]
E-Mn^{2+}-ATP-[Mn^{2+}]	7.3	—	20 ± 4[f]	Kinetics + EPR[c]
E-[Mn^{2+}]-ATP-Mn^{2+}	7.3	0.6 ± 0.1[g]	—	EPR[g]
E-Mn^{2+}-ATP-[Mn^{2+}]	7.3	—	38 ± 7[g]	EPR[g]

[a] K_D is the dissociation constant of the component in brackets from the indicated complex. [b] From the data of Figure 1. [c] K_D was calculated using eq 3. [d] From the data of Figure 2A. [e] From the data of Figure 2B. [f] From the data of Figure 3. [g] K_D values were determined by a theoretical fit to the Scatchard plot (Figure 4) calculated for two classes of noninteracting sites (Miziorko and Mildvan, 1974).

Results and Discussion

Activator Constant of Mn^{2+} in the CrATP-Activated Enolization of Pyruvate Catalyzed by Pyruvate Kinase. As previously shown qualitatively (Gupta et al., 1976) and as exemplified in Table I, CrATP can replace ATP in promoting the enolization of pyruvate only when a divalent cation such as Mg^{2+} or Mn^{2+} is present. The results obtained with the complete system (Table I) containing the enzyme, Mg^{2+}, K^+, and CrATP or ATP indicate that CrATP is a better activator of the enolization of pyruvate than is ATP at pH 6.7. When the divalent cation is omitted the exchange is reduced to 4% and the further addition of EDTA eliminates all exchange (Table I). Since both CrATP and pyruvate have very low affinities for divalent cations with dissociation constants greater than 10 mM (Gupta et al., 1976), these results indicate an absolute requirement for an enzyme-bound divalent cation for the enolization process, providing qualitative evidence for the formation of a functional enzyme-M^{2+}-ATPCr-pyruvate complex.

For the quantitative studies, Mn^{2+} was used as the divalent cation activator, rather than Mg^{2+}, since the concentration of free Mn^{2+} can be determined by EPR spectroscopy (Cohn and Townsend, 1954). To determine the dissociation constant of Mn^{2+} from the kinetically active enzyme-Mn^{2+}-ATPCr-pyruvate complex, the rate of pyruvate enolization and the concentration of free Mn^{2+} were both determined as a function of the concentration of total Mn^{2+} in a system containing a low concentration (3.5 μM) of pyruvate kinase sites, and saturating levels of pyruvate and CrATP (Figure 1). The dissociation constant of 1.2 μM for Mn^{2+} was estimated from Figure 1, according to eq 3, as the concentration of free Mn^{2+} at the half-maximal velocity (Table II). Since the concentration of free Mn^{2+} must satisfy all equilibria involving Mn^{2+} complexes, and since E-Mn^{2+}-CrATP-pyruvate is the only functional complex of Mn^{2+} present, the above determination of the dissociation constant was not complicated by the presence of kinetically inactive complexes of Mn^{2+}. Using a dissociation constant of 0.9 ± 0.4 μM, the calculated concentrations of free Mn^{2+} using eq 3 agree with those observed by EPR over the sensitive range up to a total Mn^{2+} concentration of 20 μM (Figure 1). Above this level, deviations of the calculated and observed free Mn^{2+} values are noted, which result from inaccuracies in the calculated curve as the level of Mn^{2+} approaches saturation.

It may be noted that the shape of the kinetic titration curve of Figure 1 is hyperbolic indicating that the binding of Mn^{2+} to a single type of site with a dissociation constant of 0.9 ± 0.4 μM (Table II) results in full activation of the enzyme.

A very similar dissociation constant for Mn^{2+} from the enzyme-Mn^{2+}-ATPCr-pyruvate complex (1.3 ± 0.5 μM) was obtained from an experiment at a much higher enzyme concentration (319 μM sites) in the presence of saturating pyruvate (67.3 mM) but at a limiting level of CrATP (100 μM) (Figure 2A) (Table II). While these results establish the requirement for an enzyme-bound divalent cation such as Mn^{2+} for the enolization of pyruvate in the presence of CrATP, and show a high affinity of this site for Mn^{2+}, they do not establish the requirement for the nucleotide-bound metal. To examine the latter point, parallel experiments substituting ATP for CrATP were carried out.

ATP Activated Enolization of Pyruvate Catalyzed by Pyruvate Kinase. In these experiments a high enzyme concentration was used such that most of the nucleotide and significant amounts of the Mn^{2+} were bound in the active complex. When CrATP was replaced by ATP at high enzyme concentration under conditions otherwise identical with those described above (Figure 2B) the Mn^{2+} activation of pyruvate enolization is sigmoidal rather than hyperbolic. This finding indicates that the Mn^{2+} which binds to the high affinity site is alone insufficient to activate the enzyme. In addition, a second site on the enzyme-Mn^{2+}-ATP-pyruvate complex, with a dissociation constant of 21 ± 7 μM, must be occupied before catalysis of enolization is observed (Figure 2B). This dissociation constant, calculated from the concentrations of free Mn^{2+} along the activation curve of Figure 2B using eq 3, is very similar to that of the binary Mn-ATP complex of 21 ± 11 μM calculated for pH 6.7 from an EPR determination of the dissociation constant at pH 7.5 (Table III), using a pK_a for ATP of 6.5 ± 0.4 (Dawson et al., 1969).

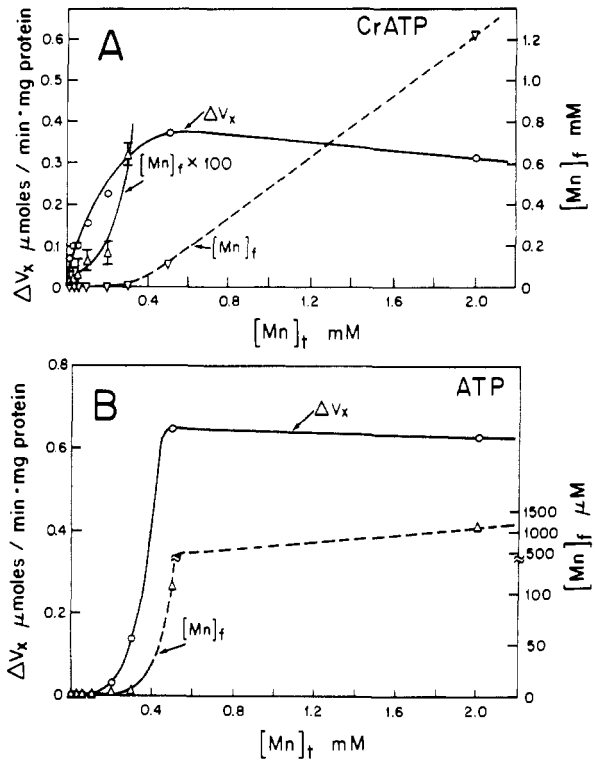

FIGURE 2: Comparison of the effects of CrATP and ATP in parallel enolization and Mn^{2+} binding studies at high levels of pyruvate kinase and limiting levels of nucleotides. In A the components present were CrATP (107 μM), pyruvate kinase (18.9 mg/ml or 319 μM sites), sodium pyruvate (67.3 mM), K^+-Pipes buffer (50 mM, pH 6.75), KCl (100 mM). The components in B were identical except that ATP (102 μM) was present instead of CrATP. $T = 23 \pm 1$ °C. In A the solid curve through the $[Mn]_f$ points was calculated by assuming a dissociation constant of 1.3 ± 0.5 μM, calculated with the use of eq 3. Similarly in B the solid curve through the $[Mn]_f$ points was calculated assuming a dissociation constant of 21 ± 7 μM for Mn^{2+} calculated with the use of eq 3.

TABLE III: Constants of Binary and Ternary Complexes of Mn^{2+} and Pyruvate Kinase at pH 7.5.[a]

Complex	K_D (μM)	Method of Determination
Mn-ATP	14 ± 6	EPR[b]
E-ATP	155 ± 15[e]	PRR[b,c]
E-[Mn]-ATP	11 ± 2	PRR[b,c]
E-Mn-[ATP]	22 ± 4	PRR[b,c]
Mn-pyruvate	$114\,000 \pm 30\,000$	EPR[d]
E-pyruvate	533 ± 100	PRR[b,c]
E-[Mn]-pyruvate	15 ± 3	PRR[b,c]
E-Mn-[pyruvate]	119 ± 15	PRR[b,c]

[a] K_D is the dissociation constant of the component in brackets from the indicated complex. [b] From Mildvan and Cohn, 1966. [c] Recalculated from proton relaxation rate (PRR) titrations of Mildvan and Cohn (1966) using the computer program of Reed et al., 1970. The uniqueness of the fit of the binding data of Mildvan and Cohn (1966) to the program of Reed et al. (1970) with standard deviations of 5.9–8.2% in ϵ_T (18.1) is indicated by the constancy of the parameters (K_3, K_S, and ϵ_T) derived from ATP titrations carried out at five enzyme concentrations ranging from 96.7 to 324 μM sites. Under these conditions the concentration of the bimetallic complex was, at most, 4.2% of the concentration of the enzyme bound Mn^{2+}. [d] From Berman and Cohn, 1970. [e] This value is an order of magnitude lower than a dissociation constant (1.25–1.95 mM) obtained by fluorescence quenching (Price, 1972). Under the conditions of high enzyme concentration used in the PRR titrations (Mildvan and Cohn, 1966) tight binding sites for ATP would predominate, whereas two types of binding sites for nucleotides, tight and weak, are known to exist on pyruvate kinase, as detected by the kinetic protection method (Mildvan and Cohn, 1966) and by x-ray diffraction (Stammers and Muirhead, 1975). The ATP and ADP sites detected by fluorescence quenching (Price, 1972) represent the weaker sites which are probably not kinetically active, since the dissociation constant of the tight-binding site (for ADP) better satisfies the rate equation for the overall phosphoryl transfer reaction than does that of the weak site (Mildvan and Cohn, 1966).

All of the above experiments were carried out at pH 6.7 for comparison with CrATP which is unstable at higher pH values (DePamphilis and Cleland, 1973). For a more detailed analysis of all of the Mn^{2+} complexes present in the ATP-activated enolization of pyruvate, a titration measuring enzymatic activity as a function of Mn^{2+} concentration was carried out at pH 7.3 where the dissociation constants of all of the relevant binary and ternary complexes are known. These values are summarized in Table III. Since ATP itself binds metal ions tightly (Mildvan and Cohn, 1966), conditions had to be chosen such that nearly all of the ATP present was complexed to the enzyme and large amounts of Mn^{2+} were bound in the active complex. High levels of the enzyme (293 μM) had to be used to achieve this. The concentration of ATP was set at 98 μM. From the known dissociation constants of ATP from the E-ATP and E-Mn-ATP complexes (Table III), it can be shown that under the experimental conditions chosen more than 80% of ATP was complexed to the enzyme. Thus at saturating concentrations of Mn^{2+}, nearly one-third of the total enzyme present was in the active complex. Raising the enzyme concentration or lowering the ATP concentration would have resulted in a lower fraction of the active complex relative to the inactive enzyme-Mn-pyruvate complex which also binds Mn^{2+} fairly tightly (Table III) and thus complicates the analysis. At the high level of pyruvate used (80 mM) all enzyme forms may be assumed to be saturated with pyruvate (Table III).

The experimental results at pH 7.3 are shown by the points in Figure 3. As at the lower pH, sigmoidal kinetic behavior is observed. The dissociation constant of Mn^{2+} from the active complex (20 ± 4 μM) calculated according to eq 3 from the concentration of free Mn^{2+} at half-maximal rate of enolization (Table II) is in agreement with the dissociation constant of the binary Mn-ATP complex (14 ± 6 μM, Table III). The concentration of free Mn^{2+} at each point of the titration curve was measured by EPR and a Scatchard plot (Figure 4) was constructed for Mn^{2+} bound only to the enzyme-ATP-pyruvate complex. This quantity was calculated by subtracting the concentrations of the enzyme-Mn-pyruvate and the Mn-pyruvate complexes from the total bound Mn^{2+}, using the dissociation constants that were determined separately (Table III). The Scatchard plot analyzed as previously described (Miziorko and Mildvan, 1974) indicates the presence of two different Mn^{2+} binding sites on the enzyme-ATP-pyruvate complex, one with a dissociation constant of 0.6 ± 0.1 μM and the other with a dissociation constant of 38 ± 7 μM (Figure 4). The former value is comparable to the dissociation constant of Mn^{2+} from the enzyme-Mn^{2+}-CrATP-pyruvate complex (Table II) and the latter value is comparable to the dissociation constant of the active complex (Table II), and also to that of the binary Mn-ATP complex (Table III). As with all Scatchard analyses of binding data involving two classes of sites, the estimated error levels in the dissociation constants are high, more so for the weaker site.

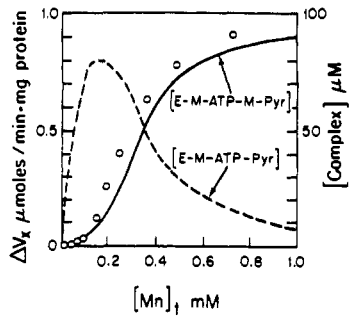

FIGURE 3: Correlation of rate of enolization of pyruvate with calculated concentrations of mono- and bimetallic pyruvate kinase complexes at pH 7.3. The open circles represent the measured values of ΔV_x the rate of enolization. The curves show the concentrations of the Mn^{2+} complexes which were calculated from dissociation constants determined under identical conditions. The dissociation constants of the Mn^{2+} complexes containing enzyme, ATP, and pyruvate were determined by Scatchard analysis of parallel Mn^{2+} binding data (Figure 4, Table II) taking into account the dissociation constants of simpler binary and ternary complexes present (Table III). Components present were ATP (98 μM), pyruvate kinase (17.4 mg/ml or 293 μM sites), sodium pyruvate (80 mM), Tris-Cl buffer (50 mM, pH 7.3), KCl (100 mM). $T = 23 \pm 1$ °C.

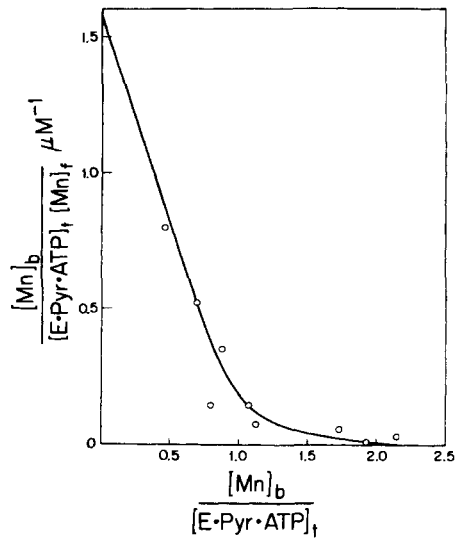

FIGURE 4: Scatchard plot of Mn^{2+} titration of the pyruvate kinase-ATP-pyruvate complex at pH 7.3. The Mn^{2+} bound only to the enzyme-ATP-pyruvate complex was calculated as described in the text using the dissociation constants of Table III. The smooth curve fitted to the data points was calculated by assuming $n_1 = 0.94 \pm 0.18$, $K_1 = 0.60 \pm 0.12$ μM, $n_2 = 1.21 \pm 0.24$, $K_2 = 38.0 \pm 7.4$ μM. Conditions are given in Figure 3.

The requirement for a second divalent cation activator at each pyruvate kinase site is thus established. The binding of Mn^{2+} to the high affinity site on the enzyme-ATP-pyruvate complex is alone insufficient to activate the enzyme as shown by the sigmoidal behavior of rate vs. Mn^{2+} concentration (Figures 2B, 3). The sigmoidal behavior is further clarified by comparing the calculated concentrations of the enzyme-ATP-pyruvate complexes containing one and two Mn^{2+} ions, as given by the curves of Figure 3, with the experimental kinetic data points of Figure 3. The catalytic activity parallels the concentration of the bimetallic complex rather than that of the monometallic complex. Alternative interpretations of the data of Figures 2 and 3, such as conformation changes induced by CrATP or allosteric behavior in the Mn-ATP system, are inconsistent with the present and previous kinetic data (Mildvan and Cohn, 1966) and with the present and previous binding and structural data (Mildvan and Cohn, 1966; Gupta et al., 1976; Sloan and Mildvan, 1976).[2]

The tight Mn^{2+} binding site represents the site on the enzyme since it remains detectable when ATP is replaced by CrATP (Figures 1, 2A), and its dissociation constant at pH 7.3 (0.6 ± 0.1 μM) is of the same order as that found with CrATP at pH 6.7 (Table II).

The second weaker site for Mn^{2+} is probably on the enzyme-bound ATP molecule itself, since it disappears when ATP is replaced by CrATP (Figures 1, 2A) and its dissociation constant (20–38 μM, Table II) agrees with the dissociation constant of the binary Mn-ATP complex (14 ± 6 μM, Table III). While the data of Figure 3 show the weaker nucleotide site for Mn^{2+} to be essential for catalysis, they afford no information on the role of the tighter Mn^{2+} site on the enzyme, since the tighter site must be occupied in order to populate the weaker site. Conversely, the data of Figures 1 and 2A show the essentiality of the tighter enzyme site but are ambiguous with respect to the nucleotide site for Mn^{2+}, since the nucleotide site is occupied by Cr^{3+}. The two experiments taken together (Figures 1–3) establish the need for occupancy of both types of Mn^{2+} binding sites in order to activate the enolization of pyruvate by pyruvate kinase.

Effect of the Nature of the Divalent Cation on the Activation of Pyruvate Kinase. Robinson and Rose (1972) previously found that the rate of enolization of pyruvate catalyzed by pyruvate kinase in the presence of ATP correlated with the electronegativity of the activating divalent cation, as measured by the pK_a of the coordinated water ligand. The higher rate of enolization observed with CrATP as compared with MgATP or MnATP (Table I) suggests that the electronegativity of the nucleotide-bound metal rather than that of the enzyme-bound metal determines the rate of enolization. This is shown more clearly by the data of Figure 5 which correlates the rate of enolization of pyruvate as a function of the pK_a of the metal-bound water using those divalent cations that activate the overall transphosphorylation reaction of pyruvate kinase as well. To insure saturation with all components, varying levels of the divalent cations were used with either ATP (2 mM) or CrATP (2 mM) and pyruvate (80.6 mM) (Figure 5). With ATP, in agreement with the results of Robinson and Rose (1972), a sevenfold variation in rates of enolization with various divalent cations is observed, and the rates decrease in the order $Co^{2+} > Ni^{2+} \sim Mn^{2+} > Mg^{2+}$, which correlates with the increasing pK_a of the water ligands of these divalent cations (Figure 5). A linear least-squares fit to these data gives a slope of $10^{-0.24\pm0.06}$ in agreement with that obtained by Robinson and Rose in the related detritiation of phosphoenolpyruvate.

On the other hand, with CrATP less than a twofold variation in the rate of enolization is observed with all of the divalent cations, and no correlation with the pK_a values of the divalent cations is detected. Moreover, the greater rates observed with CrATP in the presence of any of the divalent cations correlate with the lower pK_a value of water coordinated to trivalent Cr^{3+} (Figure 5), indicating that the electronegativity of the nucleotide-bound metal, rather than that of the enzyme-bound metal, determines the rate of enolization. The least-squares line through all of the points including those of CrATP gives a slope of $10^{-0.16\pm0.04}$ which overlaps with that obtained from the data of the divalent cations alone (Figure 5). A small

[2] Although expressed in terms of ATP and pyruvate, the theoretical curves of Figure 3 may include contributions from enzyme-bound ADP and phosphoenolpyruvate due to a shift of the equilibrium on the enzyme. Such a shift would not invalidate these conclusions.

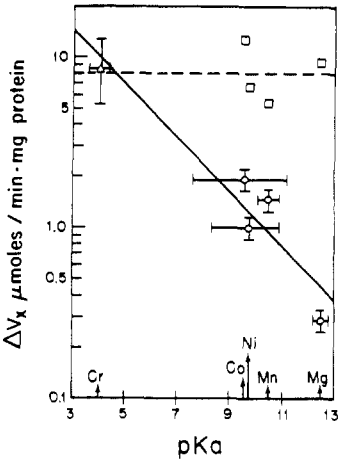

FIGURE 5: Rate of enolization of pyruvate as a function of the electronegativity of the activating metal ions in the presence of saturating amounts of either CrATP or ATP. The open squares give the rate of enolization with CrATP (2 mM) and optimum concentrations of $CoCl_2$ (2 mM), $NiCl_2$ (2 mM), $MnCl_2$ (2 mM), and $MgCl_2$ (7.5 mM). The triangle gives the average rate of enolization with all divalent cations in the presence of CrATP. The open circles give the rates of enolization with ATP (2 mM), and optimum concentrations of $CoCl_2$ (4 mM), $NiCl_2$ (4 mM), $MnCl_2$ (4 mM), and $MgCl_2$ (15 mM). Other components present were pyruvate kinase (0.839 mg/ml or 14.2 μM sites), sodium pyruvate (80.6 mM), K^+-Pipes buffer (50 mM, pH 6.7), and KCl (0.1 M). T = 23 ± 1 °C. The pK_a values for the metals are from Chaberek et al. (1952), Bjerrum (1907), Bjerrum (1910), and Denham (1908). The solid line which is a least-squares fit to the triangle and circles has a slope of $10^{-0.16\pm0.04}$. The least-squares line through the circles alone (not shown) has a slope of $10^{-0.24\pm0.06}$.

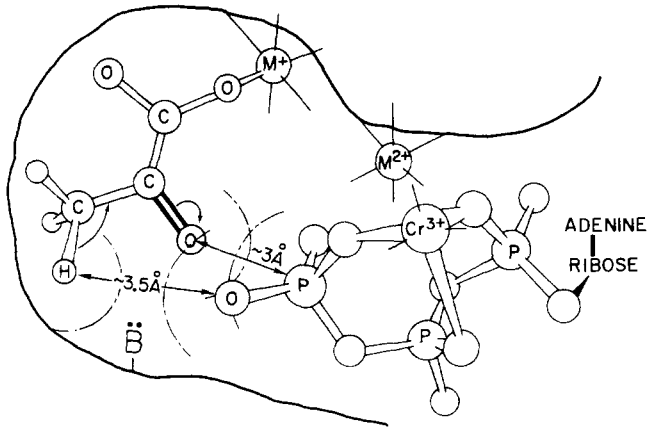

FIGURE 6: Geometry of the interaction of pyruvate and CrATP on rabbit muscle pyruvate kinase in solution, as determined by nuclear relaxation studies and model building. The substrate conformations and arrangement are based on a total of 12 measured distances from the divalent cation at site I (M^{2+}) to the substrates phosphoenolpyruvate (Melamud and Mildvan, 1975), pyruvate (Fung et al., 1973), ATP (Sloan and Mildvan, 1976), and three measured distances from Cr^{3+} at site 2 to pyruvate (Gupta et al., 1976). The indicated distances are derived from the resulting model, the broken arcs show the van der Waals radii, and M^+ indicates the probable location of the monovalent activator (Mildvan et al., 1976).

change in the exponent ρ, which cannot be excluded, could reflect a change in the rate-determining step with CrATP.

Implications of Dual Divalent Cation Requirement in the Reaction Mechanism of Pyruvate Kinase. A requirement for two divalent cations per active site has previously been suggested for phosphoenolpyruvate carboxylase (Foster et al., 1967). Although not previously appreciated for pyruvate kinase, a dual divalent cation requirement is consistent with all of the early and more recent structural and binding data on this enzyme (Mildvan, 1974) and would resolve a controversy in the interpretation of the steady-state kinetic data on the overall reaction (Cleland, 1967; Mildvan et al., 1971). Thus, the enzyme-bound divalent cation has been found by nuclear relaxation studies to form a second sphere complex with bound P-enolpyruvate (Melamud and Mildvan, 1975) and with bound ATP (Sloan and Mildvan, 1976). It is therefore not surprising that CrATP, which shows little affinity for Mn^{2+}, with a dissociation constant greater than 10 mM (Gupta *et al.*, 1976), binds to the Mn^{2+}-enzyme complex and activates the enolization of pyruvate. The role of the enzyme bound metal is probably to adjust the protein conformation (Suelter and Melander, 1963) and to orient water ligands near the phosphoryl groups of the substrate (Melamud and Mildvan, 1975; Sloan and Mildvan, 1976; Mildvan et al., 1976). The enzyme-bound metal has little direct role in polarizing the ATP, since the rate of enolization of pyruvate does not correlate with the electronegativity of this metal ion as measured by the pK_a of its coordinated water ligand (Figure 5).

The role of the nucleotide bound metal, which is estimated by NMR methods to be 7 ± 1 Å from the enzyme-bound divalent metal (Figure 6),[3] is probably to adjust the polyphosphate conformation and to polarize and thereby increase the electrophilicity of the γ-phosphoryl group of ATP which is in molecular contact with the carbonyl oxygen of pyruvate, as determined by NMR (Figure 6, Mildvan et al., 1976). The nucleotide bound metal has no direct role in adjusting the protein conformation, since its role can be filled by Cr^{3+} which is substitution inert and therefore cannot acquire ligands from the enzyme. Another role of the nucleotide-bound metal, suggested by the data of Figure 5, and by the earlier studies of Robinson and Rose (1972), is to activate the base which deprotonates pyruvate. NMR studies indicate that the nucleotide-bound metal is 0.4–1.2 Å nearer the carbon atoms and methyl protons of bound pyruvate than is the enzyme-bound metal (Gupta et al., 1976; Fung et al., 1973; James and Cohn, 1974). From a correlation of the rates of enolization with the electronegativities of the metal ions, Robinson and Rose (1972) have suggested that the base which deprotonates pyruvate may be a ligand of the divalent activator such as an amino acid residue of the protein or a hydroxyl ion. Such a hypothesis would indeed explain our unsuccessful attempts to specifically alkylate such a base on the enzyme with bromopyruvate even though bromopyruvate is a substrate in the enolization reaction.[4] However, the fact that substitution inert Cr^{3+} functions effectively as the nucleotide-bound metal ion rules out an amino acid ligand. The low ρ value, which is significantly less than 1.0, and the long 7.9-Å distance from the Cr^{3+} of CrATP to the methyl protons of pyruvate in the active complex (Gupta et al., 1976) argue against a hydroxyl ligand of the metal, although an indirect effect of a hydroxyl ligand in a charge relay to another base is possible. The only ligand of the nucleotide-bound metal appropriately positioned to directly deprotonate pyruvate is the γ-phosphoryl group of the bound metal-ATP

[3] A distance of 7 ± 1 Å between the Mn^{2+} and Cr^{3+} ions on pyruvate kinase may be estimated from a model of the substrates at the active site of the enzyme based on 15 metal-substrate distances (Figure 6, Mildvan et al., 1976). In accord with this proximity, a paramagnetic effect of Cr^{3+} on the electron spin relaxation time of Mn^{2+} is suggested by the threefold lower enhancement of the Mn^{2+}-H_2O interaction observed in the enzyme-Mn^{2+}-ATP-Cr^{3+} complex (6.0, Gupta et al., 1976), as compared to the enzyme-Mn^{2+}-ATP complex (18.1, Sloan and Mildvan, 1976).

[4] H. P. Meloche and A. S. Mildvan, unpublished observations.

complex, an oxygen of which is ~3.5 Å from the methyl protons of pyruvate (Figure 6). Although the pK_a of this group in the binary metal-ATP complex is well below that expected for a base capable of deprotonating a methyl group, an important role of the enzyme may be to increase this pK. Indeed, the pH dependence of the enolization of pyruvate indicates the participation of a basic group with a pK of 7.2 when MnATP is the activator and a $pK \geq 8.5$ when MgATP is the activator (Rose, 1960; Robinson and Rose, 1972). After deprotonating the methyl group of pyruvate, the protonated γ-phosphoryl group of the bound ATP-metal complex would thereby become more active in phosphorylating the enolate of pyruvate. If the pK_a of the γ-phosphoryl group of enzyme-bound ATP were unusually high, the correlation of the rate of enolization with the acidity rather than the basicity of the metal ligand (Figure 5) could result from the greater extent of deprotonation of this phosphoryl group with more electrophilic metal ions, although the low ρ value of 0.2 again suggests an indirect effect. Alternatively, as discussed above, the correlation of Figure 5 may indicate the predominant role of the nucleotide bound metal ion to be promotion of carbonyl polarization of pyruvate through the γ phosphorus atom, leaving open the possibility of a separate base on the enzyme to deprotonate pyruvate. Further kinetic and magnetic resonance studies are in progress to examine these alternatives more directly.

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