Glucose 6-phosphate activation of pyruvate kinase from *Mycobacterium smegmatis*

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1. Activation by glucose 6-phosphate is one of the unique properties of pyruvate kinase from *Mycobacterium smegmatis*. 2. Pyruvate kinase, partially purified from ultrasonic extracts of the mycobacteria by $(\text{NH}_4)_2\text{SO}_4$ fractionation, exhibited sigmoidal kinetics at various concentrations of phosphoenolpyruvate, with a high degree of co-operativity (Hill coefficient, $h = 3.7$) and $s_{0.5}$ value of 1.0 mM. 3. In the presence of glucose 6-phosphate, the degree of co-operativity shown by the phosphoenolpyruvate saturation curve was decreased to $h = 2.33$ and the $s_{0.5}$ value was lowered to 0.47 mM. 4. The enzyme was activated by AMP and ribose 5-phosphate also, but the activation constant was lowest with glucose 6-phosphate (0.24 mM). 5. The enzyme was strongly inhibited by ATP at all phosphoenolpyruvate concentrations. The concentrations of ATP required to produce half-maximal inhibition of enzyme activity at non-saturating (0.2 mM) and saturating (2 mM) phosphoenolpyruvate concentrations were 1.1 mM and 3 mM respectively. 6. The inhibition by ATP was partially relieved by glucose 6-phosphate. 7. The enzyme exhibited Michaelis–Menten kinetics with ADP as the variable substrate, with an apparent K_m of 0.66 mM. 8. The enzyme required Mg^{2+} or Mn^{2+} ions for activity. It was not activated by univalent cations. 9. The kinetic data indicate that under physiological conditions glucose 6-phosphate probably plays a significant role in the regulation of pyruvate kinase activity.

In mycobacteria, glucose is metabolized mainly through the Embden–Meyerhof–Parnas pathway (Jayanthi Bai *et al.*, 1976). Pyruvate kinase (EC 2.7.1.40) has been widely recognized as a key regulatory enzyme of glycolysis. The allosteric pyruvate kinase from diverse sources, notably yeast (Hess *et al.*, 1966), rat liver and kidney cortex (Tanaka *et al.*, 1967) and *Escherichia coli* (Maeba & Sanwal, 1968) is activated by fructose 1,6-bisphosphate and inhibited by ATP; this is postulated to reflect the mechanism whereby the activity of this enzyme is regulated under physiological conditions. Contrary to this, the enzyme from *Mycobacterium phlei* is unaffected by fructose 1,6-bisphosphate (Dudouet *et al.*, 1973). Preliminary studies conducted on pyruvate kinase isolated from *Mycobacterium smegmatis* in our laboratory have revealed that fructose 1,6-bisphosphate does not activate this enzyme. In an attempt to elucidate the mechanism of regulation of pyruvate kinase from *M. smegmatis*, a study of the allosteric properties of this enzyme was undertaken.

Materials and methods

Chemicals

Phosphoenolpyruvate (tricyclohexylammonium salt), dithiothreitol, Hepes [4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid], NADH, bovine serum albumin, lactate dehydrogenase (type III, ox heart), hexose monophosphates, fructose 1,6-bisphosphate, Sephadex G-25 and nucleotides (all sodium salts) were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A. All other chemicals were of analytical grade.

Organism and growth conditions

Mycobacterium smegmatis CDC 46 (originally obtained from Centre for Disease Control, Atlanta, GA, U.S.A.), a fast grower and saprophyte, was grown in the medium described by Youmans & Karlson (1947) containing 2% (v/v) glycerol. The culture medium was incubated at 37°C without shaking. The cells were harvested in the exponential phase of growth (4 days), by centrifugation at 8000 g

at 4°C, washed thoroughly with glass-distilled water and stored at -20°C until further use.

Preparation of enzyme

All procedures were carried out at 4°C. Cell-free extracts were prepared by sonic disruption of the cell suspension (40%, w/v) in 0.05 M-potassium phosphate buffer, pH 7.0, containing 1 mM-dithiothreitol (buffer I) for 5 min in a MSE ultrasonicator at 20 kHz, followed by centrifugation at 20 000 g for 30 min in an IEC refrigerated centrifuge. The enzyme was precipitated from the cell-free extracts by the addition of (NH₄)₂SO₄ between 40 and 60% saturation. The suspension was centrifuged at 20 000 g for 15 min. The precipitate was suspended in buffer I. The enzyme was stored at -20°C. For kinetic experiments, the enzyme was diluted in 0.05 M-potassium phosphate buffer, pH 7.0, containing 20% (v/v) glycerol and 1 mM-dithiothreitol (buffer II).

Pyruvate kinase assay system

Pyruvate kinase was assayed by a modification of the coupled assay procedure of Bücher & Pfeleiderer (1955). The standard assay mixture for the glucose 6-phosphate-activated pyruvate kinase contained: phosphoenolpyruvate, 2 mM; ADP, 2 mM; MgCl₂, 15 mM; NADH, 0.15 mM; Hepes buffer (pH 7.0), 50 mM; lactate dehydrogenase, 28 µg/ml; and an appropriate amount of the enzyme preparation (8–10 µg) in a total volume of 1 ml. For kinetic studies at pH 8.0, 0.05 M-Hepes buffer at pH 8.0 was used. The reaction was initiated by the addition of phosphoenolpyruvate. The enzyme was assayed by measuring the decrease in A₃₄₀ at 30°C, in a Beckman DU spectrophotometer. Lactate dehydrogenase was freed from (NH₄)₂SO₄ by passing it through a column of Sephadex G-25.

All the activators and inhibitors of pyruvate kinase discussed in this paper were tested for their effect on lactate dehydrogenase. None was found to inhibit or activate the enzyme. Whenever nucleoside triphosphates were used in assay mixtures, an equimolar quantity of MgCl₂ was added. The specific activity of the enzyme preparation was in the range 1.0–1.3 µmol of phosphoenolpyruvate utilized/min per mg of protein.

The protein concentration of the preparations was determined by the method of Lowry *et al.* (1951), with bovine serum albumin as standard.

NADH oxidase

NADH oxidase activity was determined by omitting lactate dehydrogenase, ADP and phosphoenolpyruvate from the standard assay mixture given above. The NADH oxidase activity was less than 5% of the pyruvate kinase activity.

Assay for adenylate kinase and ATPase

Adenylate kinase (EC 2.7.4.3) was assayed by the procedure described by Bergmeyer (1974). ATPase (EC 3.6.1.3) was assayed by the adenylate kinase assay system, modified by excluding AMP.

Under the standard assay conditions for the determination of pyruvate kinase activity, the interfering enzymes, namely ATPase, phosphatase and adenylate kinase, were not detectable in the enzyme preparation obtained by (NH₄)₂SO₄ fractionation.

Kinetic analysis

For the sigmoid phosphoenolpyruvate-saturation curves, Hill plots were used to evaluate phosphoenolpyruvate concentration at half saturation, [PEP]_{0.5v}, and the Hill coefficient *h* (Hill, 1910). The maximum velocity was obtained by measuring pyruvate kinase activity in the presence of saturating phosphoenolpyruvate concentration. In experiments where inhibition of enzyme activity was observed at high concentration of the substrate phosphoenolpyruvate, the exact maximal velocity could not be determined. Hence, Hill coefficients and [PEP]_{0.5v} were evaluated by the method of Endrenyi *et al.* (1975), which can be used when the maximum velocity is not known. Initial-velocity data were obtained in pairs, one reading being made at a concentration *c*, the other at *ac*, the constant *a* representing a concentration ratio fixed for all data pairs; its value by definition exceeds unity. The values of initial reaction velocities obtained at substrate concentrations *c* and *ac* were represented by *v* and *w*. A plot of 1/*w* against 1/*v* was used to evaluate the Hill coefficient and maximum velocity by using the following equations:

$$\text{Hill coefficient} = \frac{-\log \text{slope}}{\log a}$$

$$\text{Maximum velocity} = \frac{1 - \text{slope}}{\text{vertical intercept}}$$

Results

Cell-free extracts prepared from *M. smegmatis* cells grown on different carbon sources, i.e. glycerol, glucose, or fructose, showed a single band of pyruvate kinase activity on polyacrylamide-disc-gel electrophoresis and a single peak of pyruvate kinase activity on Sephadex G-200 column chromatography, thereby indicating the existence of a single form of pyruvate kinase in this organism.

The partially purified pyruvate kinase was found to lose about 60% of its original activity within 12 h, at a protein concentration of 0.1 mg/ml. The influence of various modifiers of the enzyme on the inactivation kinetics is shown in Fig. 1. Glycerol and

Fig. 1. Dilution inactivation of pyruvate kinase from *M. smegmatis*

The enzyme preparation (24 mg/ml of protein) obtained by (NH₄)₂SO₄ fractionation (40–60% saturation) of the cell-free extract was diluted, 100-fold, at zero time into different buffers. At the times indicated, appropriate samples (containing 15 μg of protein) were withdrawn from the diluted preparations and assayed for pyruvate kinase activity by the standard assay procedure at pH 7.0 as described in the Materials and methods section. Inactivation kinetics was studied at a fixed concentration of the enzyme (0.24 mg of protein/ml) in the presence of 50 mM-Hepes/KOH buffer, pH 7.0, containing 2 mM-β-mercaptoethanol (■) with the following additions: 20% glycerol (Δ); 20% glycerol and 0.1 M-KCl (□); 20% glycerol and 1 mM-phosphoenolpyruvate (●); 20% glycerol plus 0.1 M-KCl and 1 mM-phosphoenolpyruvate (○).

phosphoenolpyruvate prevented the inactivation to a great extent. Complete stabilization of the diluted enzyme (at a protein concentration of 0.24 mg/ml) could be achieved by the addition of 20% glycerol, 0.1 M-KCl, 1 mM-phosphoenolpyruvate and 2 mM-β-mercaptoethanol.

Metal ion requirements

The enzyme had an absolute requirement for a bivalent cation. The Mg²⁺-saturation curve (in the presence of saturating concentration of both substrates, ADP and phosphoenolpyruvate) was sigmoidal, maximum activity begin observed at 15 mM-Mg²⁺. The Hill plot of the data had a slope of 1.8 and a [Mg²⁺]_{0.5v} value of 2.2 mM. The Mn²⁺-saturation curve was hyperbolic, with an approximate K_m of 0.43 mM. The maximum velocity observed in the presence of Mn²⁺ was only half of the value obtained when Mg²⁺ was used. Although univalent cations were required to prevent inactivation of the enzyme on dilution, they were not necessary for activity.

Table 1. Activation of pyruvate kinase by phosphorylated compounds

The assays were carried out under standard conditions described in the Materials and methods section with 0.6 mM-phosphoenolpyruvate, 2 mM-ADP and 1 mM of the activator.

Activator	Activity (% of that without activator) at pH		
	6.0	7.0	8.0
None	100	100	100
Glucose 6-phosphate	256	476	588
AMP	149	410	130
Ribose 5-phosphate	102	181	141
Fructose 6-phosphate	102	100	106
Glucose 1-phosphate	95	119	94
Fructose 1,6-bisphosphate	108	110	100

Phosphoenolpyruvate-saturation kinetics

The pyruvate kinase of *M. smegmatis* showed sigmoidal kinetics with a high degree of cooperativity (*h* = 3.7) and *s*_{0.5} value of 1 mM, when assayed at pH 7.0, with various concentrations of phosphoenolpyruvate and ADP at saturating concentrations. The enzyme exhibited Michaelis-Menten kinetics with ADP as the variable substrate. The apparent K_m value for ADP was 0.66 mM.

Activation of the enzyme

A search was made for possible effectors of the enzyme at a non-saturating phosphoenolpyruvate concentration. Among the hexose phosphates studied, only glucose 6-phosphate activated the enzyme. The data in Table 1 show that fructose 6-phosphate, glucose 1-phosphate and fructose 1,6-bisphosphate had no effect on enzyme activity. Of the positive effectors studied, glucose 6-phosphate was found to be the most potent activator of pyruvate kinase, causing a 4-fold activation of the enzyme. Glucose 6-phosphate added to the assay system for studying the activation of the enzyme could be recovered at the end of the experiment. The activation profiles of pyruvate kinase from *M. smegmatis* in the presence of glucose 6-phosphate and AMP are shown in Fig. 2. The concentrations of glucose 6-phosphate and AMP required to produce half-maximal activation (*K*_a) were 0.24 mM and 0.5 mM respectively.

Fig. 3 shows the effects of glucose 6-phosphate and AMP on the phosphoenolpyruvate-saturation curve of the enzyme. The addition of 1 mM-glucose 6-phosphate to the assay system altered the sigmoidal nature of the phosphoenolpyruvate response curve by increasing the affinity of the enzyme for phosphoenolpyruvate, as indicated by a decrease in the Hill coefficient from 3.7 (in the absence of any effector) to 2.33 and 1.33 at pH 7.0 and pH 8.0

Fig. 2. Activation of *M. smegmatis* pyruvate kinase by glucose 6-phosphate (O) and AMP (■)

The concentration of phosphoenolpyruvate was 0.6 mM and all other assay conditions were those of the standard assay mixture described in the Materials and methods section. v_a is the velocity of the enzyme in the presence, and v_0 is that in the absence, of the activator. The change in A_{340} in the absence of the activator was 0.02 unit/min.

Fig. 3. Effect of activators on the phosphoenolpyruvate saturation kinetics

All assay conditions are described in the Materials and methods section. Enzyme activity was determined at various concentrations of phosphoenolpyruvate at pH 7.0 without additions (O) or with the following additions: (Δ) 1 mM-glucose 6-phosphate, pH 7.0; (□) 1 mM-AMP, pH 7.0; (■) 1 mM-glucose 6-phosphate, pH 8.0. The Endrenyi plots of the data are shown in the insert (same symbols as in the main panel). The parameters v and w are defined in the text.

respectively. However, the sigmoidal character of the phosphoenolpyruvate-saturation plot could not be eliminated completely. The $s_{0.5}$ value for phosphoenolpyruvate decreased from 1 mM-glucose-6-phosphate at pH 7.0 and 8.0 respectively. A similar activation was observed by 1 mM-AMP; the corresponding value for h was 1.5 and the $s_{0.5}$ value for phosphoenolpyruvate was 0.50 mM. Though the maximum velocity was not affected, the addition of AMP at pH 7.0 or glucose 6-phosphate at pH 8.0 did not prevent substrate inhibition at high phosphoenolpyruvate concentration (above 1 mM). The enzyme activated by saturating concentrations of glucose 6-phosphate could not be stimulated further by the addition of AMP.

Effect of inhibitors

The data on the nucleoside triphosphate inhibition of pyruvate kinase from *M. smegmatis* are presented in Table 2. ITP, GTP and ATP were potent inhibitors of this enzyme. ATP, a product of the pyruvate kinase reaction, has been shown to be an inhibitor of various microbial pyruvate kinases (Dudouet *et al.*, 1973; Waygood & Sanwal, 1974; Liao & Atkinson, 1971). The relationship between initial velocity and ATP concentration was determined at two different phosphoenolpyruvate concentrations (Fig. 4). The concentration of ATP giving half-maximal inhibition changed from 3.0 mM at saturating concentration of phosphoenolpyruvate (2 mM) to 1.1 mM at a non-saturating (0.2 mM) concentration. The interaction between the activator glucose 6-phosphate and the inhibitor ATP was investigated by determining their combined effect on the relationship between initial velocity and phosphoenolpyruvate concentration (Fig. 5). The addition of 1 mM-ATP to the assay system decreased both the affinity of the enzyme for phosphoenolpyruvate, as indicated by an increase in $s_{0.5}$ value for

Table 2. Nucleoside triphosphate inhibition of pyruvate kinase

The assays were carried out as described in the Materials and methods section in a mixture containing 2 mM-ADP, 0.6 mM-phosphoenolpyruvate and 1 mM of the inhibitor.

Nucleotide	Activity (% of that without inhibitor) at pH		
	6.0	7.0	8.0
None	100	100	100
ATP	50	25	36
GTP	23	22	25
ITP	25	12	15
CTP	50	78	62
UTP	48	57	31

Fig. 4. Effect of ATP on pyruvate kinase

The assays were carried out in the presence of (○) 0.2 mM- and (■) 2 mM-phosphoenolpyruvate; all other assay conditions were those of the standard assay mixture described in the Materials and methods section. v_i is the velocity of the enzyme in the presence, and v_0 that in the absence, of inhibitor.

Fig. 5. Effect of glucose 6-phosphate and ATP on phosphoenolpyruvate saturation of pyruvate kinase. Standard assays were carried out at pH 7.0 as described in the Materials and methods section. Enzyme activity was determined at various concentrations of phosphoenolpyruvate in the absence of any effector (○) and in the presence of (■) 2 mM-ATP or (△) 2 mM-ATP plus 1 mM-glucose 6-phosphate. The insert shows the Hill plots of the data (same symbols as in the main Figure).

phosphoenolpyruvate from 0.8 mM to 1.0 mM, and the maximum velocity of the reaction, without affecting the Hill coefficient. The ATP inhibition of pyruvate kinase was partially relieved by the addition of 1 mM-glucose 6-phosphate at low concentrations of the substrate, phosphoenolpyruvate, resulting in an increase in the affinity of the enzyme

for phosphoenolpyruvate, but the maximal velocity and Hill coefficient were not affected. Hill plots of the data show a decrease in $s_{0.5}$ value for phosphoenolpyruvate to 0.9 mM.

Discussion

The results presented herein represent the first definitive information on the glucose 6-phosphate activation of pyruvate kinase from the genus *Mycobacterium*. The kinetic data presented in this paper establish the allosteric nature of the pyruvate kinase from *M. smegmatis*. The sigmoid velocity-substrate-concentration relationship characteristic of regulatory enzymes suggests a co-operative binding of the substrate, phosphoenolpyruvate. Like the pyruvate kinase from *Veillonella parvula* (Ng & Hamilton, 1975), the enzyme from *M. smegmatis* exhibited a much higher degree of co-operativity with phosphoenolpyruvate (h 4.4 and 4.0 respectively) than that observed with the enzyme from yeast (2.0), *Escherichia coli* (1.3), *Brevibacterium flavum* (3.0) and *Azotobacter vinelandii* (1.8). Substrate co-operativity could have considerable regulatory significance by ensuring extreme sensitivity of enzyme activity to small changes in substrate concentration (Stadtman, 1966). In this case, phosphoenolpyruvate would then be both a substrate and a positive feedback effector.

In general, microbial pyruvate kinases (Hess *et al.*, 1966; Maeba & Sanwal, 1968) and type L and K pyruvate kinase of mammalian tissues (Tanaka *et al.*, 1967) are activated by fructose 1,6-bisphosphate, but *M. smegmatis* was found to contain only one form of pyruvate kinase, which was insensitive to fructose 1,6-bisphosphate, even at low phosphoenolpyruvate concentrations. In this respect it is similar to the pyruvate kinases isolated from *Acetobacter xylinum* (Benziman, 1969), *Brevibacterium flavum* (Ozaki & Shii, 1969), *Mycobacterium phlei* (Dudouet *et al.*, 1973), *Rhodospseudomonas sphaeroides* (Schedel *et al.*, 1975) and *Fusarium oxysporum* (Zink, 1977). It is noteworthy that the activation of yeast pyruvate kinase by fructose 1,6-bisphosphate was reported to be related to activation of the enzyme by K^+ and NH_4^+ ions (Hess & Haeckel, 1967). As noted above, the *M. smegmatis* enzyme was not only insensitive to fructose 1,6-bisphosphate, but it was also unaffected by K^+ and NH_4^+ ions. Activation by glucose 6-phosphate is one of the unique properties of pyruvate kinase from *M. smegmatis*; fructose 6-phosphate or hexose 1-phosphates did not activate the enzyme.

Glucose 6-phosphate activation of pyruvate kinase has been observed in various microorganisms (Liao & Atkinson, 1971; Somani *et al.*, 1977). Pyruvate kinase from *Streptococcus mutans*

JC 2 shows an absolute and specific requirement for glucose 6-phosphate (Yamada & Carrlson, 1975). In mycobacteria, glucose 6-phosphate was found to activate pyruvate kinase by decreasing the degree of co-operativity of the rate-concentration curve of phosphoenolpyruvate and increasing the affinity of the enzyme for phosphoenolpyruvate, with little or no effect on the maximum velocity. Liao & Atkinson (1971) have reported a similar effect of glucose 6-phosphate on the activity of pyruvate kinase from *Azotobacter vinelandii*. Glucose 6-phosphate increased the maximum velocity of pyruvate kinase in *Veillonella parvula* and *E. coli* (Somani *et al.*, 1977). In mycobacteria, the co-operativity of phosphoenolpyruvate binding is not completely eliminated by the addition of glucose 6-phosphate, and in this respect it is similar to the enzyme from *Veillonella parvula*. Since the concentration of glucose 6-phosphate *in vivo*, computed at 0.4 mM, is higher than the activation constant for glucose 6-phosphate (0.24 mM), it probably acts as an activator of pyruvate kinase under physiological conditions.

ATP inhibition of pyruvate kinase seems to be universal, except in *Bacillus subtilis* (Diesterhaft & Freese, 1972). Although ATP inhibition of some pyruvate kinases has been attributed to the binding of Mg^{2+} ions by ATP (Wood, 1968), this does not appear to apply to our system, since inhibition was observed even in the presence of excess Mg^{2+} ions. Pyruvate kinase from *M. smegmatis* exhibits product inhibition by ATP without invoking allosteric inhibition. Since pyruvate is involved in the channelling of carbohydrate via pyruvate into the tricarboxylic acid cycle, the enzyme plays an important role in the generation of ATP. A high ADP/ATP concentration ratio would favour the pyruvate kinase reaction and the oxidation pathway of the tricarboxylic acid cycle, with concurrent production of high-energy phosphate. However, a rise in ATP concentration would regulate the activity of pyruvate kinase and decrease the synthesis of high-energy phosphate. The concentration of ATP required for significant inhibition of the pyruvate kinase activity (1.1 mM, giving 50% inhibition at 0.2 mM-phosphoenolpyruvate) is comparable with those reported for other bacterial pyruvate kinases (Schedel *et al.*, 1975; Ng & Hamilton, 1975) and is within the range of concentration found *in vivo* (Kapoor *et al.*, 1979). Hence inhibition by ATP would probably provide a mechanism by which the pyruvate kinase activity could be regulated *in vivo*, as demonstrated by Wilke & Schlegel (1975) for *Alcaligenes eutrophus*.

As glucose 6-phosphate has been found partially to overcome the ATP inhibition of pyruvate kinase, at low concentrations of phosphoenolpyruvate, the interplay between allosteric activation by glucose 6-phosphate and AMP and inhibition by ATP is

likely to be an important factor for the regulation of glycolysis at the pyruvate kinase step.

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