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3-Phosphoglycerate Kinase from *Hydrogenomonas facilis*

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Phosphoglycerate kinase levels in *Hydrogenomonas facilis* were reasonably constant whether cells were utilizing or synthesizing hexose during growth. Specific enzyme activities (micromoles of 3-phosphoglycerate disappearing per minute per milligram of protein) at 30 C were 0.234, 0.391, 0.300, and 0.229 in the "soluble" fraction derived from cells grown on fructose, lactate, succinate, and glutamate, respectively. The enzyme was purified 300-fold from succinate-grown cells. The final preparation, which was not homogenous but was free from glyceraldehyde-3-phosphate dehydrogenase and adenylate kinase, had a specific activity at 30 C of 90 μmoles of 3-phosphoglycerate per min per mg of protein. K_m values for adenosine triphosphate (ATP), 3-phosphoglycerate, and Mg^{++} were 0.16, 0.83, and 0.4 mM, respectively, at *p*H 7.4 and 30 C. Adenosine monophosphate (AMP) inhibited 23% at a ratio of AMP to ATP of 2.4, and the possible physiological implications of this inhibition are discussed. No evidence was found for an enzyme which catalyzes ATP-dependent conversion of 3-phosphoglycerate to 1,3-diphosphoglycerate, AMP, and phosphate.

The hydrogen bacteria are aerobic, facultative chemoautotrophs (25, 27) which oxidize molecular hydrogen and assimilate carbon dioxide via the reductive pentose phosphate cycle (4, 14-16, 22), or grow heterotrophically on a number of carbon sources including organic acids and hexoses (27). In the latter case, the Entner-Doudoroff pathway is functional (12, 19).

During autotrophic growth or growth on hexose, phosphoglycerate kinase [EC 2.7.2.3; adenosine triphosphate (ATP):3-phospho-D-glycerate 1-phosphotransferase] is detectable (12, 28, 29). In the course of hexose degradation, it contributes to conversion of glyceraldehyde-3-phosphate to pyruvate. In contrast, during autotrophic growth and during growth on lactate, glutamate, or succinate, metabolite flow into the pathways of CO_2 fixation and hexose phosphate synthesis is in the opposite and apparently less thermodynamically favored direction, i.e., from 3-phosphoglycerate (3-*P*-glycerate) to 1,3-diphosphoglycerate (1,3-P_2-glycerate; $\Delta G_0' = +4.75$ kcal/mole; see 8). Thus, phosphoglycerate kinase may catalyze net flow towards D-glyceraldehyde-3-phosphate only if the concentration ratio of 3-*P*-glycerate to 1,3-P_2-glycerate is of the order of 1,000.

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In the face of these considerations, scrutiny of the enzyme(s) catalyzing the interconversion of 3-*P*-glycerate and 1,3-P_2-glycerate is of interest. The present communication describes studies of the kinetics of heat inactivation of phosphoglycerate kinase in the soluble fraction from *Hydrogenomonas facilis*, its extensive purification, and inhibition by adenosine monophosphate (AMP).

MATERIALS AND METHODS

Organism and cultivation. *H. facilis* is a subculture of the original strain isolated by Schatz and Bovell (26). It was grown in a 0.01% yeast extract-mineral salts medium containing, per liter: 3.6 g of Na_2HPO_4, 1.5 g of KH_2PO_4, 1.5 g of NH_4Cl, 0.2 g of $MgSO_4$, 10 mg of $CaCl_2$, 5 mg of ferric ammonium citrate, 1.5 mg of $NiSO_4$, 1 mg each of $Na_2B_4O_7 \cdot 10H_2O$, MoO_3, $CuSO_4$, $ZnSO_4 \cdot 7H_2O$, $CoCl_2 \cdot 6H_2O$, KI, and NH_4VO_3, and 0.5 g of $NaHCO_3$. For maintaining autotrophic stock cultures on slants, the medium, from which yeast extract had been omitted, was solidified with 2% agar, inoculated, and incubated at 30 C under an atmosphere of 70% H_2, 20% air, and 10% CO_2. Except as noted, heterotrophic cultures were grown at 30 C on a rotary shaker in the mineral salts medium to which 2.0 g of sodium succinate per liter or 3.0 g of lactic acid (neutralized) per liter had been added.

The cells were harvested with a Sharples centrifuge, washed with 0.01 M potassium phosphate containing 0.01 M $MgSO_4$ (*p*H 7.0), brought to a 70% cell

suspension with the same buffer, and stored at −0 C.

Preparation of extracts. Cell-free extracts were prepared by disintegrating the cells at 2 C in a French pressure cell at a pressure of 15,000 psi and centrifuging the homogenate for 60 min at 12,000 × *g*. The supernatant fluid was again centrifuged for 60 min at 105,000 × *g*, and the resulting high-speed supernatant (HSS) fraction containing about 47 mg of protein/ml (21) was used in the purification procedure.

Purification of phosphoglycerate kinase activity. The HSS fraction was subjected to heat treatment for 6 to 10 min at 50 C. After removal of the precipitate by centrifugation, 0.01 ml of $MnCl_2$ solution (1 g/ml) per ml of protein solution was added at 2 C to remove nucleic acids. The precipitate was removed by centrifugation, and the supernatant fraction of 39 ml (31 mg of protein/ml) was dialyzed overnight at 2 C against 100 volumes of 0.01 M potassium phosphate containing 0.01 M $MgSO_4$, *p*H 7.0. To the dialyzed solution at a *p*H of 7.0, crystalline ammonium sulfate was added (2.92 g/10 ml of solution) to 0.5 saturation (at 2 C), and the precipitate was removed by centrifugation and discarded. To the supernatant fraction of 45 ml, ammonium sulfate was added again (0.74 g/10 ml of solution) to bring it to 0.61 saturation (at 2 C). The precipitate containing most of the phosphoglycerate kinase activity was redissolved in 4 ml of the 0.01 M phosphate-Mg^{++} buffer, *p*H 7.0, and a small portion was used in part of the kinetic studies. The remainder (ca. 3.9 ml) was further fractionated on a Sephadex (Pharmacia, Uppsala, Sweden) G-150 column (1.5 by 80 cm) equilibrated at 4 C in the 0.01 M phosphate-Mg^{++} buffer, *p*H 7.0. Elution was conducted with the same buffer, and 3.6-ml fractions were collected. Phosphoglycerate kinase activity was recovered at an elution volume of 55 to 95 ml.

The two most active fractions were combined, and 6.0 ml was loaded onto a diethylaminoethyl (DEAE) cellulose (Carl Schleicher & Schuell Co.) column (0.9 by 30 cm) equilibrated at 4 C in 0.01 M phosphate buffer, *p*H 7.0. The protein was eluted at 4 C with the same buffer until the first ultraviolet (UV)-absorbing peak came off the column (ca. 20 ml). Then an increasing linear gradient of NaCl in 0.01 M phosphate, *p*H 7.0, was applied to a limit of 0.2 M NaCl. The phosphoglycerate kinase activity was recovered at an NaCl concentration of 0.057 to 0.068 M in a symmetrical peak of UV-absorbing effluent.

The enzyme solution through Sephadex fractionation was stored at 2 C at various stages of the procedure without loss of activity. The final enzyme preparation was less stable, losing about 70% of the activity after 11 days at 2 C.

Enzyme assay procedures. Phosphoglycerate kinase was measured routinely (29) in a test (assay I) in which it was coupled with D-glyceraldehyde-3-phosphate dehydrogenase. The oxidation of reduced nicotinamide adenine dinucleotide (NADH) resulting in a decrease in optical density at 340 nm was followed with a Gilford-modified Beckman DU spectrophotometer and was recorded by a Photovolt recorder. The composition of an optimal assay mixture was established to contain: 125 μmoles of triethanolamine (TEA)-hydrochloride buffer, *p*H 7.4; 1.35 μmoles of ethylenediaminetetraacetate, *p*H 7.3; 20 μmoles of $MgSO_4 \cdot 7H_2O$; 0.7 μmole of NADH; 7.2 μmoles of ATP; 31 μmoles of 3-*P*-glycerate; excess muscle D-glyceraldehyde-3-phosphate dehydrogenase (about 8 units, from Sigma Chemical Co.); and 5 to 20 μliters of enzyme solution in a total volume of 3.0 ml. The temperature was maintained at 30 C.

The formation of 1,3-P_2-glycerate from 3-*P*-glycerate was also measured (assay II) by converting the acyl phosphate to the hydroxamate, which was determined as the ferric ion-hydroxamic acid complex at 490 nm (2, 20). The assay mixture contained, in a total volume of 2.0 ml: 65 μmoles of TEA-hydrochloride buffer, *p*H 7.4; 1,000 μmoles of hydroxylamine-HCl, *p*H 7.4; 15 μmoles of $MgSO_4 \cdot 7H_2O$; 15.5 μmoles of 3-*P*-glycerate; 12 μmoles of ATP; and 10 to 50 μliters of enzyme solution. After 30 min of incubation at 30 C, 3 ml of a mixture of equal volume of 2.5 N HCl, 12% (w/v) trichloroacetic acid, and 5% (w/v) $FeCl_3$ solution was added, the suspension was clarified by centrifugation, and the optical density of the supernatant fluid was measured at 490 nm against a control from which only the protein had been omitted.

D-Glyceraldehyde-3-phosphate dehydrogenase was measured at 30 C in a coupled optical test (3) with 3-*P*-glycerate and ATP as substrate and an excess of yeast phosphoglycerate kinase obtained from Boehringer Mannheim Corp.

Adenylate kinase was measured at 30 C by the method of Oliver (24). α-Glycerophosphate dehydrogenase was assayed in a reaction coupled with commercial triosephosphate isomerase and aldolase acting upon fructose-1,6-diphosphate (3).

NADH oxidase was measured under the conditions of phosphoglycerate kinase assay I except that D-glyceraldehyde-3-phosphate dehydrogenase and 3-*P*-glycerate were omitted from the reaction mixture and a 10-fold higher protein concentration was used.

Enzyme units. For all activities specified, a unit of enzyme catalyzes the formation of 1 μmole of product per minute, and specific activity equals units per milligram of protein.

RESULTS

Levels of 3-P-glycerate kinase after different modes of growth. When *H. facilis* was grown under the conditions described upon 0.6% fructose, 1.2% lactic acid (neutralized), 0.8% disodium succinate, or 0.8% glutamate, the HSS fraction contained 3-*P*-glycerate kinase at specific activities of 0.234, 0.391, 0.300, and 0.229, respectively. Each of these values is corrected for specific activities of NADH oxidase, which ranged from 0.009 to 0.024 in the HSS fraction.

Heat treatment of 3-P-glycerate kinase in HSS from succinate-grown H. facilis. The phosphoglycerate kinase activity in the HSS fraction from *H. facilis* was shown to be heat-labile. In early experiments, 95% of the ac-

tivity was lost after 3 min at 55 C in 0.05 M phosphate, *p*H 7.0. However, higher recoveries were obtained after 30 min at 50 C. Under the latter conditions, kinetics of inactivation were biphasic.

In a more detailed study, we tried to find conditions under which the enzyme was completely stable at 50 C. Samples of HSS fraction that had been equilibrated with 0.01 M TEA-hydrochloride, *p*H 7.5, or 0.01 M phosphate, *p*H 7.5, were heated to 50 C for 10 min in the presence of Mg^{++}, 3-*P*-glycerate, or ATP, and combinations of these three components, each at 10 mM. To control the concentrations accurately, the HSS fraction was dialyzed to equilibrium in the appropriate Mg^{++}-free or Mg^{++}-containing buffers. 3-*P*-glycerate, ATP, or both were added to samples of the dialyzed protein solution before heating. After heating, denatured protein was removed by centrifugation and the activity recovered (assay I) in the supernatant fluid was estimated and compared with the original activity. The results listed in Table 1 demonstrate a cumulative protection against heat inactivation by Mg^{++}, phosphate, 3-*P*-glycerate, and ATP. When all four factors were present simultaneously during heating at 50 C for 10 min, about 70% of the activity was recovered.

Inactivation at 50 C of 3-*P*-glycerate kinase (assay I) in the HSS fraction (Fig. 1) was rapid and was complete in 30 min when the protein was dissolved in 0.01 M TEA-hydrochloride buffer (curve A). When Mg^{++} was added to this buffer (curve B) or a phosphate buffer containing Mg^{++} was used in its place (curve C),

TABLE 1. *Recovery of 3-phosphoglycerate kinase activity in the HSS function[a] from succinate-grown H. facilis after heating to 50 C for 10 min in various media*

Buffer	Supplements (each at 10 mM)			Recovered activity (%)
TEA-HCl buffer, *p*H 7.5, 0.01 M	—	—	—	8
	Mg^{++}	—	—	23
	Mg^{++}	3-PGA[b]	—	36
	Mg^{++}	—	ATP	51
	Mg^{++}	3-PGA	ATP	55
Phosphate buffer, *p*H 7.5, 0.01 M	Mg^{++}	—	—	44
	Mg^{++}	3-PGA	—	59
	Mg^{++}	—	ATP	65
	Mg^{++}	3-PGA	ATP	68

[a] Protein concentration, 30 mg/ml.
[b] 3-Phosphoglycerate.

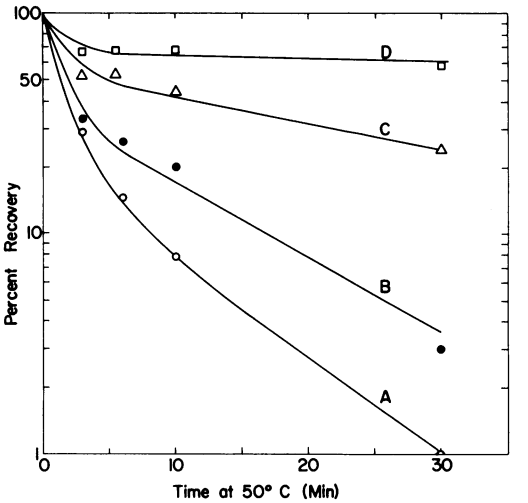

FIG. 1. *Kinetics of heat inactivation at 50 C and pH 7.5 of 3-phosphoglycerate kinase in the HSS fraction (30 mg of protein/ml) from succinate-grown H. facilis. Buffers at 0.01 M contained additional components each at 10 mM. Relevant compositions were as follows: curve A, TEA-HCl buffer; B, TEA-HCl buffer, Mg^{++}; C, phosphate buffer, Mg^{++}; and D, phosphate buffer, Mg^{++}, ATP, 3-phosphoglycerate.*

the rates of activity decline were smaller. The greatest protection was obtained in the presence of Mg^{++}, ATP, and 3-*P*-glycerate (curve D). Indeed, a fraction of the activity was relatively stable in the presence of these three components.

One plausible explanation for these inactivation kinetics is that there are at least two active components. Accordingly, one would be rapidly inactivated under all conditions tested, and the second would be heat-labile in the absence of Mg^{++} but increasingly stabilized by the presence of Mg^{++}, phosphate, 3-*P*-glycerate, and ATP. We have tried without success to separate two phosphoglycerate kinase activities in extracts from *H. facilis* grown on succinate or lactate using fractionation of HSS directly by acetone, ammonium sulfate, or Sephadex G-150.

Purification of phosphoglycerate kinase. The 3-*P*-glycerate kinase activity (assay I) of succinate-grown *H. facilis* was purified by means of ultracentrifugation, heat treatment, precipitation of nucleic acids by Mn^{++}, ammonium sulfate fractionation, gel filtration, and DEAE cellulose chromatography (Table 2).

Precipitation of nucleic acid with Mn^{++} was finally employed because treatment with protamine sulfate or streptomycin sulfate failed to improve the ratio of protein to nucleic acid.

TABLE 2. *Purification of 3-phosphoglycerate kinase from 60 g (wet packed) of succinate-grown H. facilis cells*

Fraction	Specific activity[a]	Purification	Units	Recovery (%)
I. High speed supernatant	0.31	1.0	720	100
II. Heat treatment, 50 C for 6 min	0.39	1.3	464	64
III. Mn^{++} precipitation, dialysis, precipitation with $(NH_4)_2SO_4$ (0.51 to 0.61 saturation)	1.1	3.5	252	35
IV. Sephadex G-150 fractionation	3.0	9.7	388	54
V. DEAE cellulose chromatography	90	290	140	20

[a] The values shown (in micromoles per minute per milligram of protein) were obtained after correction as necessary for the presence of NADH oxidase.

The supernatant fluid from the Mn^{++} precipitation was dialyzed immediately and then used in ammonium sulfate fractionation. The precipitate obtained in the range of 0.51 to 0.61 saturation with respect to ammonium sulfate contained the bulk of the 3-*P*-glycerate kinase activity, although the next precipitate (0.61 to 0.69 saturation) contained approximately half the amount of activity of the major fraction. Gel filtration of the major fraction resulted in an increase in enzyme units, suggesting the removal of a macromolecular inhibitor. At that stage, 10-fold purification with an enzyme recovery of 54% (388 units) was obtained considering the contents of five peak tubes which ranged in specific activity from 2.4 to 3.3. Further chromatography on DEAE cellulose resulted in added purification of about 30-fold with a recovery of 140 units (overall recovery, 20%). Polyacrylamide gels loaded with the peak kinase-containing fractions from chromatography on DEAE cellulose and submitted to electrophoresis at *p*H 9.5 (10) revealed two protein bands.

During the course of purification, loss of several other enzyme activities from the 3-*P*-glycerate kinase fractions was observed. NADH oxidase, which was present in the HSS fraction with a specific activity of 0.003 to 0.01, was only removed during the fractionation on Sephadex gel. Fraction III contained traces of adenylate kinase and the final product (V) was free from that activity. NAD^+-linked α-glycerophosphate dehydrogenase was not detected in the HSS fraction. D-Glyceraldehyde-3-phosphate dehydrogenase was closely associated with phosphoglycerate kinase activity through early purification steps. However, Sephadex filtration separated both activities almost completely. The preparation resulting from DEAE cellulose chromatography contained D-glyceraldehyde-3-phosphate dehydrogenase in amounts less than 1% of the kinase activity.

It is important to note that with the HSS fraction omission of the hydroxylamine during incubation (assay II) followed by hydroxylamine addition yielded less than 1% as much hydroxamate as was found with the normal assay in which hydroxamate was formed continually. This provides evidence against the presence of an enzyme in the HSS fraction which catalyzes phosphorylation of 3-*P*-glycerate with formation of AMP and inorganic orthophosphate instead of adenosine diphosphate (ADP).

Kinetic studies. AMP was shown to inhibit the reaction:

$$3\text{-}P\text{-}glycerate^{-3} + ATP^{-4} \rightarrow 1,3\text{-diphosphoglycerate}^{-4} + ADP^{-3}$$

catalyzed by the final phosphoglycerate kinase preparation from the DEAE cellulose column known to be adenylate kinase-free. Results with the hydroxylamine procedure (assay II) are shown in Table 3.

Inhibition by AMP was confirmed by use of assay procedure I which involved coupling of endogenous kinase with exogenous excess D-glyceraldehyde-3-phosphate dehydrogenase. At various AMP to ATP ratios, the degree of inhibition was similar to that observed with assay II. For example, at a ratio of 10, 60% inhibition was observed under conditions where the rate was dependent upon endogenous kinase units, establishing that the locus of inhibition was 3-

TABLE 3. *Adenylate inhibition of the reaction catalyzed by phosphoglycerate kinase*

Molar ratio of AMP to ATP	Activity[a] (%)
0	(100)
0.2	104
0.6	109
1.0	96
2.4	77
3	74
6	60
12	26

[a] The activity was measured as ΔA_{490}/30 min as described with the use of 0.64 μg of protein and an ATP concentration of 6 mM.

P-glycerate kinase. Figure 2 shows a double reciprocal plot of ATP concentration versus velocity obtained with the final preparation (fraction V) in the presence and absence of AMP by use of assay procedure I. The inhibition is not competitive. The Michaelis constant (K_m) for ATP is 0.16 mM (11). At ATP concentrations of 10 mM, an apparent substrate inhibition was observed both in the presence and in the absence of AMP (Fig. 2), which may have only reflected removal of Mg^{++} (present at 5 mM).

A double reciprocal plot of 3-*P*-glycerate concentrations versus velocity at several AMP concentrations is given in Fig. 3. The inhibition is of a mixed type and increases with increasing AMP concentration. The K_m for 3-*P*-glycerate is 0.83 mM.

A double reciprocal plot relating concentration of Mg^{++} to velocity is linear (not shown), and a K_m of 0.4 mM at 30 C and *p*H 7.4 is obtained for Mg^{++}.

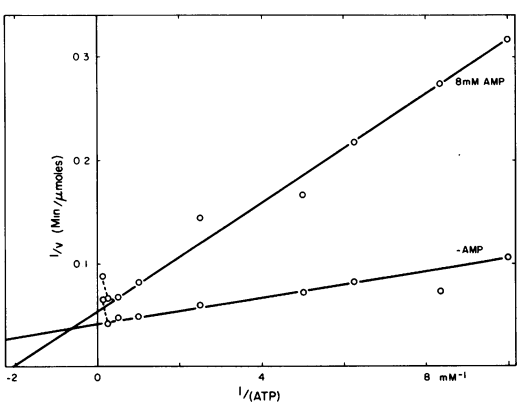

FIG. 2. *Double reciprocal plot of phosphoglycerate kinase activity (assay I) against ATP concentration in the absence and presence of AMP. The assay mixture contained, in 3 ml: 125 μmoles of TEA-HCl buffer, pH 7.4; 1.35 μmoles of ethylenediaminetetraacetate, pH 7.3; 15 μmoles of Mg^{++}; 31 μmoles of 3-phosphoglycerate; 0.7 μmoles of NADH; 200 μg of D-glyceraldehyde-3-phosphate dehydrogenase; AMP and ATP as indicated; and 1.6 and 3.2 μg of protein in mixtures without AMP and with AMP, respectively. The activity was measured as ΔA_{340}/min, and rates are corrected for the differences in protein content. For other details see Materials and Methods.*

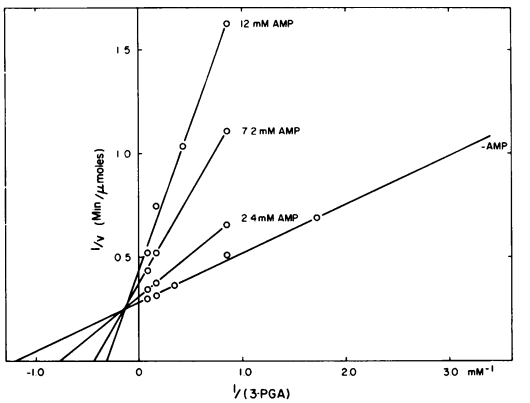

FIG. 3. *Double reciprocal plots of phosphoglycerate kinase activity against concentration of 3-phosphoglycerate (3-PGA) in the absence and presence of AMP at the concentration shown. The enzyme employed had been purified fivefold from lactate-grown cells and corresponded to fraction III. For assay conditions, see legend for Fig. 2. The concentration of ATP was 2.4 mM.*

DISCUSSION

3-*P*-glycerate kinase has been purified from several sources. It has been crystallized from yeast (6, 7), human erythrocytes (13), and skeletal muscle (30) after 60-, 350-, and 38-fold purification, respectively. The pure yeast and skeletal muscle enzymes have been compared (18).

In spite of the central importance to carbon dioxide assimilation and sugar metabolism, investigations of the enzyme purified from bacteria have been largely lacking. The 300-fold purification from a facultative autotroph, *H. facilis*, described here results in an enzyme preparation free from common contaminating enzymes such as D-glyceraldehyde-3-phosphate dehydrogenase and adenylate kinase. The apparent K_m values at 30 C for ATP, 3-*P*-glycerate, and Mg^{++} are 0.16, 0.83, and 0.4 mM, respectively, at a *p*H of 7.4 at which phosphorylated substrates are almost completely ionized. These values are similar to those at 25 C for the yeast enzyme (6), but in neither case have the kinetic constants been corrected for metal ion complexes with substrates.

In the present work, no evidence was found for appreciable variation in the level of 3-*P*-glycerate kinase in cells utilizing or synthesizing hexose during heterotrophic growth. Moreover, no evidence was found for additional enzymes catalyzing the ATP-dependent conversion of 3-*P*-glycerate to 1,3-P_2-glycerate under conditions that would have specifically identified the formation of the diphosphate in an uncoupled reaction. Thus, it seems unlikely that there is a 1,3-P_2-glycerate synthase in *Hydrogenomonas* analogous to phosphoenolpyruvate synthase (5, 9). Instead, it seems likely that the concentration ratio of 3-*P*- glycerate to 1,3-P_2-glycerate fluctuates sufficiently when the metabolism shifts from hexose degra-

dation to gluconeogenesis to insure thermodynamic feasibility. In this connection, the inhibition of the kinase in the direction of 1,3-P_2-glycerate synthesis by AMP may be of physiological significance. Thus, as the cellular energy charge (1) decreases, compensatory conservation of ATP would be ensured regardless of the mechanism of action of AMP, which was not defined in the present work. Although significant inhibition by AMP occurred at a ratio of 2.4 for AMP to ATP, a value well removed from apparent physiological ratios (23), Atkinson and co-workers have discovered profound sensitization to decreasing energy charge of a biosynthetic enzyme, phosphoribosyladenosine triphosphate synthetase, in the presence of its end product histidine (17). It will be interesting to examine terminal intermediates of gluconeogenesis to probe for analogous synergism with energy charge in regulating the activity of 3-*P*-glycerate kinase.

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