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Isolation and Kinetic Properties of Pyruvate Kinase Activated by Fructose-1,6-Bisphosphate from *Salmonella typhimurium* LT-2.I.

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Pyruvate kinase, activated by fructose-1,6-bisphosphate from *Salmonella typhimurium* LT-2, has been isolated and purified to homogeneity. The enzyme, similar to that from *Escherichia coli*, is a tetramer with an approximate molecular weight of 240,000. The native enzyme shows optimum pH 6.8 (T = 30° C). The enzymatic reaction does not require K^+ ions; while Mg^{2+} or Mn^{2+} are essential for its activity. The non-activated enzyme shows sigmoid kinetics to phosphoenolpyruvate with a Hill coefficient of 2.73; the activated enzyme becomes michaelian with K_s^{ADP} y K_s^{PEP} 0.25 and 0.08 mM, respectively. Both substrates excess and ATP cause enzyme inhibition. In agreement with the experimental results a steady-state random-ordered hybrid Bi-Bi mechanism with two dead-end complexes is proposed.

Pyruvate kinase (EC 2.7.1.40) is a key enzyme in the cellular metabolism, for it catalyzes the substrate-level phosphorylation reaction leading to adenosine 5'-triphosphate and pyruvate from phosphoenolpyruvate and adenosine 5'-diphosphate. Both compounds are located in a nodal point of metabolism, since phosphoenolpyruvate is the first substrate in the anaplerotic way to Krebs cycle from glycolysis, and it is also a precursor for aromatic aminoacids and the cell-wall peptidoglycan. Moreover, pyruvate can be oxidized via acetyl-CoA through the Krebs cycle or act as precursor for aminoacids classified as «pyruvate group» or for pantothenic acid, N-acetylneuraminic acid or lactate. Therefore, the regulation of pyruvate kinase activity could have a major physiological importance driving the intracellular levels of phosphoenolpyruvate and pyruvate and channelling the fluxes of these compounds to distinct pathways according to the cell's needs: catabolic, anaplerotic of anabolic.

In most of the organisms studied, both eukaryotic and prokaryotic, the enzyme has been shown as two kinds of pyruvate kinase potentially able to perform the

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transformation from phosphoenolpyruvate to pyruvate. One of these types can be activated by fructose-1,6-bisphosphate, the other one in not sensitive to this compound (8). The existence of a single type of enzyme has been reported in a lot of biological sources, but both enzymes are present at the same time in very different organisms such as mammalians (1, 13) fungi (19, 20) and enterobacteriaceae (2, 15). Fructose-1,6-bisphosphate activated pyruvate kinase is called pyruvate kinase B in *Escherichia coli;* an additional pyruvate kinase in this cell can be activated by adenosine 5'-monophosphate and it is known as pyruvate kinase A (7). The first enzyme is inducible by glycolitic substrates, showing sigmoid kinetics towards the substrate, phosphoenolpyruvate. Pyruvate kinase A is a constitutive one and shows normal Michaelis-Menten kinetics (15, 16).

From the fact that two distinct enzymes catalyzing the same key reaction in the amphibolic Embden-Meyerhof pathway exist, and from the fact a multiplicity of operative controls act on these enzymes, the validity of this subject as a mean of understanding their physiological significance and mechanism of action can be seen. The importance of pyruvate kinase B has been emphasized by studies using defective mutants in *E. coli.* Mutants *pyk* A^- *pyk* B^+ grown under laboratory conditions, grow like the wild strain in all glycolytic and gluconeogenic substrates assayed (7). However, *pyk* A^- *pyk* B^+ mutants are unable to grow on those glycolytic substrates which are not transported via sugar —phosphotransferase system—. Finally, *pyk* A^+ *pyk* B^- mutants do not grow on any glycolytic substrate under anaerobic conditions (COOPER, personal communication).

Kinetic (1, 8, 12, 13) and regulatory (3, 10, 14, 16, 22) studies have been carried out with different organisms for the phosphoenolpyruvate—pyruvate reaction. Nevertheless, in spite of the psysiological importance of pyruvate kinase B in the bacterial cell, no bibliography has been found dealing with kinetic mechanism neither for pyruvate kinase B nor A from *Enterobacteriaceae.* Although one can think of similarities between *Escherichia* and *Salmonella* with respect to the pass from phosphoenolpyruvate to pyruvate up to 1979, when we described the existence of two distinct forms of pyruvate kinase in *Salmonella typhimurium* (2), nobody, had discovered this. Hence, in order to obtain some information on the role of pyruvate kinase B in this microorganism we have isolated the enzyme to homogeinity and studied its kinetic properties.

Materials and Methods

Organism. The strain utilized has been *Salmonella typhimurium* LT-2, obtained from Dr. R. A. Cooper, Departament of Biochemistry, University of Leicester, U.K.

Growth conditions and preparation of enzymatic extracts. The cells were grown aerobically at 37° C in minimum medium M-63 (17) containing 10 mM glucose. The growth rate was followed by measuring optical density of the culture at wavelength of 680 nm. In the second half of the log phase (OD_{680} approx. eq. to 0.8), the cells were harvested, buffer-washed (50 mM Tris-HCl [1] pH 7.5) and their wet weight measured; they were con-

(1) Abbreviations used:
ADP: Adenosine 5'-diphosphate; ATP: Adenosine 5'-triphosphate; EDTA: Ethylenediaminetetraacetic acid; FBP: Fructose-1,6-bisphosphate; GTP: Guanosine 5'-triphosphate; HEPES: N-2-hydroxyethylpiperazine-N'-2-ethanosulfonic acid; LDH: Lactate dehydrogenase; $NADH_2$: Nicotinamide adenine dinucleotide (reduced form); PEP: Phosphoenolpyruvate. Tris: Tris (hydroxymethyl) aminomethane.

served at —20° C. To obtain the crude extract the cells were suspended in a volume (ml) of the above buffer twice their weight value, expressed in grams. The cell suspension was disrupted by exposure to ultrasonic oscillations in a Branson B-12 sonifier (70-80 Watts) at ice temperature for 10-s periods with cooling between for 1/2 min. The suspension was centrifugated at 25,000 × *g* for 15 min at 4° C to remove cell debris.

Enzyme purification. The purification steps were carried out according to the following procedure and are summarized in table I. The centrifuged extract prepared from around 20 g of cells was heated at 55° C for 10 min and afterwards centrifuged 25,000 × *g* 15 min. To the clear supernatant was added 10 % v/v glycerol and it was subsequently diluted with 50 mM Tris-HCl pH 7.5 plus 1 mM EDTA and 2 mM mercaptoethanol to around 50 ml. This solution was fractionated by addition of ammonium sulphate, and the protein precipitating between 40-70 % saturation was resuspended in 5 ml of the same buffer and applied to a Sephacryl S-200 gel-filtration column (40 × 2.6 cm) previously equilibrated overnight with buffer. Elution flux was 10 drops every 30-40 seconds; a fraction was formed with 80 drops (2.5 ml).

The highest pyruvate kinase B activity tubes were carefully applied to a small (28 ml) DEAE-Sephacel anion-exchange column previously washed with buffer, maintaining fraction collecting conditions as above. Elution was pursued by discontinuous changes in the ionic strength, ranging from 0 to 200 mM KCl. Pyruvate kinase B is normaly eluted with 150 mM KCl, but best recoveries are achieved using the anion-exchange column on the first use in which elution appears at 200 mM KCl. These elution KCl concentrations are all included in the Tris-EDTA-mercaptoethanol buffer above quoted.

Electrophoresis. The enzyme purity was tested by means of electrophoresis in 10 % polyacrylamide gels according to WEBER *et al.* (24). The molecular weight of the enzyme monomer was calculated from polyacrylamide-gel electrophoresis with SDS using the Shandon-Southern SDS-Polygel kit with protein markers ranging between 53 and 265 Kdaltons of molar mass.

Molecular weight of the enzyme. The gel-filtration column conditions used for purification of the enzyme were employed to determine the molecular weight. Reference proteins were catalase, rabbit muscle lactate dehydrogenase, bovine serum albumin and ovalbumin (5); blue dextran was used to obtain Vo.

Table I. *A summary of the purification of pyruvate kinase B from* Salmonella typhimurium.

Purification step	Volume (ml)	Protein (mg)	Activity (units)	Specific activity (units mg protein^{-1})	Yield	Purifi. factor
1. Crude extract	19	798.0	519.3	0.65	«100»	—
2. Heated extracts	13	114.4	345.0	3.01	66.5	4.62
3. $(NH_4)_2SO_4$ fractionation	3.6	30.0	173.0	5.81	33.1	8.92
4. Gel filtration chromat.: Sephacryl S-200	12	6.74	100.1	14.8	19.3	22.7
5. Ion exchange chromat.: DEAE-Sephacel	3.0	0.04	43.4	1004.8	8.4	1544

Enzyme assays. The pyruvate kinase reactions was measured the decrease in $OD_{340\,nm}$ which corresponds to the conversion of $NADH_2$ to NAD^+ as result of the pyruvate-lactate reduction catalized by LDH. In all cases initial enzyme velocities were measured. One enzyme unit catalyzes the conversion of 1 μmol of $NADH_2$ to NAD^+ per min under standard conditions (1 cm optical way, 30° C, pH and effectors defined elsewhere). The final concentration in cuvette of each component of the reaction mixture was 30 mM HEPES, 0.16 mM $NADH_2$, 5 mM $MgCl_2$, 1 mM FBP and 10 u.i. LDH in a volume of 1 ml. In kinetic measurements the substrate PEP and ADP were added in variable concentrations; the enzyme amount used corresponds to 72 μg protein, unless otherwise indicated. In the extracts, the reaction was iniciated by the addition of PEP (1 μmol). The activity observed in this assay was taken to be due to pyruvate kinase A. The assay was repeated with the additional presence of FBP (1 μmol), and the difference in rate between the two assays was assumed to be due to pyruvate kinase B. The ratio of $NADH_2$ oxidized to PEP utilized was assumed to be unity. One enzyme unit corresponds to 1 μmol of substrate transformed per min, and specific activity is enzyme units per milligram of protein.

Protein estimation. Protein was measured either colorimetrically, by the modified Folin method (11) with crystalline bovine serum albumen as standard, or spectrophotometrically (22).

Reagents. Sodium ATP was obtained from Aldrich-Europe. The tricyclohexylammonium salt of PEP was purchased from Merck. PEP (monopotassium and trisodium salts), HEPES and the tetracyclohexylammonium salt of FBP were adquired from Sigma, as well as pyruvate kinase-free LDH. Other reagents were of the highest commercial purity available.

Data analysis. Kinetic data were processed by linear regression routine of a Texas Instruments TI-59 programmable calculator and programs designed to ellucidate kinetic mechanism of the enzyme and the concentrations of the different binding species of substrate and activator with Mg^{2+} and H^+ ions recording the equations by MACFARLANE and AINSWORTH (12). These programs are available in our Department.

Results and Discussion

General properties. The pyruvate kinase has been purified until homogeneity as described in the «Materials and Methods» section. The purity of the preparation has been tested by the results obtained by polyacrylamide gel electrophoresis. The molecular weight of this protein and its subunits appears to be 240,000 ± 10,000 and 60,000 ± 4,000, respectively, as judged by gel filtration cromatography (fig. 1) and sodium dodecyl sulphate polyacrylamide gel electrophoresis. The value obtained for the holomer is on the range

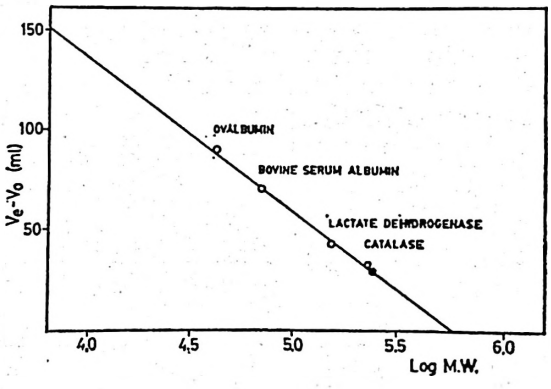


Fig. 1. *Calibration curve for the determination of the molecular weight of* S. typhimurium *pyruvate kinase B by Sephacryl S-200 gel filtration.*
The used standard proteins are indicated. The distinct symbol corresponds to the pyruvate kinase B value.

obtained for yeast pyruvate kinase (12) and close with that from *Pseudomonas citronellolis* (3); likewise, this molecular weight value is very similar to that obtained from *E. coli*, according to WAYGOOD and SANWAL (23), but a little higher than obtained in this organism by VALENTINI *et al.* (21). In a similar way to pyruvate kinase B in *E. coli* (16), the enzyme from *S. typhimurium* is thermoestable up to 70° C. When it is store at 5° C in the presence of 10 % glycerol and 2 mM dithiotreitol, the enzyme maintains its activity for almost one month.

Kinetic properties. Conditions for optimum enzyme activity. Different buffer solutions (Tris-HCl, imidazole, phosphate) have been used, and the best enzymatic activities are obtained using HEPES. The enzyme shows optimum activity at pH 6.8; this is the constant pH value used in this study. Although the optimum temperature is over 45° C, the enzymatic assays were carried out at the standard temperature of 30° C Monovalent ions like Na^+ and K^+ are not apparently required for catalytic activity. This behaviour is similar to the analogous enzyme from *E. coli*, but differs of the pyruvate kinase isolated from most sources (8, 9, 12), where K^+ ions are essential for activity. Nevertheless, either Mg^{2+} or Mn^{2+} ions are required for *S. typhimurium* pyruvate kinase B to act, within a reduced range of ion concentrations (fig. 2). The specific activity *versus* total (free and substrate- and FBP-linked) Mg^{2+} concentration plot shows positive homotropic interactions and the optimum total Mg^{2+} concentration was estimated to be 5 mM. The calculated value for the optimum free-Mg^{2+} concentration was around 2 mM. The conversion from total-Mg^{2+} to free-Mg^{2+} dependence of the specific activity is shown by the dotted curve on figure 2, and it was the basis for the correction of the results concerning the enzyme mechanism, as will be explained below. Using

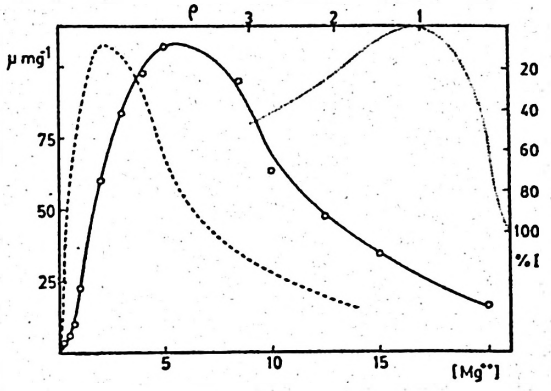


Fig. 2. *Magnesium dependence of the pyruvate kinase B reaction.*
The solid line shows the dependence of the specific activity of pyruvate kinase B on the total magnesium concentration. The concentration of both ADP and PEP was hold to 3 mM, FBP concentration was 1 mM and pH 6.8. Coupling factors as described in «Materials and Methods». The reaction was started with the addition of 24.4 μg protein. The dotted line gives the free-magnesium dependence of the specific activity. The free-magnesium concentration was calculated from the total magnesium added by the use of a program based in the dissociation constants for the different binding species of PEP, ADP and FBP with magnesium and hydrogen ions (12). The dashed line is a plot of the actual free Mg-optimum free Mg ratio (ρ) *versus* the percent inhibition of the reaction with respect to the optimum enzyme activity (% I). The curve was taken as pattern for the correction to 2 mM of free Mg from the experimental kinetic data obtained with 5 mM total Mg concentration.

a constant level of total Mg^{2+} (5 mM) and optimum pH (6.8), the rate-concentration plot for FBP shows an essential activation curve with a maximum point at 1 mM, holding constant the PEP and ADP concentrations at 1 and 2 mM, respectively (data not shown).

Substrate-dependence studies. Pyruvate kinase B shows sigmoid kinetics when phosphoenolpyruvate is used as variable substrate, with a K (1/2) value of 5.2 mM

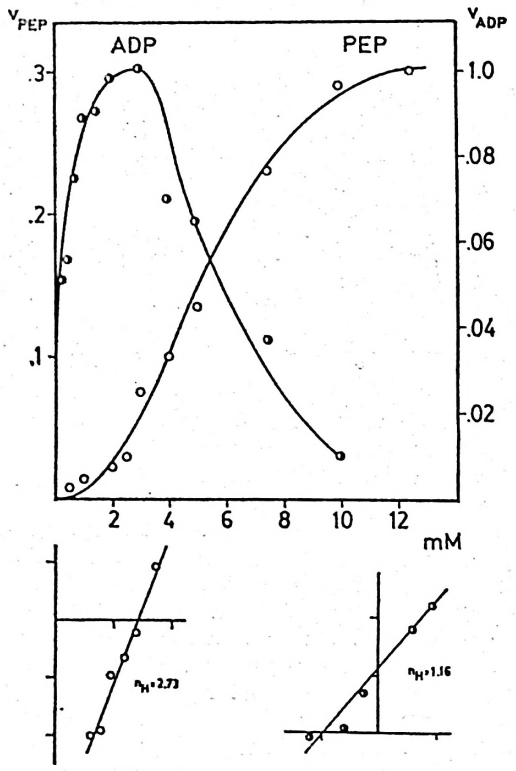
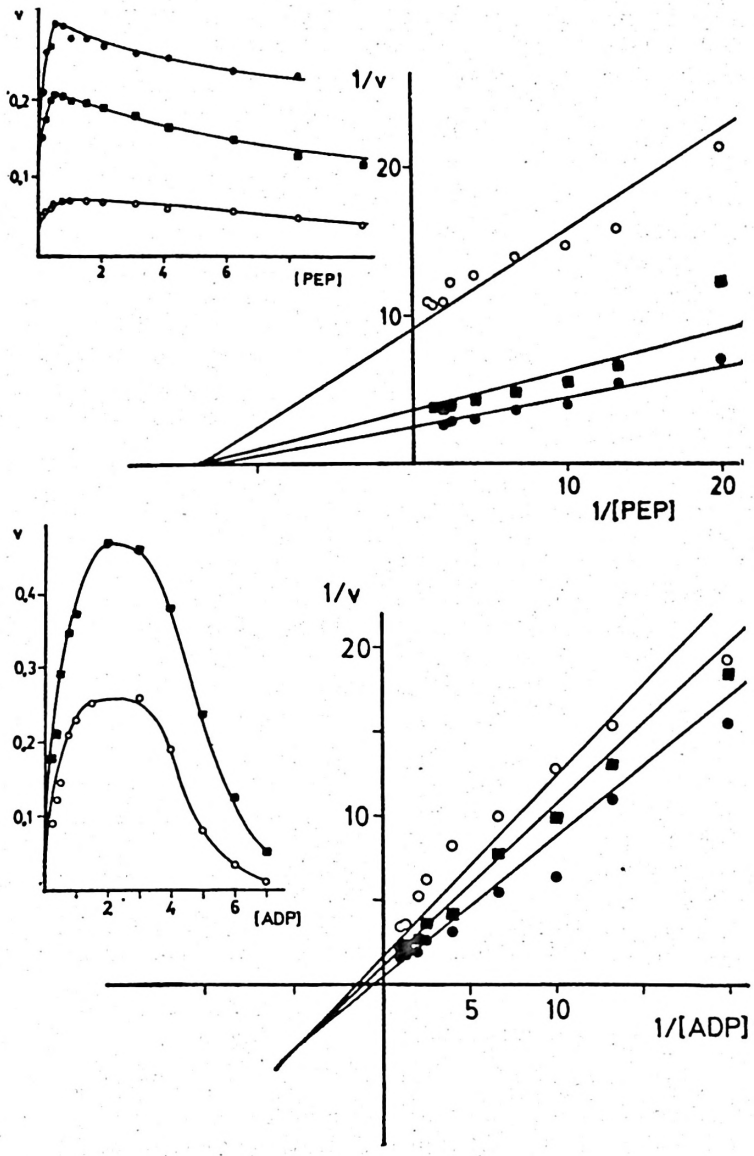


Fig. 3. *Substrate-behaviour of the* S. typhimurium *pyruvate kinase B in the absence of activator.*

The enzyme velocities are expressed as absorbance units per minute. The abscissa concentrations refers to the variable substrate. Below is the Hill representation of the upper curves; each unit represents 0.5 logarithm units, abscisses are logarithm of the substrate concentration, and ordinates are log v/(V-v), Hill coefficients are 2.73 for PEP and 1.16 for ADP.

(fig. 3) and Hill coefficient 2.73; on adding FBP, the kinetics become hyperbolic (fig. 4 *a*). When ADP is the variable substrate the rate-concentration plot are hyperbolic regadless of the presence or absence of the activator FBP, with K(1/2) = 0.25 mM. This behaviour is quite analogous to the *E. coli* pyruvate kinase B (23). The use of free rather than total bivalent-ion concentrations in the study of enzyme reactions involving nucleoside-phosphates is now recommended instead of the early maintenance of a total bivalent- ion concentration (18). As the range of substrate concentrations used (fig. 4) is very small (0-1 mM), the variations in the total-Mg^{2+} concentrations to add to the cuvette to get 2 mM free Mg^{2+} as optimum concentration (fig. 2) can also be shown to be small; the error implied in the manipulation of such concentrations could lead to uncertain results. Therefore, to overcome those difficulties the dashed line on figure 2 was constructed, which served as pattern for the correction at optimum Mg^{2+} conditions of the experimental points obtained at 5 mM total Mg^{2+}. Using 5 mM total Mg^{2+}, 1 mM FBP and pH 6.8 the usual approach to the study of a bisubstrate reaction has been followed, that is, to determine for both substrates the dependence of the reaction rate agains one substrate, holding the concentration of the other constant.

Figure 4 *a* shows the effect of varying the PEP concentration by keeping ADP concentration at three fixed values. In the upper graphic there is an apparent high-level substrate inhibition, which becomes even more pronounced with the increase of the ADP concentration. This can suggest a preferential kinetic way (6) and/or proper high-level PEP inhibition. The apparent Michaelis constant value for PEP, obtained by plotting the data in double reciprocal form, is 0.08 mM; if the results are corrected for 2 mM free Mg^{2+} (fig. 4 *a*, main graph), K_a^{PEP} appears as 0.07 mM, in the same range as *E. coli* pyruvate kinase B (23) and yeast enzyme (12). The reaction rate-ADP concentration relationship is shown on figure 4 *b*. Similarly to the PEP action, there is a clearly hyperbolic shape with strong inhibition as ADP concentration increases. The corrected double reciprocal plot (figure 4 *b*, main graph) leads to lines intersecting at the third quarter; the abscissa of the crosspoint gives a K_a value for



Statistical parameters from these representations are as follows (mathematical straight lines are assumed to be in the form $y = mx + b$; a is the abscissa in origin. r is the regression coefficient):

a)

[ADP]	0.1	0.3	0.5
b	9.136	3.739	2.529
a	—13.692	—13.862	—12.883
m	0.667	0.27	0.196
r	0.998	0.984	0.997

b)

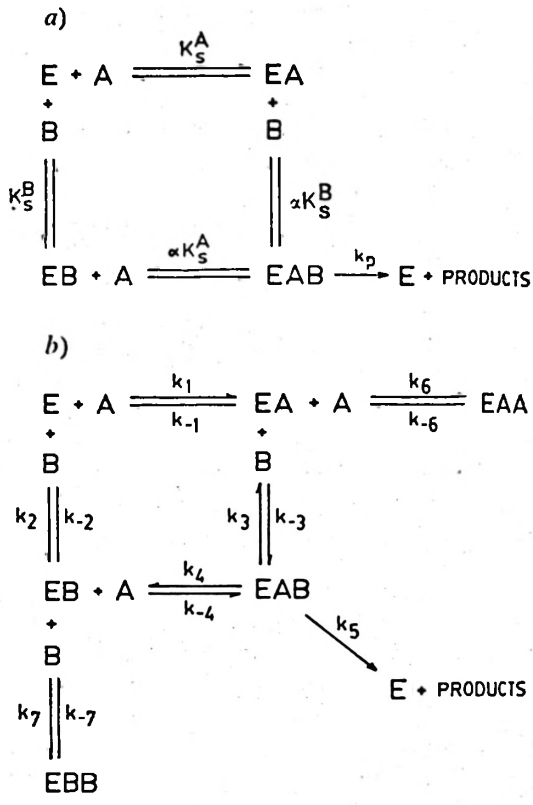
[PEP]	0.1	0.3	0.5
b	1.664	0.971	0.447
a	—1.373	—1.050	—0.531
m	1.212	0.924	0.841
r	0.993	0.999	0.998

Fig. 4. *Substrates-dependence of the reaction rate.*
The data were obtained at 5 mM total Mg and corrected for 2 mM free Mg by using the curve on figure 2. The reaction was started with the addition of 72 μg protein. Velocity is expressed as absorbance units per minute. All the concentrations are millimolar. The upper graphs are the direct rate-concentration representations. *a*) PEP-dependence of the reaction rate in the presence of 0.1 (○), 0.3 (■) and 0.5 (●) mM ADP. FBP concentration, pH and coupling factors as already described. Lower graph is the double reciprocal representation from which K_s^{PEP} value is obtained. *b*) ADP-dependence of the reaction rate in the presence of 0.1 (○), 0.3 (■) and 0.5 (●) mM PEP. Lower graph is the double reciprocal representation from which K_s^{ADP} value is obtained. In the upper graph on *b*) there was omitted the curve at 0.5 mM for more clarity. In both graphs, points corresponding to 0.01, 0.025, 0.05 and 0.075 mM PEP or ADP are not shown, but they were included in the analysis.

ADP of 0.25 mM, similar to that of *E. coli* pyruvate kinase B (23) but less than yeast enzyme (12).

Kinetic mechanism of pyruvate kinase B. Figure 4 *a* shows that ADP binds non-competitively to the enzyme with respect to PEP, i.e. ADP seems to be an activator that can bind either free enzyme or enzyme-PEP complex without any change in K_s^{PEP}. From figure 4 *b* a mixed type activation pattern is deduced: PEP behaves with respect to ADP as an essential activator that decreases the affinity of enzyme-PEP complex to ADP. Therefore, the binding of PEP to the enzyme causes an increase in the dissociation constant for ADP on a factor $\alpha > 1$ (scheme 1 *a*). This intersecting pattern excludes a ping-pong and ordered Bi-Bi type mechanism. Following the rapid-equilibrium random Bi-Bi system, one can readily see that figures 4 *a* and 4 *b* should be symmetrical, in the sense that the intersection points of the lines can both be located above, on or below the (-x)-axis. The present results, therefore, exclude a rapid-equilibrium mechanism, but they support at least qualitatively a steady-state mechanism (scheme 1 *b*) with the existence of a kinetically preferred way and one or two dead-end complexes. According to KING and ALTMAN'S procedure (4) to obtain the initial rate equations, this steady-state random-ordered hybrid Bi-Bi mechanism has proved to be the only one suitable for the experimental results whenever the following conditions are satisfied: i) the kinetically preferred way to the ternary complex involves the formation of enzyme-ADP rather than enzyme-PEP complex; ii) the enzyme-ADP-ADP dead-end complex must be formed more rapidly than enzyme-PEP-PEP, this latter being optional. The model has been tested by theoretical simulation.

These results reveal the possible role of pyruvate kinase B in glycolytic conditions. PEP can be channeled either to the oxalacetate formation by means of the anaplerotic reaction of phosphoenolpyruvate carboxylase, or the formation of



Scheme I. *Kinetic mechanisms for the reaction ADP + PEP → Pyr + ATP.*

a) Kinetic pathway according to a rapid equilibrium random Bi-Bi system. E represents free enzyme, A means ADP and B, PEP; EA, EB and EAB are the respective enzyme-ADP and ternary enzyme-ADP-ADP complexes. K_s^{ADP} and K_s^{PEP} are the dissociation constants for EA and EB complexes, respectively. K_s- modifying factor is α and it should be noted that α can modify either K_s^{ADP} or K_s^{PEP}, since the rate equation set up assuming constant concentration of ADP or PEP have the same shape (4). *b*) Kinetic pathway according to the proposed hybrid steady-state system. The subscript «minus» in the rate constant means reverse rate constants. EAA and EBB are the respective enzyme-ADP-ADP and enzyme-PEP-PEP dead-end complexes.

pyruvate by pyruvate kinase B. It is a significant fact that both enzymes are located in a dual metabolic point and that, in *S. typhimurium*, as phosphoenolpyruvate carboxylase activity is independent of FBP and activated by ATP (3), pyruvate kinase B is activated by FBP and product-inhibited by ATP (data not shown). This ATP modulation of both enzymes can contribute to control the PEP distribution to the different metabolic pathway, given the energetic status of the cell. In *E. coli*, a similar regulatory situation appears to exist (23), but in this case the kinase inhibition and carboxylase activation are due to ATP. Moreover, the strong increase in the reaction rate at low concentrations of ADP, that is, at little changes in the cell energy level, and the fact that the kinetically preferred way of the proposed mechanism involves the primary formation of enzyme-ADP complex, suggest a direct implication of pyruvate kinase B in the energy production.

Resumen

Ha sido aislada y purificada hasta homogeneidad la piruvato quinasa activable por fructosa-1,6-bisfosfato de *Salmonella typhimurium* LT-2. La enzima, semejante a la de *Escherichia coli*, es un tetrámero con peso molecular aproximado de 240.000. El pH óptimo de la enzima se estimó en 6,8 (T = 30° C). La reacción enzimática no requiere iones K^+, siendo el Mg^{2+} ó Mn^{2+} esenciales para la actividad. La enzima sin efector muestra cinética sigmoidal frente a fosfoenolpiruvato, con un coeficiente de Hill de 2,73; activada con fructosa-1,6-bisfosfato presenta comportamiento hiperbólico con K_s^{ADP} y K_s^{PEP} de 0,25 y 0,08 mM, respectivamente. Se ha observado inhibición de la enzima frente a ATP y exceso de ambos substratos. De acuerdo con los resultados experimentales se postula un mecanismo Bi-Bi de estado estacionario híbrido aleatorio-ordenado con dos complejos de vía muerta.

References

1. AINSWORTH, S. and MACFARLANE, N.: *Biochem. J.*, **131**, 223-236, 1973.
2. ALONSO, J. M., GARCÍA-OLALLA, C. and GARRIDO-PERTIERRA, A.: 8.° Congreso de la SEB. Murcia, Spain, 1979.
3. CHUANG, D. T. and UTTER, M. F.: *J. Biol. Chem.*, **254**, 8434-8441, 1979.
4. DIXON, M. and WEBB, E. C.: «Enzymes» (3rd ed.). Longman Group Ltd., London, 1979.
5. DONNELLY, M. T. and COOPER, R. A.: *Eur. J. Biochem.*, **113**, 555-561, 1981.
6. FERDINAN, W.: *Biochem. J.*, **113**, 278-283, 1966.
7. GARRIDO-PERTIERRA, A. and COOPER, R.A.: *J. Bacteriol.*, **129**, 1208-1214, 1977.
8. GILES, I. G. and POAT, P. C.: *Biochem. J.*, **185**, 289-299, 1980.
9. GUDERLEY, H. and CARDENAS, J. M.: *J. Exp. Zool.*, **211**, 185-198, 1980.
10. LIAO, C. L. and ATKINSON, D. E.: *J. Bacteriol.*, **106**, 37-44, 1971.
11. LOWRY, R. H., ROSEBROUGH, N. J., FARR, A. L. and RANDALL, R. J.: *J. Biol. Chem.*, **193**, 265-275, 1951.
12. MACFARLANE, N. and AINSWORTH, S.: *Biochem. J.*, **129**, 1035-1047, 1972.
13. MACFARLANE, N. and AINSWORTH, S.: *Biochem. J.*, **139**, 499-508, 1974.
14. MAEBA, P. and SANWAL, B. D.: *J. Biol. Chem.*, **243**, 448-450, 1968.
15. MALCOVATI, M. and KORNBERG, H. L.: *Biochim. Biophys. Acta*, **178**, 420-423, 1969.
16. MALCOVATI, M., VALENTINI, G. and KORNBERG, H. L.: *Acta Vitamin. Enzymol.*, **27**, 96-111, 1973.
17. MILLER, J. H.: In «Experiments in molecular genetics». Cold Spring Harbor, N.Y. Laboratory, Cold Spring Harbor, N.Y., 1972.
18. MORRISON, J. F.: *Methods in Enzymology*, **63**, 261, 1979.
19. PASSERON, S. and ROSELINO, E.: *FEBS Lett.*, **18**, 9-12, 1971.
20. PAZNOKAS, J. L. and SYPHERD, P. S.: *J. Bacteriol.*, **130**, 661-666, 1977.
21. VALENTINI, G. IADAROLA, P., LAL SOMANI, B. and MALCOVATI, M.: *Biochim. Biophys. Acta*, **570**, 248-258, 1979.
22. WARBURG, O. and CHRISTIAN, W.: *Biochem. Z.*, **310**, 384-392, 1941.
23. WAYGOOD, E. B. and SANWAL, B. D.: *J. Biol. Chem.*, **249**, 265-274, 1974.
24. WEBER, K., PRINGLE, J. R. and OSBORN, M.: *Methods in Enzymology*, **26**, 3-27, 1972.