Catalytic and Regulatory Properties of Pyruvate Kinases in Tissues of a Marine Bivalve*

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**SUMMARY**

Pyruvate kinase (EC 2.7.1.40) from oyster tissues occurs in at least two electrophoretically and kinetically distinguishable forms in mantle tissue and in adductor muscle. In mantle, pyruvate kinase activity appears as a single band having a pI value of 6.35 while adductor pyruvate kinase appears as a “doublet” having pI values of 5.6 and 6.5.

Both forms of the enzyme have an essential requirement for Mg++, and a monovalent cation (K+ or NH4+). The activation of oyster enzymes was more efficient with K+. Neither of the enzymes is inhibited by Cu++. In the absence of fructose-1,6-diphosphate, the $K_m(ADP)$ for adductor muscle pyruvate kinase is several-fold higher than the $K_m(ADP)$ for the mantle enzyme. In the presence of fructose-1,6-P2, the $K_m(ADP)$ for both enzymes are identical. The $K_m$ of the substrate phosphoenolpyruvate differs for the two enzymes, $K_m(PEP)$ being several-fold lower for the adductor enzyme. In the absence of fructose-1,6-P2, the $K_m(PEP)$ for both enzymes decreases markedly with increasing pH; the $K_m(PEP)$ is strikingly decreased by FDP and becomes insensitive to pH in presence of fructose-1,6-P2.

For oyster pyruvate kinases fructose-1,6-P2 stimulation is maximal at acidic pH.

Both forms of the enzymes are subject to feedback inhibition by ATP, l-alanine, and phenylalanine, and these inhibitory effects are reversed by fructose-1,6-P2. $K_i$ values of these metabolites for adductor pyruvate kinase are lower than the $K_i$ values of mantle enzyme. ATP inhibition differs for the two enzymes, being noncompetitive for the mantle form and competitive for the adductor enzyme. $K_i$-Alanine inhibition of both enzymes is of the mixed competitive type. Phenylalanine inhibition is competitive with respect to P-enolpyruvate for the mantle enzyme, but not for the adductor pyruvate kinase.

The data in this study suggest that, as in the mammalian case, the enzyme pyruvate kinase in the oyster occurs in tissue specific multimolecular forms and that the kinetic properties of each isozyme seem to gear in well with the over-all metabolism of the tissue.

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In theory, pyruvate occupies a central crossroads position in energy metabolism since it may be metabolized by a number of different pathways. At least six such pathways are present in oyster tissues. However, not all are equally functional in all tissues (1). In oyster adductor muscle, the major source of pyruvate is storage glycogen. The pyruvate, which is produced in high quantities during periods of anaerobiosis, can have several metabolic fates, the most important one being conversion to malic acid. The latter is readily metabolized to succinate, which is a major end product of anaerobic metabolism in oysters. Lactate, in contrast, does not accumulate (1). Under aerobic conditions pyruvate can be oxidized by the usual Krebs cycle reactions. In mantle, on the other hand, the pyruvate branching point is more complex and the degree to which these pathways operate differs from that in the adductor muscle. During anaerobic metabolism the gluconeogenic nature of the tissue is evident from the formation of glycogen. Hence if the data are correct, there are special requirements for holding pyruvate kinase in a "shut off" conformation in this tissue.

From the above consideration it is evident that the requirements for the regulation of the pyruvate kinase reaction in adductor and mantle tissues are quite distinct from each other. One obvious way of meeting these different requirements is the elaboration of tissue-specific variants of the enzyme. We therefore initiated this study by examining mantle and adductor tissues for different electrophoretic forms of pyruvate kinase. In adductor muscle homogenates, two forms of pyruvate kinase can be resolved by electrophoresis on cellulose acetate or by n-nitrocellulose. In mantle tissue, a single form of pyruvate kinase is present. The pyruvate kinase activities from these tissues are more efficient with K+. Nei-
two tissues differ in catalytic and regulatory properties. A preliminary report of this work was presented previously (4).

MATERIALS AND METHODS

All substrate, cofactors, reagents, and the coupling enzyme, lactate dehydrogenase, were purchased from Sigma.

Oysters (Crassostrea gigas) used in this study were collected from Chukumut Bay, Bellingham, Washington, at low tides by the kind permission of Dr. Wallace Heath, Department of Biology, Western Washington State College, Bellingham. The oyster is a facultative anaerobe and experiences wide variations in environmental factors. Animals were brought to the laboratory in an icebox and opened quickly to excise the tissues. All tissues were washed thoroughly with cold homogenizing medium to remove exogenous algae and other microorganisms.

Mantle and adductor tissues were homogenized in a Sorvall Omnimixer for 1 to 2 min with 3 to 4 volumes of ice-cold 0.01 M Tris-HCl buffer, pH 7.5, containing 2 mM EDTA. The homogenate was stirred for 1 hour at 4°C and then centrifuged at 12,000 × g for 15 min and the pellet was discarded. The supernatant was filtered through glass wool and then brought to 40% saturation with solid ammonium sulfate and stirred for 1 hour at 4°C. The suspension was then centrifuged as above, the pellet was discarded, and the supernatant was brought to 75% saturation with solid ammonium sulfate. After 1 hour with stirring, the solution was centrifuged at 37,000 × g for 20 min. The pellet was dissolved in a minimal volume of 0.01 M Tris-HCl buffer, pH 7.5. The dissolved pellet was further centrifuged at 84,000 × g for 90 min in refrigerated Beckman model L preparative ultracentrifuge to remove glycogen and the high speed supernatant was used as the source of pyruvate kinase. Portions of enzymes were dialyzed before use against 0.05 M Tris-HCl buffer, pH 7.5. The enzyme was stable at 0-4°C for a few days and if frozen was stable for several weeks without causing any change in the K_{m} of the substrate, P-enolpyruvate. Mantle enzyme was somewhat unstable to dialysis, showing a loss of activity of approximately 10% within 2 hours of dialyzing.

Enzyme was assayed by the methods of Bucher and Pfleiderer (5). Pyruvate formation was coupled to lactate dehydrogenase and the rate of pyruvate kinase activity was measured as the decrease in E_{650} due to NADH. Tris-HCl buffers were used in all assay reactions. Standard assay mixtures contained the following in a final volume of 2 ml: 50 mM Tris-HCl buffer, Mg**, K+, ADP, P-enolpyruvate, NADH, and excess of dialyzed Sigma lactate dehydrogenase at concentrations specified in the figure legends. Saturating concentrations for each of the reactants for both mantle and adductor enzymes were 6 mM Mg**, 50 mM K+, 0.2 mM ADP, 1.4 mM P-enolpyruvate, 5 × 10^{-4} M fructose-1,6-P_{2}, 5 mM ATP, 3 mM t-alanine (adductor pyruvate kinase), 8 mM t-alanine (mantle pyruvate kinase), and 10 mM phenylalanine. All reactions were started by the addition of pyruvate kinase preparation. All experiments were performed at 20° since K_{m}(PEP) was found temperature-independent over a temperature range of 5-30°C.

Mantle, gill, and adductor muscle pyruvate kinases were prepared and studied electrophoretically to determine whether tissue-specific forms are present in the oyster. These experiments were performed in collaboration with Dr. Walter Susor by the procedure given by Susor and Rutter (6).

The technique of electrofocusing was used to elaborate further the results of electrophoresis and to determine whether isoenzymes having different pi values were present. Electrofocusing experiments were performed according to the method of Haglund (7). Both mantle and adductor enzymes were run at pH 5 to 8 (LKB-8133) at 900 volts for 53 hours. The temperature of the apparatus was maintained at 3° ± 0.05. For enzyme activity all fractions were assayed in presence of 2.5 × 10^{-4} M fructose-1,6-P_{2} at pH 8.5.

RESULTS

Electrophoresis and Electrofocusing—Electrophoretic resolution of pyruvate kinase activity in three different tissues is shown in Fig. 1. Mantle pyruvate kinase activity appears as a single band, moving toward the cathode; pyruvate kinase in gill tissue displays a similar electrophoretic mobility. Adductor pyruvate kinase, showing a different pattern, moves as a “doublet” toward the cathode. The electrophoretic differences were confirmed by electrofocusing experiments (Fig. 2). Mantle pyruvate kinase
appears as a single major activity peak with a pI value of 6.35. In contrast, the adductor pyruvate kinase appears as two distinct peaks having pI values of 5.6 and 6.5. Further, it was noted that mantle pyruvate kinase is more unstable than the adductor pyruvate kinase. It should be mentioned here that in all of the kinetic experiments performed on adductor pyruvate kinase the two components were not separated because the tissue is small and it is difficult to obtain large quantities of material.

**Cation Requirements**—In common with pyruvate kinase from other species, oyster pyruvate kinases show absolute requirements for divalent and monovalent cations. Mg++ can satisfy the former requirement (K_a values at pH 8.5 are 1.25 and 2.21 mM for mantle and adductor pyruvate kinases, respectively) and K^+ or NH_4^+ can satisfy the latter requirement (K_a of K^+ at pH 8.5 is 7.7 mM for both enzyme systems). High K^+ concentrations inhibited the oyster enzymes, in contrast to pyruvate kinases from other sources (8). Cu^{2+} at concentrations of up to 10 mM does not affect these enzymes.

**Effect of Fructose-1,6-P_2 on pH Optima**—As shown in Fig. 3 in the absence of fructose-1,6-P_2, both forms show pH optima at pH 8.5 but the shape of the activity curves differs for both, with adductor pyruvate kinase being more sensitive to pH changes. In the presence of fructose-1,6-P_2, the activity of the mantle enzyme is approximately constant between pH 7.5 and 9 while the pH optimum for the adductor enzyme is displaced toward pH 7.

**Effect of fructose-1,6-P_2 on the pH optima**

![Graph of pH optima](http://www.jbc.org/)

**Fig. 3. Effect of fructose-1,6-P_2 on the pH optima** (□, △ no fructose-1,6-P_2; ■, ▲, 2.5 × 10^{-6} M fructose-1,6-P_2). Reaction contents were: 50 mM Tris-HCl buffer at different pH values, 6 mM Mg++, 50 mM K+, 0.2 mM ADP, 0.5 mM P-enolpyruvate, 0.15 mM NADH, and excess of lactate dehydrogenase.

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**Fig. 4. Effect of fructose-1,6-P_2 on the K_m of ADP of mantle and adductor pyruvate kinases.** Reactant concentrations are 50 mM Tris-HCl (pH 8.5), 6 mM Mg++, 50 mM K+, 0.1 mM P-enolpyruvate, increasing concentrations of ADP, 0.15 mM NADH, and excess of lactate dehydrogenase.

**Fig. 5. Effect of pH and fructose-1,6-P_2 on the K_m of P-enolpyruvate (PEP) of mantle pyruvate kinase (PyK).** Reactant concentrations are: 50 mM Tris-HCl (□, pH 6.5; △, pH 7.5; ○, pH 8.5, ■, ▲, ●, with 2.5 × 10^{-6} M fructose-1,6-P_2), 6 mM Mg++, 50 mM K+, 0.2 mM ADP, 0.15 mM NADH, increasing concentrations of P-enolpyruvate and excess lactate dehydrogenase.

**Fig. 6. Effect of pH and fructose-1,6-P_2 on the K_m of P-enolpyruvate (PEP) of adductor pyruvate kinase (PyK).** Reactant concentrations are the same as described under Fig. 5 (○, pH 6.5; □, pH 7.5; △, pH 8.5; ●, ■, ▲, with 2.5 × 10^{-6} M fructose-1,6-P_2).
Effect of Fructose-1,6-P₂ on Kₘ of ADP—The effect of increasing ADP concentration at a fixed P-enolpyruvate concentration on the activity of the two enzymes in the absence and presence of 2.5 × 10⁻⁶ M fructose-1,6-P₂ at pH 8.5 are given in Fig. 4. Both enzymes require 2 × 10⁻⁴ M ADP for maximal activity at 1 × 10⁻⁴ M P-enolpyruvate. For both enzymes the curves for the reaction velocity against ADP in the presence and absence of fructose-1,6-P₂ have the Michaelis-Menten form. For mantle pyruvate kinase the Kₘ(ADP) in the absence of fructose-1,6-P₂ is 6.1 × 10⁻⁴ M while fructose-1,6-P₂ increases the Kₘ(ADP) at least by 6-fold. In contrast to this, fructose-1,6-P₂ has no effect on the Kₘ(ADP) of adductor pyruvate kinase.

Effect of pH and Fructose-1,6-P₂ on Kₘ of P-enolpyruvate—Double reciprocal plots for the initial velocities of P-enolpyruvate reaction rates at different pH values in the presence and absence of fructose-1,6-P₂ are shown in Fig. 5 (right panel). For mantle enzyme (Fig. 5, left panel), the Kₘ in the absence of fructose-1,6-P₂ and at pH 8.5 is about 2 × 10⁻⁴ M; the Kₘ increases markedly below pH 8.5 to a value of about 6 × 10⁻⁴ M at pH 6.5. In the presence of fructose-1,6-P₂ the Kₘ for the mantle enzyme is markedly lowered to about 7 × 10⁻⁴ M and remains constant over the pH range of 6.5 to 8.5. Fig. 6 shows the same kind of
response for the adductor pyruvate kinase. Fructose-1,6-P_2 increases the V_{max} of P-enolpyruvate for both enzymes. These results are in contrast to those reported by Rozengurt, Jiménez De Asúa, and Carminatti (9) for mouse liver pyruvate kinase. Rozengurt et al. demonstrated that, as the pH of the assay medium is lowered, susceptibility to fructose-1, 6-P_2 activation correspondingly decreases. In the case of oyster pyruvate kinases both enzymes are much more susceptible to fructose-1, 6-P_2 activation at acidic pH than at alkaline pH.

ATP inhibition—Like pyruvate kinases from other sources (9, 10) ATP also inhibits both forms of the oyster enzymes. While ATP inhibits enzymes at both pH values examined, several pH differences can be noted. With 1 mM P-enolpyruvate at pH 8.5 both enzymes are less inhibited than at pH 7.5. The K_I values for the mantle enzymes are 4 mM and 2.65 mM at pH 8.5 and 7.5, respectively. The adductor enzyme shows a similar behavior, although the K_I values for the adductor pyruvate kinase are lower, about 2.8 mM and 1.9 mM at pH 8.5 and 7.5, respectively.

The nature of inhibition is also different. Double reciprocal plots of the velocity of the pyruvate kinase reaction at different ATP concentrations (Fig. 7) indicate that the ATP inhibition of the mantle enzyme is noncompetitive with respect to P-enolpyruvate. Thus for mantle enzyme ATP decreases the calculated V_{max}, but does not affect the K_m(PEP). This noncompetitive inhibition of mantle pyruvate kinase is relatively unique, having been reported only once previously for the mouse brain enzyme by Lowry and Passonneau (11). Since P-enolpyruvate in mantle is not likely to reach saturating concentrations, these results suggest that ATP is not an important modulator of mantle enzyme. In contrast, the adductor enzyme shows competitive inhibition (Fig. 8) and is inhibited by lower concentrations of ATP. Thus, 2 mM ATP increases the K_m(PEP) at least 10-fold with little or no effect on the V_{max}. Further, it was noted that the nature of inhibition of either enzyme could not be altered by increasing or decreasing the Mg^{2+} concentration of the assay medium, although the degree of inhibition clearly depends upon the amount of the Mg^{2+} present. However, Mg^{2+} even at very high concentrations does not completely abolish ATP inhibition.

Interacting Effects of ATP and Fructose-1,6-P_2—Rozengurt et al. (9) and Tanaka, Sue, and Morimura (10) have reported that ATP inhibition of liver pyruvate kinase is reversed by fructose-1, 6-P_2. Studies of this kinetic property with the oyster mantle enzyme at two different pH values at varying concentrations of P-enolpyruvate indicate a behavior similar to that of mouse liver pyruvate kinase. Figs. 9 and 10 show double reciprocal plots of P-enolpyruvate reaction rates of the mantle enzyme in the presence of fructose-1, 6-P_2 and ATP at two pH values. At pH 8.5 (Fig. 9) 10^{-6} M fructose-1, 6-P_2 releases the ATP inhibition (caused by 3 mM ATP) by decreasing the K_m to two-thirds of control and increasing the V_{max} by 2-fold; thus, fructose-1, 6-P_2 not only releases the ATP inhibition but overrides it. At pH 7.5 (Fig. 10) under similar conditions fructose-1, 6-P_2 lowers the K_m to nearly one-fifth of control and again doubles the V_{max}. In the presence of ATP and fructose-1, 6-P_2, together the K_m(PEP) is similar at both pH values. In the presence of
ATP and fructose-1,6-P<sub>2</sub> the behavior of adductor pyruvate kinase closely parallels that of the mantle enzyme.

Search for Other Modulators—Since pyruvate occupies a central crossroads in oyster tissue metabolism, we felt it necessary to study the effects of other metabolites on pyruvate kinase activities. Of the various compounds tested, 5'-AMP, acetyl-CoA, citrate, succinate, malate, and oxaloacetate have neither stimulatory nor inhibitory effects on the oyster enzymes. Only L-alanine and phenylalanine were found to affect the enzyme in an inhibitory manner.

With 5 × 10<sup>-4</sup> M P-enolpyruvate, both oyster enzymes were found more susceptible to alanine inhibition at pH 7.5 than at 8.5 (Fig. 11). For mantle enzyme, the <i>K<sub>i</sub></i> values for alanine inhibition are 7.6 mM and 2.7 mM at pH 8.5 and 7.5, respectively. The adductor enzyme (Fig. 11A) is inhibited at lower concentrations of alanine (<i>K<sub>i</sub></i> values 3.6 and 0.6 mM, respectively). Fig. 12 shows <i>K<sub>i</sub></i> determination of phenylalanine for mantle and adductor pyruvate kinase activities. Phenylalanine inhibition of mantle pyruvate kinase is pH-independent, having a <i>K<sub>i</sub></i> value around 6 mM at both pH values examined; again the <i>K<sub>i</sub></i> values for the adductor enzyme are somewhat lower.

**Nature of Alanine and Phenylalanine Inhibition**—L-Alanine is known to inhibit pyruvate kinase (12-14) in a manner competitive with respect to P-enolpyruvate. In contrast, in the case of oyster pyruvate kinases, L-alanine inhibition of both enzymes involves changes in the apparent <i>K<sub>m</sub></i>(PEP) and the <i>V<sub>max</sub></i> at both pH values examined. As in the case of ATP inhibition, fructose-1,6-P<sub>2</sub> reverses alanine inhibition. For mantle enzyme, with 6 mM alanine at pH 8.5 (Fig. 13, upper panel), fructose-1,6-P<sub>2</sub> completely overcomes alanine inhibition. At pH 7.5 under similar conditions (Fig. 13, lower panel) the activity returns only to 70% of the control. The nature of phenylalanine inhibition differs for the two enzymes, being competitive for the mantle enzyme (Fig. 14, upper panel) and mixed competitive (involving changes in apparent <i>K<sub>m</sub></i>(PEP) and <i>V<sub>max</sub></i>) for the adductor enzyme (Fig. 14, lower panel). For the mantle enzyme, 6 to 10 mM phenylalanine doubles the <i>K<sub>m</sub></i>(PEP) while <i>V<sub>max</sub></i> remains
almost unaffected. Again fructose-1,6-P_2 protects the mantle enzyme against phenylalanine inhibition, 2.5 x 10^{-4} \text{m} fructose-1,6-P_2 reversing the inhibition caused by 6 mM phenylalanine.

**DISCUSSION**

The data in this study suggest that, as in the mammalian case, the enzyme pyruvate kinase in the oyster occurs in tissue-specific multimolecular forms and that the kinetic properties of each isozyme seem to gear in well with the over-all metabolism of the tissue in which it occurs. Thus, the \( K_m \) values of P-enol-pyruvate and ADP for mantle pyruvate kinase are 3 and 6 times higher than the corresponding values for adductor pyruvate kinase. Under conditions of gluconeogenesis, when P-enolpyruvate is being produced from pyruvate and C-4 acids of the Krebs cycle, any significant simultaneous pyruvate kinase activity would serve merely to recycle carbon at the expense of ATP (15). In oyster, this kind of recycling would not be favored at low P-enolpyruvate concentrations because of the high Michaelis constant for the mantle pyruvate kinase. Also, the enzyme is strongly activated by fructose-1,6-P_2 (causing a large decrease in the \( K_m^{(PEP)} \)). This may reflect a physiological mechanism whereby pyruvate kinase activity can be increased during glycolysis and markedly decreased during gluconeogenesis, when fructose-1,6-P_2 concentration may be reduced. An entirely analogous situation occurs in mammalian tissues. Thus in liver, a major gluconeogenic tissue, the \( K_m^{(PEP)} \) for pyruvate kinase is an order of magnitude higher than in the highly glycolytic muscle (15, 16) and indeed is comparable to the \( K_m^{(PEP)} \) for the mantle enzyme. In this circumstance, too, P-enolpyruvate conversion to pyruvate would not be favored in the gluconogenic tissue when P-enolpyruvate concentrations are low. Also, in this case, fructose-1,6-P_2 may act as a specific "on-off" switch on the liver enzyme, but it does not affect the mammalian muscle enzyme. Thus both liver and mantle enzymes seem well adapted for function in a metabolism that involves glycolytic and gluconeogenic function within a single tissue.

The Michaelis constants for P-enolpyruvate and ADP for pyruvate kinases of adductor muscle, fish muscle, and mammalian muscle are rather similar to each other and, as pointed out, are distinctly lower than for the mantle and liver enzymes. Thus, these enzymes would compete favorably for quite low concentrations of P-enolpyruvate for conversion to pyruvate. The adductor muscle pyruvate kinase differs from the mammalian muscle pyruvate kinases, however, in being strongly feed forward-activated by fructose-1,6-P_2. This has been observed for fish muscle as well, and may be a general characteristic of muscle pyruvate kinase in poikilothermic organisms (17).

The role of fructose-1,6-P_2 protection of both mantle and adductor pyruvate kinases is of interest. In all cases thus far examined, fructose-1,6-P_2 is able to reverse ATP inhibition of pyruvate kinase. In addition, in the oyster, fructose-1,6-P_2 protects both enzyme forms against alanine and phenylalanine inhibition. Thus far there is no adequate explanation available for these effects and this is clearly an important area for further research.

In mammalian systems, fructose-1,6-P_2 activation is greatest at alkaline pH values (9). An opposite pH dependence of the fructose-1,6-P_2 activation of the oyster pyruvate kinases is observed. For both enzymes, fructose-1,6-P_2 lowers the \( K_m^{(PEP)} \) and this effect is particularly striking at lower pH values (at pH 6.5 the \( K_m \) is reduced from 5.8 x 10^{-4} \text{m} to 8 x 10^{-5} \text{m}; at pH 8.5 the \( K_m \) is reduced from 1.9 x 10^{-4} \text{m} to 6.6 x 10^{-5} \text{m}). In consequence, in the presence of fructose-1,6-P_2 the \( K_m^{(PEP)} \) is essentially pH-independent. Since the oyster is a facultative anaerobe, the physiological function of fructose-1,6-P_2 activation may be to allow P-enolpyruvate conversion to pyruvate during extended periods of anaerobiosis, when intracellular pH might be reduced.

The adductor enzyme appears to be under tight ATP regulation. Thus, 2 mM ATP, a value probably within the physiological range (18), causes a 10-fold increase in the \( K_m^{(ATP)} \). Under conditions of low P-enolpyruvate concentrations, it is evident that adductor pyruvate kinase would be unusually sensitive to ATP. In this characteristic, the adductor enzyme resembles mammalian muscle pyruvate kinase (16) and adipose pyruvate kinase (19), all of which have similar \( K_i^{(ATP)} \) values, but it differs from mammalian brain pyruvate kinase (11) and the mantle enzyme. In both of the latter, ATP inhibition is noncompetitive. Because of the high \( K_i^{(ATP)} \) for the mantle enzyme, and because ATP does not alter the apparent \( K_m^{(PEP)} \), ATP would not be an efficient inhibitor of this enzyme.

In this connection, it is interesting that both mantle enzyme and mammalian brain pyruvate kinase (20, 21) are competitively inhibited by phenylalanine and the \( K_i \) values are again similar for the enzymes from the two tissue types. In the mantle, pyruvate kinase activity is fairly sensitive to phenylalanine (at \( K_i \) concentrations) produces quite large increases in the \( K_m^{(PEP)} \). Since phenylalanine concentrations are known to be unusually high in mollusc tissues (22), this amino acid may be an important physiological feedback inhibitor of pyruvate kinase activity in this tissue as it is in mammalian brain.

From the data presented it is speculated that coordinated changes in intracellular concentrations of H^+, P-enolpyruvate, fructose-1,6-P_2, ATP, alanine, and phenylalanine control pyruvate kinase activity in vivo.

**REFERENCES**

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