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Purification and Properties of Pyruvate Kinase from *Streptococcus mutans*

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Pyruvate kinase (EC 2.7.1.40) from *Streptococcus mutans* strain JC2 was purified, giving a single band on sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The molecular weight of the native enzyme was 180,000 to 190,000, and the enzyme was considered to consist of four identical subunits. This enzyme was completely dependent on glucose 6-phosphate for activity, and the saturation curve for activation by glucose 6-phosphate was sigmoidal. In the presence of 0.5 mM glucose 6-phosphate, the saturation curves for the substrates phosphoenolpyruvate and ADP were hyperbolic, and the K_m values were 0.22 and 0.39 mM, respectively. GDP, IDP, and UDP could replace ADP, and the K_m for GDP (0.026 mM) was 0.067 of that for ADP. The enzyme required not only divalent cations, Mg^{2+} or Mn^{2+}, but also monovalent cations, K^+ or NH_4^+, for activity, and it was strongly inhibited by P_i. When the concentration of P_i was increased, the half-saturating concentration and Hill coefficient for glucose 6-phosphate increased. However, the enzyme was immediately inactivated in a solution without P_i. The intracellular concentration of glucose 6-phosphate, in cooperation with that of P_i, may regulate pyruvate kinase activity in *S. mutans*.

The acids which are generated through the fermentation of sugar by the microorganisms in dental plaque decalcify the tooth surface (13, 17). *Streptococcus mutans* is considered to be one of the most important cariogenic agents (5, 8, 15). Therefore, the regulation of glycolysis by this microorganism attracts considerable attention in connection with the initiation of dental caries.

Iwami and Yamada have recently reported that the reaction catalyzed by pyruvate kinase (EC 2.7.1.40) is a rate-limiting step of the glycolytic pathway in *S. mutans* (12). This reaction is also important as one of the two or three ATP-regenerating steps in this organism, and the substrate for pyruvate kinase, phosphoenolpyruvate (PEP), is essential for the sugar transport system in *S. mutans* (25, 26).

Pyruvate kinases from *Escherichia coli* (9, 19, 33), *Saccharomyces carlsbergensis* (10), *Streptococcus lactis* (6, 7), and *Streptococcus sanguis* (36) are activated by fructose 1,6-bisphosphate, and those from *Azotobacter vinelandii* (16), *Veillonella parvula* (23), *Streptococcus cremoris* (29), and *Pseudomonas citronellolis* (4) are activated by a variety of glycolytic intermediates. Pyruvate kinases from these microorganisms have some catalytic activity without these activators. Yamada and Carlsson have partially purified pyruvate kinase from *S. mutans* and have reported that it is completely dependent on glucose 6-phosphate (36).

The present study was undertaken to purify pyruvate kinase from *S. mutans* and to provide a more detailed kinetic characterization of this unique enzyme by using a purified preparation.

MATERIALS AND METHODS

Chemicals. The following enzyme and reagents were obtained from Boehringer Mannheim Corp., Mannheim, West Germany: lactate dehydrogenase (rabbit muscle, EC 1.1.1.27), NADH, ADP, GDP, IDP, PEP, glucose 6-phosphate, fructose 1,6-bisphosphate, glucose 1,6-bisphosphate, fructose 6-phosphate, and glyceraldehyde 3-phosphate. ATP, UDP, CDP, 5'-AMP, 5'-GMP, 5'-IMP, 2',3'-AMP, and 2',3'-UMP were purchased from Yamasa Shoyu Co., Choshi, Japan. DEAE-cellulose was supplied by Brown Co., Elkhart, Ind. Sephadex G-200 was from Pharmacia Fine Chemicals, Uppsala, Sweden, and ribulose 5-phosphate, TDP, and reactive blue 2-agarose were from Sigma Chemical Co., St. Louis, Mo. Morpholinepropanesulfonic acid (MOPS) and protamine sulfate were obtained from Wako Pure Chemical Industries Ltd., Osaka, Japan, and ribose 5-phosphate was from Kyowa Hakko Co., Tokyo, Japan.

Microorganism and growth conditions. *S. mutans* strain JC2 (3) was grown at 35°C in a medium containing glucose (10 g), NH_4HCO_3 (2 g), dried extract of yeast (Daigo, Eiyokagaku Ltd., Osaka, Japan; 2 g), L-cysteine-HCl (100 mg), $MgSO_4 \cdot 7H_2O$ (200 mg), NaCl (10 mg), $MnSO_4 \cdot 4H_2O$ (10 mg), and $FeSO_4 \cdot 7H_2O$ (10 mg), in 1 liter of 100 mM potassium phosphate buffer, pH 7.0. The cells were harvested at the logarithmic growth phase and washed three times with 40 mM

potassium phosphate buffer (pH 7.0)–10 mM 2-mercaptoethanol. The cells were then stored at −20°C.

Purification of pyruvate kinase. (i) Cell extract. The frozen cell paste (88 g, wet weight) was thawed and suspended in 396 ml of 40 mM potassium phosphate buffer (pH 7.0)–10 mM 2-mercaptoethanol. The cells were disrupted by sonic oscillation for 15 min at 0°C (200 W, 2 A). The extract was centrifuged at 17,500 × *g* for 30 min at 4°C, and the supernatant was used for further purification of the enzyme. All of the following purification procedures were carried out at 0 to 4°C.

(ii) Protamine sulfate precipitation. Freshly prepared 2% (wt/vol) protamine sulfate solution (39 ml) was added dropwise to the cell extract (390 ml) and then stirred. After standing for 40 min, the mixture was centrifuged at 17,500 × *g* for 30 min, and the precipitate was discarded. Solid ammonium sulfate was then added with constant stirring to 75% saturation (516 g/liter of supernatant fluid). The resultant precipitate was collected by centrifugation at 17,500 × *g* for 30 min and suspended in a small volume of 40 mM potassium phosphate buffer (pH 7.0)–10 mM 2-mercaptoethanol. The suspension was then dialyzed overnight against the same buffer.

(iii) DEAE-cellulose chromatography. The dialyzed sample was applied to a DEAE-cellulose column (2.3 by 35 cm) equilibrated with 10 mM potassium phosphate buffer (pH 7.5)–10 mM 2-mercaptoethanol. Pyruvate kinase was eluted with a linear gradient created by mixing 250 ml of 10 mM potassium phosphate buffer (pH 7.5)–10 mM 2-mercaptoethanol and 250 ml of 40 mM potassium phosphate buffer (pH 7.0)–0.5 M KCl–10 mM 2-mercaptoethanol. Fractions with high activity were pooled. The enzyme was precipitated by ammonium sulfate (561 g/liter). The precipitate was then collected by centrifugation and suspended in 2 ml of 40 mM potassium phosphate buffer (pH 7.0)–10 mM 2-mercaptoethanol. The suspension was then dialyzed overnight against the same buffer.

(iv) Sephadex G-200 gel chromatography. The dialyzed preparation was placed on a Sephadex G-200 column (1.7 by 84 cm) equilibrated with 40 mM potassium phosphate buffer (pH 7.0)–10 mM 2-mercaptoethanol, and the column was eluted with the same buffer. Fractions with high activity were pooled. The enzyme was collected by the addition of ammonium sulfate to 80% saturation (561 g/liter) and suspended in 2 ml of 10 mM potassium phosphate buffer (pH 6.2)–10 mM 2-mercaptoethanol. The suspension was dialyzed overnight against the same buffer.

(v) Reactive blue 2-agarose affinity chromatography. A reactive blue 2-agarose column (1.7 by 17 cm) was equilibrated with 10 mM potassium phosphate buffer (pH 6.2)–10 mM 2-mercaptoethanol. The dialyzed preparation was applied to the column. After being washed with the same buffer, the column was developed with a linear gradient created by mixing 150 ml of 10 mM potassium phosphate buffer (pH 6.2)–10 mM 2-mercaptoethanol and 150 ml of 40 mM potassium phosphate buffer (pH 7.0)–1 M KCl–10 mM 2-mercaptoethanol. Fractions eluted from 0.25 to 0.35 M KCl were pooled and precipitated by the addition of 80% saturated ammonium sulfate (561 g/liter). The precipitate was collected by centrifugation and stored at 4°C.

Assay of pyruvate kinase activity. The standard assay mixture contained in 3 ml: 50 mM Tris-hydrochloride buffer, (pH 7.5), 100 mM KCl, 10 mM $MgCl_2$, 0.12 mM NADH, 10 mM 2-mercaptoethanol, 1.5 mM ADP, 1 mM PEP, 0.5 mM glucose 6-phosphate, 30 μg of lactate dehydrogenase and enzyme sample. The activity was estimated spectrophotometrically by recording the rate of oxidation of NADH at 340 nm. One unit of pyruvate kinase was defined as the amount of enzyme which catalyzed the formation of 1 μmol of pyruvate per min.

Assay of protein. The protein concentration was measured by the method of Lowry et al. with bovine serum albumin as the standard (18).

SDS-polyacrylamide gel electrophoresis. The purified enzyme and standard proteins were incubated at 100°C for 5 min in the presence of 1% SDS and 1% 2-mercaptoethanol and were applied to SDS-polyacrylamide gels at 8 mA per tube for 4 h by the method of Weber and Osborn (34). The gel rods were stained with Coomassie brilliant blue and calibrated with molecular weight standards: cytochrome *c* (molecular weight, 12,500), chymotrypsinogen A (molecular weight, 25,000), ovalbumin (molecular weight, 45,000), and bovine serum albumin (molecular weight, 68,000).

Gel filtration. The molecular weight of the native enzyme was estimated by gel filtration on a Sephadex G-200 column equilibrated with 40 mM potassium phosphate buffer, pH 7.0. The standard proteins used for calibration were cytochrome *c* (molecular weight, 12,500), bovine serum albumin (molecular weight, 68,000), aldolase (molecular weight, 158,000), catalase (molecular weight, 240,000), and ferritin (molecular weight, 450,000).

RESULTS

Purification of pyruvate kinase. Pyruvate kinase from *S. mutans* strain JC2 was purified 138-fold, with a recovery of 9.1% of the original activity (Table 1). The pyruvate kinase prepared by the above-mentioned procedure gave a single band on electrophoresis in SDS-polyacrylamide gels (Fig. 1). The purified enzyme could be stored at 4°C in the 40 mM potassium phosphate buffer (pH 7.0)–10 mM 2-mercaptoethanol without detectable loss of the activity for 3 months.

Optimum pH and the effect of P_i. The enzyme activity was measured in the pH range 5.5 to 8.3 with the standard assay mixture except 50 mM Tris-maleate buffer (pH 5.5 to 8.3) or 50 mM Tris-hydrochloride buffer (pH 6.8 to 8.0). The pH of the reaction mixture was examined immediately after the assay of the enzyme activity. The optimum activity was observed between pH 6.8 to 7.7 under the experimental conditions.

The stability of the enzyme was examined by dialyzing against various buffers containing 10 mM 2-mercaptoethanol at 4°C. Both the fully and partially purified enzyme preparations were completely inactivated by dialysis against 40 mM Tris-hydrochloride buffer (pH 7.0), 40 mM Tris-maleate buffer (pH 7.0), and 40 mM morpholinepropanesulfonic acid NaOH buffer (pH

TABLE 1. Summary of the purification procedure of pyruvate kinase from *S. mutans* strain JC2

Purification step	Total activity (U)	Total protein (mg)	Sp act (U/mg)	Purification (fold)
Cell extract	1,128	1,312	0.86	
Protamine salfate treatment	1,105	526.2	2.1	2.4
DEAE-cellulose eluate	445	13.3	33.4	38.8
Sephadex G-200 eluate	297	3.18	93.4	109
Reactive blue 2-agarose eluate	103	0.87	118	138

7.0) for 20 h, respectively. The activities of both of these preparations, however, were retained even after a 20-h dialysis against 40 mM potassium phosphate buffer, pH 7.0. Moreover, P_i partially reactivated the inactivated enzyme both in the crude and partially purified preparations although the fully purified enzyme was not reactivated at all. When the inactivated enzyme from the partially purified preparation (Sephadex G-200) had been incubated with 80 mM P_i, 40% of the initial activity was recovered after a 2-h incubation at room temperature.

In this study, the concentration of P_i introduced into the enzyme assay mixture by the pyruvate kinase preparation was less than 0.3 mM. In the presence of 0.5 mM glucose 6-phosphate, addition of 0.13 and 1.0 mM P_i resulted in the same activity. Thus, inhibition by <1.0 mM P_i is considered to be negligible.

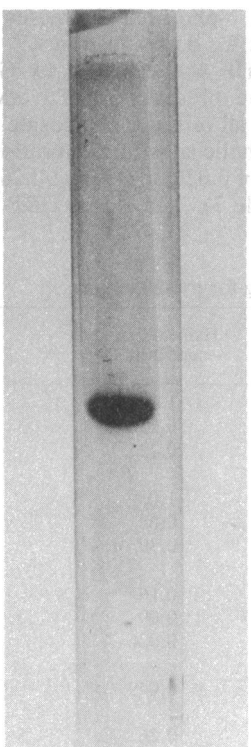

FIG. 1. SDS-polyacrylamide gel electrophoresis of the purified pyruvate kinase from *S. mutans* strain JC2. Experimental details are in the text. A sample containing 12 μg of protein was applied to the gel.

In addition to its stabilizing effect, P_i (at high concentrations) strongly inhibited pyruvate kinase. This inhibitory effect was largely influenced by the concentration of the activator glucose 6-phosphate (Fig. 2). When the concentration of P_i was increased, more glucose 6-phosphate was required for activation. A high concentration of P_i (3.3 mM) altered the half-saturating concentration of glucose 6-phosphate from 0.04 mM (at 0.13 mM P_i) to 0.28 mM and altered the Hill coefficient from 1.7 to 2.6.

Molecular weight and subunit structure. The molecular weight of the native enzyme was estimated to be 180,000 to 190,000 by molecular sieving through a Sephadex G-200 column.

The mobility of the dissociated protein on the electrophoresis in SDS-polyacrylamide gels corresponded to a subunit molecular weight of 44,000. Thus, the enzyme was considered to consist of four identical subunits.

Activation of pyruvate kinase. Purified pyruvate kinase from *S. mutans* strain JC2 had a specific requirement for glucose 6-phosphate. Without glucose 6-phosphate, no activity was

TABLE 2. Requirements of monovalent and divalent cations for pyruvate kinase activity

Monovalent cations (100 mM)	Divalent cations (10 mM)	Relative activity[a] (%)
KCl	$MgCl_2$	100
NH_4Cl	$MgCl_2$	88.9
none	$MgCl_2$	0.0
NaCl	$MgCl_2$	0.0
LiCl	$MgCl_2$	0.0
RbCl	$MgCl_2$	73.3
KCl	$MnCl_2$	94.4
none	$MnCl_2$	0.0
KCl	$CaCl_2$	0.0
KCl	none	0.0
NH_4Cl	none	0.0

[a] The standard assay mixture was used, except that overnight-dialyzed lactate dehydrogenase against 50 mM Tris-hydrochloride buffer (pH 7.5) was used, and some components were deleted and added as indicated. The purified enzyme (0.12 μg/ml) was present in the reaction mixture.

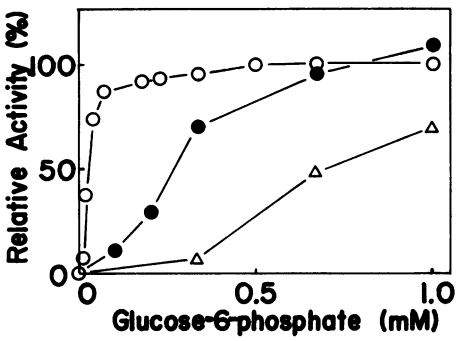

FIG. 2. Pyruvate kinase dependence upon glucose 6-phosphate in the presence of 0.13 mM Pi (○), 3.3 mM Pi (●), and 6.7 mM Pi (△). The assay mixture was the same as described in the text except for glucose 6-phosphate and P_i. The purified enzyme (0.12 μg/ml) was contained in the reaction mixture. The ordinate shows the percentage of activity, with the activity with 1 mM glucose 6-phosphate and 0.13 mM Pi (118 U/mg) as 100.

found (Fig. 2). One millimolar each fructose 1,6-bisphosphate, glucose 1,6-bisphosphate, ribulose 5-phosphate, glyceraldehyde 3-phosphate, 5'-AMP, 5'-GMP, 5'-IMP, 2', 3'-AMP, 2', 3'-GMP or 2', 3'-UMP did not affect the purified enzyme. Ribose 5-phosphate partially activated the purified enzyme. The fructose 6-phosphate reagent also activated pyruvate kinase, but this could be explained by the presence of 2% glucose 6-phosphate in that reagent.

The half-saturation concentrations of glucose 6-phosphate and ribose 5-phosphate in the presence of 1 mM ADP were 0.04 and 0.41 mM, respectively. GDP was the best phosphate acceptor among the nucleoside diphosphates studied (Table 3). When 1 mM GDP replaced 1 mM ADP, 0.011 mM glucose 6-phosphate, and 0.095 mM ribose 5-phosphate, respectively, it gave half of the maximum velocity. In either case, glucose 6-phosphate was 9 to 10 times more effective than ribose 5-phosphate.

Effect of divalent and monovalent cations. Pyruvate kinase completely required K^+ (K_m, 37 mM) or NH_4^+ and Mg^{2+} or Mn^{2+} (Table 2), but it was not activated by either Na^+ or Ca^{2+}. Rb^+ was not as effective as K^+ or NH_4^+.

Substrate kinetics of pyruvate kinase. The substrate saturation curve for PEP was sigmoidal (Hill coefficient, 2.3; half-saturating concentration, 1.1 mM) at a low glucose 6-phosphate concentration (0.05 mM), but it changed to a hyperbolic shape (Hill coefficient, 1.0; K_m, 0.22 mM) at a high glucose 6-phosphate concentration (0.5 mM) (Table 3). Similarly, with 0.05 mM glucose 6-phosphate, the substrate saturation curve for ADP was sigmoidal (Hill coefficient, 2.5; half-saturating concentration, 1.0 mM) and at a saturating concentration of 0.5 mM glucose 6-phosphate, the enzyme showed hyperbolic kinetics (Hill coefficient, 1.0) against ADP, with a K_m of 0.39 mM (Table 3). These results indicate that glucose 6-phosphate increases the affinity of the enzyme for both substrates.

When ADP was replaced by GDP, IDP, or UDP in the presence of 0.5 mM glucose 6-phosphate, all of these nucleoside diphosphates gave hyperbolic substrate saturation curves with K_m values of 0.026, 0.049, and 0.26 mM, respectively (Table 3). The K_m of GDP was 0.067 of

TABLE 3. Kinetic parameters of substrate binding for pyruvate kinase[a]

Variable substrate	Glucose 6-phosphate (mM)	Hill coefficient[b]	Half-saturating concn (mM)	V_{max}[c] (U/mg)
PEP	0.05	2.3	1.1	39
	0.1	1.7	0.65	64
	0.5	1.0	0.22	125
ADP	0.05	2.5	1.0	17
	0.1	1.4	0.68	55
	0.5	1.0	0.39	120
GDP	0.05	1.8	0.16	73
	0.1	1.4	0.09	98
	0.5	0.9	0.026	149
IDP	0.5	0.9	0.049	138
UDP	0.5	1.0	0.26	125

[a] The standard assay mixture was used, except that substrates and glucose 6-phosphate were used as indicated. The purified enzyme (0.12 μg/ml) was contained in the reaction mixture.
[b] Hill coefficients were determined graphically from Hill plots.
[c] The maximum velocities were obtained from Lineweaver-Burk plots.

that of ADP. When TDP or CDP (1.5 mM each) was substituted for ADP, an activity of 2.3 and 16.7%, respectively, was observed.

At 0.5 mM ADP, pyruvate kinase was only 20% inhibited by 3 mM ATP.

DISCUSSION

E. coli has two forms of pyruvate kinase (9, 14, 20). One form is activated by fructose 1,6-bisphosphate (31, 33), and the other form is activated by AMP or ribose 5-phosphate (28, 32). In contrast, *S. mutans* strain JC2 had only one pyruvate kinase, which was completely dependent on glucose 6-phosphate for activity (Fig. 2; 36). The enzyme from *S. mutans* was not affected by any nucleoside monophosphate studied.

Fructose 1,6-bisphosphate-activated pyruvate kinases from *E. coli* (33) and *S. lactis* (7) have a molecular weight of about 240,000, but the molecular weight of the enzyme from *S. mutans* strain JC2 (180,000 to 190,000) was similar to that of ribose 5-phosphate-activated pyruvate kinase from *E. coli* (28, 32). The enzyme from *S. mutans* was found to be composed of four identical subunits, in accordance with other bacterial pyruvate kinases, including the ones activated by ribose 5-phosphate.

Similar to ribose 5-phosphate-activated pyruvate kinase from *E. coli* (22) and other bacterial pyruvate kinases (6, 16, 27, 32), the enzyme from *S. mutans* was strongly inhibited by P_i (Fig. 2), but it should be noted that the enzyme was immediately inactivated in a solution without P_i. When a crude preparation of the inactivated enzyme was incubated with P_i, the activity was partially recovered with time, but the purified enzyme was not recovered by the addition of P_i. The mechanism of reactivation was not clarified.

Various nucleoside diphosphates could be substituted for ADP as a phosphate acceptor. Pyruvate kinase from *Propionibacterium shermanii* is also activated by glucose 6-phosphate, but has some catalytic activity without an activator and also differs in a number of other properties; that is, it does not require monovalent cations for activity, and GDP is an inferior substitute for ADP (27). Thus, pyruvate kinase from *S. mutans* seemed to have unique regulatory characteristics among pyruvate kinases from microorganisms.

Like the fructose 1,6-bisphosphate–activated enzymes from *E. coli* (33) and *S. lactis* (7), the enzyme from *S. mutans* could use GDP as an effective phosphate acceptor (Table 3). IDP or UDP could also replace ADP, but the best phosphate acceptor for ribose 5-phosphate–activated pyruvate kinase is ADP (32). These results suggest that pyruvate kinase may be involved in the regeneration of not only ATP but also other nucleoside triphosphates including GTP, ITP, and UTP in *S. mutans*. Regeneration of GTP could be especially important because it is required for protein synthesis. *S. mutans* has no citric acid cycle (2, 35), and GTP should not be regenerated by succinyl coenzyme A synthetase (GTP-forming) in this microorganism.

Divalent cations, Mg^{2+} or Mn^{2+}, are known to be essential to pyruvate kinase activity, but monovalent cations are not necessarily essential for any of the two types of pyruvate kinases from *E. coli* (32, 33) and other bacterial pyruvate kinases (4, 27). Pyruvate kinase from *S. mutans* required not only divalent cations, Mg^{2+} or Mn^{2+}, but also monovalent cations, K^+ or NH_4^+ (Table 2).

Yamada and Carlsson have reported that the intracellular level of PEP is high and the level of glucose 6-phosphate is low when *S. mutans* JC2 is grown in a glucose-limited continuous culture (36). On the contrary, the level of PEP is low and the level of glucose 6-phosphate is high when *S. mutans* JC2 is grown under nitrogen limitation in an excess of glucose. Moreover, the level of P_i in *S. mutans* is more than 20 mM (12). Therefore, pyruvate kinase of *S. mutans* is expected to be exposed to inhibition by P_i in vivo. Strong inhibition by P_i (Fig. 2) will result in PEP accumulation in cells growing under glucose limitation, and the accumulated PEP will facilitate the transport of carbohydrates into the cells by the PEP-phosphotransferase system (25, 26, 30). This regulation of glycolysis may be an ecological advantage to *S. mutans* in dental plaque when low concentrations of sugar are available. In the presence of an excess of sugar, an increase of the intracellular pool of glucose 6-phosphate may overcome inhibition by P_i. In this manner, the intracellular concentration of glucose 6-phosphate in cooperation with that of P_i may efficiently regulate pyruvate kinase activity in *S. mutans*. Actually, Iwami and Yamada have reported that the mass action ratio of the reaction catalyzed by pyruvate kinase is far from equilibrium, and the reaction is considered to be the most important rate-limiting step in the glycolytic pathway (12).

The inhibition of the glycolytic rate of *S. mutans* by P_i (11) is sometimes ascribed to the inhibitory effect of P_i on lactate dehydrogenase (1), but the inhibition of P_i has been shown to be more powerful on pyruvate kinase than on lactate dehydrogenase of *S. mutans* (1, 37). Therefore, a high concentration of P_i is considered to decrease the glycolytic rate by inhibiting pyruvate kinase rather than lactate dehydrogenase in *S. mutans*.

However, further intensive research is required to clarify the role of P_i in the regulation of

sugar metabolism in *S. mutans*. During the preparation of this manuscript Mason et al. have also proposed the importance of P_i in the regulation of glucose metabolism of *S. lactis* (21).

Since the phosphotransferase system is responsible for catabolite repression in *E. coli* (24), it might be involved in the induction of pyruvate formate-lyase (38), for example, in *S. mutans*. So, pyruvate kinase is considered to be involved not only in the rate of glycolysis but also in the transport of sugar and the induction of enzymes in *S. mutans*.

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