The Correlation of Reaction Kinetics and Substrate Binding with the Mechanism of Pyruvate Kinase*

A. M. REYNARD,† L. F. HASS,‡ D. D. JACOBSEN, AND P. D. BOYER

From the Department of Physiological Chemistry, University of Minnesota, Minneapolis 14, Minnesota

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Applications of isotopic techniques have contributed considerably to the understanding of kinase mechanisms. Studies with O¹⁸ (1-3) have shown that various kinase reactions proceed by transfer of a phosphoryl group without exchange of the phosphoryl oxygens with water or substrates. Evidence that the kinase reactions do not involve formation of a phosphoryl enzyme intermediate is given by the inability of pyruvate kinase to catalyze an exchange of pyruvate-C¹⁴ with phosphoenolpyruvate (1, 3, 7) and that the data for 3-phosphoglycerate kinase are likewise consistent with such a direct transfer mechanism (2277). The findings presented herein give strong support to a direct transfer mechanism for pyruvate kinase, and indicate that the data for 3-phosphoglycerate kinase are likewise consistent with such a mechanism.

EXPERIMENTAL PROCEDURE

Reagents—The pyruvate kinase used during most of these studies was prepared by the procedure of Tietz and Ochoa (9) except with the 5-minute heating time reduced to the time required to form a maximum of visible precipitate (approximately 3 minutes). Longer heating caused loss of pyruvate kinase activity. The preparations used for most of the studies reported herein had specific activities estimated to be approximately equal to the best preparations of Tietz and Ochoa (9) and of Kupieccki and Coon (10). Paper electrophoresis experiments with such enzyme preparations, at pH 4.5, at which point the enzyme shows definite instability, have shown up to three protein bands, and Tietz and Ochoa (9) found that prolonged solution electrophoresis at pH 7.1 showed the presence of several components with varying pyruvate kinase activity. The apparent electrophoretic heterogeneity may reflect, in part, changes in protein structure during the analysis. Traces of adenylate kinase and ATPase activities have been found in all preparations examined. Limited attempts at further purification of pyruvate kinase by chromatographic techniques were not successful. Pyruvate kinase was completely inactivated when solutions at low ionic strength near neutrality were added to diethylaminoethyl cellulose or Dowex 50-Na⁺ columns, and appeared in the dead volumes of phosphoryl columns. The specific activity of typical preparations used in these experiments was approximately equal to the best preparations of Tietz and Ochoa (9) and of Kupieccki and Coon (11). Adenylate kinase and AgBa-P-enolpyruvate were purchased from C. F. Boehringer and Soehne, Mannheim, Germany. The AgBa-P-enolpyruvate was converted to the soluble K⁺ salt by

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The specific activity of typical preparations used in these experiments was approximately 120 µmoles of pyruvate formed per mg of enzyme per minute at 25°C and pH 9.0 with 0.6 mM ADP, 0.6 mM P-enolpyruvate, 7 mM MgSO₄, and 0.08 M total ammonia as NH₃·H₂O. When measured at 25°C and pH 7.0 with 0.1 M imidazole buffer, 0.1 M KCl, 4 mM MgCl₂, 1 mM P-enolpyruvate, and 4 mM ADP as in the coupled assay of Tietz and Ochoa (9), adapted from Bücher and Pfleiderer (10), the specific activity was approximately 220. The activities reported by Tietz and Ochoa (9) and by Kupieccki and Coon (11) are based on the A280 of the oxidase (12, 13), namely, about 1.30 X A280 = milligrams of enzyme per ml when calculated with the factor reported for pyruvate kinase (10). A280 X 1.35 = milligrams of enzyme per ml, their best specific activities were approximately 268 (9) and 280 (11) in their coupled assay system.
mixing approximately 200 mg with 2 ml of a slurry containing roughly 1 ml of Dowex 50-H+, and passing through a column of Dowex 50-H+, 0.8 X 10 cm, followed by neutralization with KOH. If smaller columns were used, Ag+ ions apparently appeared in the eluate as shown by the activity increase upon the addition of EDTA or serum albumin to the enzyme assay mixtures. Sodium salts of ADP and ATP were purchased from Pabst Laboratories. Stock solutions of all phosphate compounds were made approximately neutral with KOH.

Reaction Velocity and Substrate Binding Determinations—Pyruvate kinase activity measurements were based on the amount of pyruvate produced. The reaction was initiated by addition of enzyme, the sample incubated 5 minutes, and the reaction stopped with 0.2 ml of 2.5 M pyruvate kinase activity measurements were based on the amount of pyruvate produced. The reaction was initiated by addition of enzyme, the sample incubated 5 minutes, and the reaction stopped with 0.2 ml of 2.5 M pyruvate after hydrolysis facilitated by Hg++ (16)

To a 1-ml aliquot was added 0.2 ml of 0.5 M phosphate buffer, 0.1 M EDTA was added, and the color was read at 410 mp. In many of the earlier trials EDTA was not added, but routine use of EDTA was instituted to prevent precipitation of Mg(OH)2 which was variable in presence of different concentrations of ATP; this variation in Mg(OH)2 precipitation was found to interfere with the colorimetric assay.

The rates of pyruvate production were linear at the extremes of concentrations of the various substrates and inhibitors used for the K_m values and competition studies, thus allowing use of single time measurements for most velocity determinations.

ADP was determined as the amount of pyruvate produced in the pyruvate kinase reaction with excess P-enolpyruvate in the following way. To a 2.0-ml aliquot containing approximately 0.025 mM ADP were added 0.25 ml of a buffer-salt solution (MgSO_4, 0.11 mM; KCl, 1.10 mM; glycylglycine, 0.11 mM, pH 8.5); 0.1 ml of P-enolpyruvate, 0.010 mM; and 0.2 ml of pyruvate kinase, 2.5 mg per ml. The reaction was allowed to proceed for 5 minutes at 0°C, and pyruvate was determined by the 2,4-dinitrophenylhydrazine procedure. The use of large excesses of pyruvate kinase or long time periods for the reaction was avoided because of the possibility of a side reaction catalyzed by the pyruvate kinase preparations.

P-enolpyruvate was determined in the equilibrium binding studies as pyruvate after hydrolysis facilitated by Hg++ (16). To a 1-ml aliquot was added 0.2 ml of 0.5 M Hg(Ac)_2 in 0.5 M NaAc, pH 4.0, and the mixed solution was allowed to stand 5 minutes at room temperature. Then 0.2 ml of Na_2S (approximately 1 m) was added with mixing, the precipitate of HgS was removed by centrifugation, and the pyruvate was determined on an aliquot of the supernatant fluid.

Measurements of P-enolpyruvate and of nucleotide binding with the ultracentrifugal technique were performed essentially as described by Velick et al. (17). Equilibrium dialysis measurements of pyruvate kinase binding were made with approximately 1 X 12 cm glass-stoppered vessels with the use of washed dialysis tubing in which a glass rod was inserted to reduce the time required for dialysis equilibrium. With an inside and outside volume of 2 and 3 ml, respectively, a 12-hour dialysis with slow rocking sufficed for equilibration with pyruvate. The kinetic and binding studies were conducted near 0°C because of increased stability of the enzyme and because this was the temperature at which it was possible to control the ultracentrifuge.

RESULTS

Reaction Velocity Measurements

Velocity measurements under various conditions were made to determine whether the apparent Michaelis constants for ADP and P-enolpyruvate were influenced by P-enolpyruvate and ADP concentration, respectively. In addition, the measurements allowed assessment of whether competitive relationships existed between ADP and ATP and between P-enolpyruvate and ATP, as predicted for a direct transfer mechanism. Possible competition between AMP and ADP, and between pyruvate and P-enolpyruvate was also evaluated.

To find if variation in Mg++ concentration, pH value, or buffer composition would have any marked and thus possible complicating effects on the kinetic studies, evaluations of the apparent K_m values for ADP at various Mg++ concentration (1.7 to 12.7 mM), and with ammonia (pH 9.2) and imidazole (pH 7.2) buffers were made. The results showed less than a 2-fold variation in the apparent K_m values, and further study of any small interactions thus did not appear necessary or warranted.

Increase in the Mg++ concentration up to 0.01 mM did not result in any inhibition of reaction velocity with ADP limiting. If free ADP were required as a substrate, inhibition by excess Mg++ would be expected. The presence of Mg++ in considerable excess over ADP and ATP concentrations in most experiments had the advantage that variation in the adenine nucleotide essentially represented variation in the Mg++-nucleotide concentration. In addition, at pH 8 to 9, nearly all ADP or ATP is present in one ionic form; with excess Mg++ present, the forms are MgADP-2 and MgATP-2 (18).

P-enolpyruvate-Velocity Relationships—Plots of 1/v against 1/ADP at different P-enolpyruvate concentrations are given in Fig. 1. The corresponding plots of 1/v against 1/P-enolpyruvate at different ADP concentrations are given in Fig. 2. These data show that the apparent K_m values for ADP and P-enolpyruvate, under the assay conditions used, are independent of the concentrations of P-enolpyruvate and ADP, respectively. Clearly, ADP and P-enolpyruvate do not compete for binding to the enzyme. McQuate and Utter (19) have previously reported, from data obtained under different assay conditions, small increases in the apparent K_m values of ADP and P-enolpyruvate with increase in the concentration of the other substrate.

The results given in Figs. 1 and 2 conform to the general kinetic equation (20-22) describing interaction of systems with two substrates and two products, as follows,

\[ v = \frac{V}{1 + \frac{K_m}{[A]} + \frac{K_m}{[R]} + \frac{K_m}{[AR]} \} } \]
where $A$, $B$, $K_A$, and $K_B$ are ADP and P-enolpyruvate and their respective Michaelis constants, $K_{AB}$ is a complex constant, $V$ is the theoretical maximal velocity, and $v$ the observed velocity. The constancy of the ratios of slope to intercept for the data given in Figs. 1 and 2 shows that for pyruvate kinase under the conditions used, $K_{AB} = K_A K_B$. Thus, the values of $K_A$ and $K_B$ are given directly by the ratios of slope to intercept of the $1/v$ against $1/substrate$ plots. These values for the Michaelis constants from the data of Figs. 1 and 2 are for ADP, $2.1 \times 10^{-4} M$, and for P-enolpyruvate, $3.2 \times 10^{-5} M$.

The lack of a discernible effect of P-enolpyruvate on the $K_v$ value for ADP and of ADP on the $K_v$ value for P-enolpyruvate eliminates the possibility that the reaction proceeds by a compulsory pathway in which the various steps before the interconversion of the ternary complexes (enzyme-P-enolpyruvate-ADP $\rightarrow$ enzyme-pyruvate-ATP) are adjusted rapidly. Further, the possibility that the reaction occurs by a compulsory pathway even if the more complicated steady state kinetics applies is definitely unlikely because the apparent equality, $K_{AB} = K_A K_B$, would require a quite fortuitous relationship among the various kinetic constants defining $K_A$, $K_B$, and $K_{AB}$ under such conditions. In addition, the kinetics of the ATP inhibition is incompatible with compulsory pathways, as noted in the next section. The possibility that the reaction occurs by a random addition of substrates with "equilibrium" kinetics applicable is unlikely because this leads to the prediction of nonlinear relationships between $1/v$ and $1/A$ and $1/B$.8

**Velocity Inhibition by ATP**—Relatively low concentrations of ATP inhibited considerably the rate of reaction of P-enolpyruvate with ADP even in the presence of $0.01 M$ Mg++. Clearly, the inhibition did not result from Mg++ removal. The unlikely possibility that the inhibition reflected the occurrence of the reverse reaction was eliminated by the lack of inhibitory effects of pyruvate at concentrations up to twice those formed in the 5-minute incubation, as well as by the linearity of the reaction with time in the presence of inhibitory concentrations of ATP.

The ATP inhibition was found to be overcome by increase in either the P-enolpyruvate or ADP concentration, as demonstrated by the data in Figs. 3 and 4. As noted previously, the velocity-P-enolpyruvate and velocity-ADP relationships are described by the equation for random addition of substrates with "equilibrium" kinetics applicable. On this basis, the expected equation governing the effect of an inhibitor which could compete for both ADP and P-enolpyruvate is

$$
\frac{v}{V} = \frac{K_A}{A} + \frac{K_B}{B} + \frac{K_{AB}}{AB} \left( 1 + \frac{1}{K_i} \right)
$$

The data of Figs. 3 and 4 conform closely to this equation, and average values for $K_i$, the dissociation constant for $E$-ATP, may be computed to be $1.2 \times 10^{-4} M$ for the three levels of ATP used for the experiments reported in Fig. 3, and, correspondingly, $1.4 \times 10^{-4} M$ for the data of Fig. 4.

If "steady state" kinetics are applied to a two-substrate sys-

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8 All the forms of the enzyme that would be assumed to be present in solution with P-enolpyruvate, ADP, and ATP, where binding of ATP blocked binding of either ADP or P-enolpyruvate can be represented by the following equilibria, where $A = ADP$, $B = PEP$, and $C = ATP$,

$$
A + E \rightleftharpoons AE \quad K_A = \frac{(A)(E)}{(AE)} = \frac{(A)(BE)}{(ABE)}
$$

$$
B + E \rightleftharpoons BE
$$

$$
A + BE \rightleftharpoons ABE \quad K_B = \frac{(B)(E)}{(ABE)} = \frac{(D)(BE)}{(ABE)}
$$

$$
B + AE \rightleftharpoons ABE \quad K_C = \frac{(C)(E)}{(CE)}
$$

and

$$
C + E \rightleftharpoons CE
$$

Assuming that rapid equilibrium conditions pertain and that the affinity of each the substrates, $A$ and $B$, are independent of the binding of the other substrate to the enzyme, the enzyme conservation equation and respective equilibrium equations lead to the relationship given.
FIG. 3. Competitive interaction of P-enolpyruvate and ATP with pyruvate kinase. Assays were made in the presence of 2.1 mM ADP and with conditions as given in Fig. 1. ATP concentrations were 0.0, ○; 2.5, ○; 5.0, ●; or 7.5, ●, × 10^{-3} M.

FIG. 4. Competitive interaction of ADP and ATP with pyruvate kinase. Assays were made in the presence of 0.1 mM P-enolpyruvate and with conditions as given in Fig. 1. ATP concentrations were 0.0, ○; 2.5, ○; 5.0, ●; or 7.5, ●, × 10^{-3} M.

tern in which substrate A must bind before B, competition of an inhibitor with A leads to the following velocity expression:

\[ v = \frac{V}{1 + \frac{K_B}{B} + \frac{K_A}{A} \left( 1 + \frac{1}{K_1} \right) + \frac{K_{AB}}{AB} \left( 1 + \frac{1}{K_1} \right)} \]  

where \( K_A \) and \( K_B \) are Michaelis constants for substrates A and B, respectively. \( K_{AB} \) is a complex constant which is equal to \( K_A' K_B' \) where \( K_A' \) is the dissociation constant of the EA complex (22, 24). For "equilibrium" kinetics the expression is similar, but with \( K_{AB} \) equal to \( K_A' K_B' \) which are dissociation constants. If competition of the inhibitor with either A or B is assumed, with dissociation constants \( K_1 \) and \( K_1' \), respectively, the velocity relationship for the "steady state" is:

\[ v = \frac{V}{1 + \frac{K_B}{B} \left( 1 + \frac{1}{K_1} \right) + \frac{K_A}{A} \left( 1 + \frac{1}{K_1} \right) + \frac{K_{AB}}{AB} \left( 1 + \frac{1}{K_1} \right)} \]  

Inspection of these equations shows that neither of them gives relationships corresponding to the experimental data for the reciprocal plots shown in Figs. 3 and 4.

The estimation of the binding of ATP from competition with P-enolpyruvate and ATP has a feature which deserves mention. If the competition of both ADP and P-enolpyruvate with ATP were not recognized, and calculations for \( K_1 \) were based on the observed competitive effects for one substrate with the other held constant, an erroneously high value for \( K_1 \) would be obtained. Caution is thus indicated in evaluations of \( K_1 \), and, in particular, the equating of such values to dissociation constants, in multiple substrate systems unless possible competition with all substrates is appropriately considered.

Other Competition Experiments—The possible competition between AMP and ADP was measured at pH 9.0 with 8 mM NH₃ buffer, an excess of P-enolpyruvate, and a limiting amount of ADP, 0.17 mM, so that any action of AMP in displacement of ADP would be readily evident. Addition of 1.1 mM AMP, 6.5 times the amount of ADP present, produced no discernible inhibition. This shows that the presence of the β-phosphate makes a strong contribution to the binding of ADP and probably also to that of ATP.

Experiments on competition of pyruvate with P-enolpyruvate were carried out at pH 9.0 with 8 mM NH₃ buffer, a slight excess of ADP, 0.86 mM, and a limiting concentration of P-enolpyruvate, 0.076 mM. Addition of 0.033 mM pyruvate showed no discernible inhibition but 0.33 mM gave a 12% inhibition. These results are in harmony with binding of pyruvate and P-enolpyruvate at the same catalytic site, but with a much stronger binding for P-enolpyruvate. Assuming strictly competitive inhibition, the dissociation constant of pyruvate from this result may be estimated as approximately \( 1 \times 10^{-3} \) M.

Equilibrium Binding Studies

Direct measurements of substrate binding were made to further assess competition between substrates for pyruvate kinase, and to allow comparison of Michaelis constants with dissociation constants. The best data were obtained with P-enolpyruvate, which fortunately bound rather strongly to the enzyme in the absence of other substrates. Sufficient data were obtained on binding of ADP and ATP to check their interaction with each other and the effect of ATP on phosphopyruvate binding.

Binding of P-enolpyruvate—Results of binding measurements with the ultracentrifuge technique are given in Fig. 5. The results are plotted according to the equation \( \bar{v} = v - \bar{v} \) (25) where \( \bar{v} \) is the moles of P-enolpyruvate bound per 237,000 g of protein, the estimated molecular weight (26), \( C_f \) is the concentration of free P-enolpyruvate, \( n = \bar{v} \) at \( C_f = 0 \), and \( K_d \) is the intrinsic dissociation constant of the P-enolpyruvate-enzyme complex.
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2.0 -

1.5 -

1.0 -

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0 1.0

20 30

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FIG. 5. The binding of P-enolpyruvate by pyruvate kinase. The binding was determined by the ultracentrifuge technique at 0 °C with 10 mM MgSO₄, 0.1 M KCl, 10 mM glycylglycine, pH 8.5, and 21 mg of pyruvate kinase per ml. The points are averages of duplicate determinations.

FIG. 6. The binding of pyruvate by pyruvate kinase. The binding was determined by equilibrium dialysis with conditions as given in Fig. 5. The dotted line shows the theoretical binding for the combined effect of two types of binding sites, one type with n = 120 and Kᵣ = 35 mM, and one type with n = 2.4 and Kᵣ = 0.625 mM.

equilibrium dialysis procedure; the results were considerably more variable than by the ultracentrifuge method but indicated an n value of slightly greater than 2 and a Kᵣ of approximately 3 × 10⁻⁴ M.

**Binding of ADP**—This measurement was hampered by the weak binding of ADP compared to P-enolpyruvate and was further complicated by the presence of traces of adenylate kinase activity sufficient to change the concentrations of added nucleotides during the time of the ultracentrifuge measurements. For this reason the experiments were limited to finding if the presence of ATP interfered with the binding of ADP as predicted by a direct transfer mechanism. To obviate somewhat the effects of adenylate kinase, sufficient adenylate kinase was added to allow the system to come to equilibrium before the ultracentrifuge measurements, and the concentrations of added ADP and ATP were adjusted sufficiently to give the needed information. Measurement of the amount of free ADP by the pyruvate kinase assay in presence of excess ATP was sufficiently accurate to allow experiments with considerable excess ATP present. The presence of 4 mM ATP caused at least a 40 to 80% decrease in the extent of ADP binding at 0.25 to 0.5 mM ADP. This substantiates the competitive inhibition shown by the kinetic data. The binding data were not sufficiently accurate to justify calculation of binding constants or n values for ADP.

**Binding of Pyruvate**—Results of equilibrium dialysis studies on the binding of pyruvate are shown in Fig. 6. Two regions of binding are evident, one a weakly binding region with very roughly 120 sites per 237,000 g of protein discernible at high pyruvate concentration, and a region of stronger binding evident at lower pyruvate concentration. The data suffice to show some relatively strong binding of pyruvate, with a Kᵣ value of approximately 2.5 to 8 × 10⁻⁴ M and an n value of 2 to 4.

**DISCUSSION**

The results presented herein together with other findings (1-3, 6, 7) give strong support to a mechanism for the pyruvate kinase reaction with the following characteristics: (a) direct transfer of the phosphoryl group from the donor to the acceptor; (b) participation of one catalytic site which can bind either ADP or ATP and a second site which can bind either pyruvate or P-enolpyruvate; (c) competition of P-enolpyruvate and ATP for binding on the enzyme because of overlap of their transferable phosphoryl groups; and (d) random combination of P-enolpyruvate and ADP with the enzyme with “equilibrium kinetics” governing. The substrate relationships and a transition state for the transfer are schematically indicated in Fig. 7.

Some studies of P-enolpyruvate binding were also made by an complex. The binding data correspond reasonably to a simple mass action relationship with independent binding sites for P-enolpyruvate with 1 dissociation constant, as determined by the slope of the plot, of 7.5 × 10⁻⁴ M. The results indicate that the minimal number of binding sites for P-enolpyruvate is at least two per mole of enzyme. The presence of 3 mM ATP markedly decreased the binding of P-enolpyruvate (less than 10% of P-enolpyruvate bound under conditions as in Fig. 5 with approximately 0.6 mM P-enolpyruvate present). Sufficient binding studies were not completed to warrant calculation of a Kᵣ for ATP.

Some studies of P-enolpyruvate binding were also made by an

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FIG. 7. Schematic illustration of relations among substrates and a transition state for pyruvate kinase.
The results of both the kinetic and direct binding studies show that in the pyruvate kinase reaction a competitive relationship exists between the binding of P-enolpyruvate and ATP and of ADP and ATP. The kinetic studies show no competition between ADP and P-enolpyruvate but indicate a competition between P-enolpyruvate and pyruvate. The other possible competitions, namely, between pyruvate and ADP and between pyruvate and ATP, were not assessed, but are considered quite unlikely in view of the reaction catalyzed and the results with other substrates.

As mentioned in "Results," the kinetics of the reaction of P-enolpyruvate and ADP conform closely to those predicted for independent combination of the substrate with the enzyme with "equilibrium kinetics" applicable. Other experimental findings give considerable support to such a reaction pattern. The kinetic results definitively eliminate a compulsory pathway with "equilibrium kinetics" applying, and make unlikely the possibility that a compulsory substrate binding occurs with "steady state" kinetics applicable. The ability of P-enolpyruvate, or ADP, and of pyruvate to combine independently with the enzyme, as demonstrated by the direct binding data, give additional evidence against a compulsory binding order. For P-enolpyruvate, the only substrate for which accurate direct binding data was obtained, the $K_d$ value, $7.5 \times 10^{-5}$ M, is not far different from the $K_e$ value, $3.2 \times 10^{-5}$ M. With ADP, ATP, and pyruvate, the roughly estimated binding constants, as measured by direct binding, were within an order of magnitude of the kinetically determined constants. Thus "equilibrium kinetics" very likely governs the substrate-velocity relationships of P-enolpyruvate.

The data of Bücher for phosphoglycerate kinase (8) are compatible with a mechanism corresponding to that presented herein for pyruvate kinase if appropriate consideration is given to the competition between the transferable phosphoryl groups of ATP and 1,3-diphosphoglycerate. Such competition was indicated by Bücher's data. The equation Bücher used for assessing if ADP would overcome the ATP inhibition was of the form

$$v = \frac{V}{1 + \frac{K_A}{A} \left(1 + \frac{1}{K_I}ight)}$$

(5)

With the values for $K_A$ for ADP and $K_I$ for ATP as estimated for his experiments, a calculated ratio of velocity in the presence to that in the absence of inhibitor was 0.30, whereas the experimental value was 0.83. Bücher's equation does not adequately consider the competitions of ATP with both ADP and 1,3-diphosphoglycerate for the enzyme. With use of Bücher's data (8), but with Equation 2 of this paper as a basis for calculation, a ratio of velocity in the presence to that in the absence of inhibitor of 0.89 is obtained, in good agreement with the experimental value. This suggests that the P-glycerate and pyruvate kinase mechanisms, like those of other closely related enzymes, may indeed be quite similar, but additional data for the P-glycerate kinase is necessary before such a conclusion is warranted.

The manner by which the enzyme may favor phosphoryl transfer, other than by appropriate positioning of the reactants, remains obscure. Data of Rose (27) suggest that binding of various anions on the site for the terminal phosphoryl group of ATP may favor enolization of the pyruvate. Formation of an enol before phosphate transfer is not essential, however. As the carbonyl oxygen of pyruvate approaches the phosphorus of the terminal phosphoryl group of ATP, electrons may shift from the methyl to form the CH$_2$ group of the P-enolpyruvate. Mg$^{2+}$ may be associated with the phosphoryl group transferred on the enzyme surface, thereby increasing its susceptibility to nucleophilic attack, as suggested by Boyer et al. (1, 2) and by Westheimer (28). The role of K$^+$ remains obscure; it may be that this ion is necessary for the protein to assume its proper configuration and has no direct role in the catalysis. Such a role is consistent with the previous studies showing that the $K_a$ value for P-enolpyruvate is independent of the K$^+$ concentration (15).

**SUMMARY**

The observed Michaelis constants for adenosine diphosphate (ADP), $2.1 \times 10^{-4}$ M, and for phosphoenolpyruvate, $3.2 \times 10^{-5}$ M, in the pyruvate kinase reaction at pH 8.5 and with excess Mg$^{2+}$ are independent of the concentration of the other substrate. Adenosine triphosphate (ATP) inhibits the rate of phosphoryl transfer to ADP not because of reaction reversal but in a manner which is overcome competitively by either ADP or P-enolpyruvate. Adenosine monophosphate (AMP) does not inhibit the pyruvate causes a weak inhibition with P-enolpyruvate limiting. The kinetic studies in particular, as supported by direct binding studies, show that the observed Michaelis constants for ADP and P-enolpyruvate approximate their dissociation constants.

Direct measurement of P-enolpyruvate binding by ultracentrifuge techniques indicates presence of at least two binding sites per mole of enzyme with a $K_d$ value of $7.5 \times 10^{-4}$ M. Other direct binding measurements show that pyruvate and ADP combine with the enzyme independently of the presence of other substrates, and that ATP decreases the binding of both P-enolpyruvate and ADP.

These results together with other findings lead to the conclusion that phosphoryl transfer by pyruvate kinase occurs by a direct transfer of the phosphoryl group from the donor to the acceptor, with participation of one binding site for ATP and ADP, and another site for pyruvate and P-enolpyruvate, but with competition between ATP and P-enolpyruvate for binding because the transferable phosphoryl groups of ATP and of P-enolpyruvate bind to the same locus. Previously reported data for phosphoglycerate kinase are compatible with a similar mechanism if appropriate consideration is given to competition between ATP and both ADP and 1,3-diphosphoglycerate.

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