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Phosphorylation of Glycerol and Dihydroxyacetone in *Acetobacter xylinum* and Its Possible Regulatory Role

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Extracts of *Acetobacter xylinum* catalyze the phosphorylation of glycerol and dihydroxyacetone (DHA) by adenosine 5'-triphosphate (ATP) to form, respectively, L-α-glycerophosphate and DHA phosphate. The ability to promote phosphorylation of glycerol and DHA was higher in glycerol-grown cells than in glucose- or succinate-grown cells. The activity of glycerol kinase in extracts is compatible with the overall rate of glycerol oxidation in vivo. The glycerol-DHA kinase has been purified 210-fold from extracts, and its molecular weight was determined to be 50,000 by gel filtration. The glycerol kinase to DHA kinase activity ratio remained essentially constant at 1.6 at all stages of purification. The optimal pH for both reactions was 8.4 to 9.2. Reaction rates with the purified enzyme were hyperbolic functions of glycerol, DHA, and ATP. The K_m for glycerol is 0.5 mM and that for DHA is 5 mM; both are independent of the ATP concentration. The K_m for ATP in both kinase reactions is 0.5 mM and is independent of glycerol and DHA concentrations. Glycerol and DHA are competitive substrates with K_i values equal to their respective K_m values as substrates. D-Glyceraldehyde and L-Glyceraldehyde were not phosphorylated and did not inhibit the enzyme. Among the nucleotide triphosphates tested, only ATP was active as the phosphoryl group donor. Fructose diphosphate (FDP) inhibited both kinase activities competitively with respect to ATP (K_i = 0.02 mM) and noncompetitively with respect to glycerol and DHA. Adenosine 5'-diphosphate (ADP) and adenosine 5'-monophosphate (AMP) inhibited both enzymic activities competitively with respect to ATP [K_i (ADP) = 0.4 mM; K_i (AMP) = 0.25 mM]. *A. xylinum* cells with a high FDP content did not grow on glycerol. Depletion of cellular FDP by starvation enabled rapid growth on glycerol. It is concluded that a single enzyme from *A. xylinum* is responsible for the phosphorylation of both glycerol and DHA. This as well as the sensitivity of the enzyme to inhibition by FDP and AMP suggest that it has a regulatory role in glycerol metabolism.

Glycerol and dihydroxyacetone (DHA) can be utilized by *Acetobacter xylinum* as a source of carbon and energy for growth and cellulose synthesis. Glycerol utilization by resting cells of *A. xylinum* is accompanied by the formation of DHA, cellulose, CO_2, and small amounts of acetate (21). These cells operate a pentose cycle as well as a citric acid cycle, and the carbon of glycerol and DHA are most probably introduced into these cycles at the level of triose phosphate (2, 20). Condensation of triose phosphate to the hexose phosphate level has been indicated in *A. xylinum* as a step in gluconeogenesis from citrate cycle intermediates (3), and the oxidation of triose phosphate is a primary reaction in this organism for the channeling of sugar carbon from the pentose cycle into the citric acid cycle (8).

In view of its possible regulatory role, the mechanism of glycerol and DHA phosphorylation was studied. The results indicate that in *A. xylinum* glycerol and DHA are phosphorylated by a single adenosine 5'-triphosphate (ATP)-dependent kinase. The succeeding sections describe some of the properties of the enzyme and the reactions which it catalyzes as well as their possible physiological significance.

(A preliminary report of this work has been published [27].)

MATERIALS AND METHODS

Cells. The cellulose-synthesizing strain of *A. xylinum* was the same as that used by Schramm and Hestrin (22). Cells were grown and harvested after 24 h as previously described (1). A celluloseless mutant of *A. xylinum* was obtained as described by

Schramm and Hestrin (22) and grown as described by Schramm et al. (23). The culture media for small scale growth were as follows: substrate (glycerol, succinate, or glucose), 2%; yeast extract (Difco Laboratories, Detroit, Mich.), 0.5%; peptone (Difco), 0.5% and monopotassium phosphate, 0.3%. The succinate medium was brought to pH 4.0 with NaOH and the glucose and glycerol media were brought to pH 5.0 with HCl. For growth on a large scale, a 200-liter culture of the celluloseless mutant was grown in a fermentor at 30°C with vigorous aeration. The medium contained 2% succinate, 2% yeast extract, and 0.05% $MgSO_4$ and was maintained at pH 4.7 by addition of succinic acid. Cells were harvested at the end of their exponential growth.

Preparation of extracts. Freshly harvested cells suspended in 20 mM imidazole-hydrochloride buffer, pH 7.0, containing 5 mM ethylenediaminetetraacetate and 3 mM β-mercaptoethanol (IEM buffer) were lysed by stirring with lysozyme (1 mg per g of wet cells) for 1 h at 30°C. The lysate was centrifuged at 20,000 $\times$ *g* for 20 min, and the sediment was discarded. The resulting supernatant is termed "crude extract."

Enzyme assays. Enzyme assays involving the measurement of absorbance changes were performed at room temperature (about 22°C) in a Gilford model 240 recording spectrophotometer with 10-mm lightpath cells.

Glycerokinase was assayed by the radiochemical method of Newsholme et al. (16), which is based on the adsorption of the ^{14}C-labeled glycerol-3-phosphate formed from ^{14}C-labeled glycerol and ATP to diethylaminoethyl-cellulose paper disks. The reaction mixture (0.2 ml) contained: 100 mM tris(hydroxymethyl)aminomethane (Tris)-hydrochloride, pH 7.4, 10 mM $MgCl_2$, 5 mM ATP, 5 mM [U-^{14}C]glycerol (1.0×10^6 to 5.0×10^6 cpm/μmol) and enzyme. The mixture was incubated at 22°C for 5 min. The reaction was terminated by the addition of 0.2 ml of ethanol. Samples (20 to 50 μl) were applied to diethylaminoethyl-cellulose paper disks (Whatman DE-81, 2.5-cm diameter) which were subsequently washed with 150 ml of water. The disks were dried, placed in 10 ml of toluene-based scintillation liquid [toluene containing 0.3% diphenyloxazole and 0.01% 1,4 bis(4-methyl-5-phenyloxazole-2-yl) benzene] and counted in a Packard Tri-Carb scintillation counter. Controls were made with boiled enzyme or in the absence of ATP, and there was no detectable activity under either of these conditions. Retention of ^{14}C-labeled glycerol phosphate was unaffected by concentrations of ATP up to 15 mM. The assay was linear with respect to enzyme concentration and with time up to an incubation period of 10 min.

DHA kinase was assayed spectrophotometrically by measuring the rate of decrease in absorbance at 340 nm concomitant with the oxidation of reduced nictonamide adenine dinucleotide (NADH) in the presence of glycerol-3-phosphate dehydrogenase (EC 1.1.1.8). The complete assay system (1.0 ml) contained: 100 mM Tris-hydrochloride buffer (pH 7.4), 10 mM $MgCl_2$, 5 mM ATP, 50 mM DHA, 0.3 mM NADH, 3 units of crystalline glycerol-3-phosphate dehydrogenase, and enzyme. The reaction was started by the addition of ATP. In some experiments DHA kinase activity was assayed in reaction mixtures from which NADH and the dehydrogenase were excluded, and the DHA phosphate formed was determined after termination of the reaction as described below.

The enzyme was also assayed by measuring the rate of the glycerol- or DHA-dependent formation of adenosine 5'-diphosphate (ADP) from ATP by measuring the decrease in absorption at 340 nm in a reaction coupled with phosphoenolpyruvate, pyruvate kinase (EC 2.7.1.40), and lactate dehydrogenase (EC 1.1.1.27). The complete reaction mixture (1 ml) contained 100 mM Tris-hydrochloride (pH 7.4), 10 mM $MgCl_2$, 5 mM ATP, 50 mM KCl, 5 mM glycerol or 50 mM DHA, 0.3 mM NADH, 5 mM phosphoenolpyruvate, 5 units each of pyruvate kinase and lactate dehydrogenase, and enzyme. The reaction was started by the addition of phosphoryl acceptor. All enzyme units are expressed as the amount of enzyme catalyzing the transformation of 1 μmol of substrate per min under the conditions described. Specific activity is defined as number of units per milligram of protein. Results obtained with the various assay methods were in good agreement.

Other enzymes tested were assayed by the following procedures: adenosinetriphosphatase (EC 3.6.1.3.) and adenylate kinase (EC 2.7.4.3.) as previously described (4); fructose diphosphatase (EC 3.1.3.11.), aldolase (EC 4.1.2.13.), triosephosphate isomerase (EC 5.3.1.1.), glycerol-3-phosphate dehydrogenase, and glyceraldehyde-phosphate dehydrogenase (EC 1.2.1.12.) according to Bergmeyer et al. (5).

Analytical methods. Adenosine 5'-monophosphate (AMP), ADP, and ATP were assayed as previously described (4). Glycerol and DHA were determined with yeast glycerokinase and glycerol-3-phosphate dehydrogenase (28, 29). Glycerol was also determined with *Aerobacter aerogenes* glycerol dehydrogenase (6). Glycerol phosphate and DHA phosphate were determined with glycerol-3-phosphate dehydrogenase (14). Fructose diphosphate (FDP) was determined with aldolase, triophosphate isomerase, and glycerol-3-phosphate dehydrogenase (14). Protein was estimated by the method of Lowry et al. (13) with crystalline bovine serum albumin as standard. Growth of cells was monitored with a Klett-Summerson colorimeter using a no. 66 filter.

Assay of intracellular FDP. Samples (30 ml) of bacterial culture of measured turbidity were rapidly injected into 10 ml of cold 8 N H_2SO_4. The mixture was frozen and thawed three times with intermittent mixing. The denatured protein was removed by centrifugation and reextracted with 2 ml of 2 N H_2SO_4. The supernatants were combined and then brought to pH 6.0 with K_2CO_3. Samples were assayed for FDP in a system containing aldolase, triosephosphate isomerase, L-glycerol-3-phosphate dehydrogenase, and NADH by measuring the decrease in fluorescence in a Turner Fluorometer (17). The FDP level in cell-free medium was determined in other samples of the culture from which the cells were removed by rapid filtration on Millipore mem-

brane filters (0.45-μm pore diameter). The level of FDP in such filtrates was less than 10% of that of the whole culture. The internal concentration of FDP was calculated on the assumption that 1 mg of dry weight is associated in the cell with 4 μl of water (17).

Molecular weight determination. The apparent molecular weight of the enzyme was determined by gel filtration (7) with a column of BioGel A-0.5 (5 by 100 cm) previously calibrated with the following protein standards: horseradish peroxidase (EC 1.11.1.7) (molecular weight, 40,000), bovine serum albumin (molecular weight, 68,000), yeast hexokinase (EC 2.7.1.1) (molecular weight, 105,000), rabbit muscle lactate dehydrogenase (molecular weight, 150,000), rabbit muscle pyruvate kinase (molecular weight, 237,000), and *Escherichia coli* β-galactosidase (EC 3.2.1.23) (molecular weight, 515,000).

Chemicals. Nucleotides, sugar phosphates, glycerol phosphate, dihydroxyacetone phosphate, dihydroxyacetone, phosphoglycerates, and most of the enzymes were purchased from Sigma Chemical Co., St. Louis, Mo. [U-^{14}C]glycerol was obtained from the Radiochemical Corp., Amersham, England.

RESULTS

Phosphorylation of glycerol and DHA with ATP. Cell-free extracts of *A. xylinum* catalyzed the phosphorylation of glycerol and DHA by ATP. Under optimal assay conditions glycerol was phosphorylated at a rate about 1.5 times faster than DHA. The specific activities of the ATP-dependent phosphorylation of glycerol and DHA were virtually the same in extracts obtained from cells of the cellulose-forming strain of *A. xylinum* and from a celluloseless mutant of this organism. The glycerol and DHA phosphorylating activities of cells grown on glycerol (0.13 and 0.08 units per mg of dry cells) were about four times greater than those of cells grown on glucose or succinate as carbon source (0.03 and 0.02 units per mg of dry cells).

Purification of glycerol-DHA kinase. The steps reported below, except for the heating step, were carried out at 2 to 4°C. A crude extract of succinate-grown cells of the celluloseless mutant was applied to a diethylaminoethyl-cellulose column (5- by 100-cm fibrous Whatman DE-22) previously equilibrated with IEM buffer containing 0.02 M KCl. Effluent fractions of 250 ml were collected manually, and the protein and enzyme activity were monitored. The column was washed with 5 liters of IEM buffer containing 0.05 M KCl, then with 5 liters of IEM buffer containing 0.15 M KCl, and finally with 4 liters of buffer containing 0.25 M KCl. The enzyme began to elute at about 0.18 M KCl, and fractions rich in enzyme activity were pooled and precipitated at 50% saturation with $(NH_4)_2SO_4$. The suspension was placed on a column (5 by 20 cm) of cellulose powder (Whatman SF-10) equilibrated with IEM buffer 50% saturated with $(NH_4)_2SO_4$. The column was next operated at a descending linear gradient of $(NH_4)_2SO_4$ in IEM buffer. The reservoir and mixing chamber contained 4 liters each of buffer saturated 25 and 50% with ammonium sulfate, respectively. The enzyme was eluted between 33 and 38% saturated $(NH_4)_2SO_4$, and the fractions were pooled and precipitated with 50% saturated $(NH_4)_2SO_4$. Portions of the precipitate were collected by centrifugation, dissolved in a minimal volume of IEM buffer, and then chromatographed on a Bio-Gel A-0.5 column (5 by 100 cm) equilibrated with IEM buffer containing 0.2 M KCl. The enzyme appeared between 1,300 and 1,400 ml and was precipitated immediately with 50% $(NH_4)_2SO_4$. The precipitate was dissolved in IEM buffer containing 5 mM glycerol to a concentration of 3 to 5 mg of protein per ml and then heated for 20 min at 50°C. The precipitate was removed by centrifugation. The supernatant contained 43% of the glycerokinase and DHA kinase activities of the crude extract. The total purification achieved was 220-fold. The purification procedure is summarized in Table 1.

The purified enzyme preparation was free of adenosinetriphosphatase, fructose diphosphatase, aldolase, L-glycerol-3-phosphate dehydrogenase, adenylate kinase, triosephosphate isomerase, and glyceraldehydephosphate dehydrogenase activities. When stored at 4°C in the presence of glycerol, the purified enzyme retained most of its activity for at least 1 month.

Apparent molecular weight. The apparent molecular weight of the enzyme was determined by gel filtration on Bio-Gel A-0.5. It was found to be 50,000 by comparing its elution volume with that of known protein standards (see Methods and Materials). The same value was obtained both before and after heating. This is a rather low molecular weight compared to the molecular weights reported for glycerokinases from *E. coli* (molecular weight, 217,000), yeast (molecular weight, 251,000), and *Neurospora crassa* (molecular weight, 140,000) (26).

Stoichiometry. The stoichiometry of glycerol and DHA phosphorylation as catalyzed by the purified enzyme is shown in Table 2. The disappearance of equimolar amounts of glycerol and ATP was accompanied by the production of equivalent amounts of L-α-glycerophosphate and ADP. In the DHA kinase reaction the DHA phosphate and ADP formed were equivalent to the DHA and ATP utilized.

Effect of pH. The dependence of glycerol and DHA phosphorylating activities on the pH was identical. Maximal activity was obtained at pH 8.4 to 9.2 and about half-maximal activity at

TABLE 1. *Purification of glycerol-DHA kinase*

Fraction	Volume (ml)	Total protein (g)	Total activity (units)[a]	Sp act (units/mg of protein)	Recovery (%)	Activity ratio (glycerokinase/DHA kinase)
Crude extract	2,000	25.4	1,780	0.070	100	1.6
Diethylâminoethyl-cellulose combined fractions	4,000	3.3	1,221	0.370	70	1.6
Reverse $(NH_4)_2SO_4$ extraction	3,000	0.73	832	1.14	47	1.7
Bio-Gel A-0.5 combined fractions	150	0.10	765	7.65	43	1.6
Heat treated	10	0.05	765	15.3	43	1.6

[a] Micromoles of glycerol phosphorylated per minute under standard assay conditions.

TABLE 2. *Stoichiometry of reactions catalyzed by glycerol-DHA kinase*

System[a]	Component measured[b]					
	Glycerol	DHA	ATP	ADP	L-α-glycerol-phosphate	DHA phosphate
I	−3.24		−3.04	+3.20	+2.96	
II		−2.35	−2.15	+2.20		+2.45

[a] Reaction mixtures (1 ml) contained for system I (in micromoles): Tris-hydrochloride (pH 7.4), 100; glycerol, 5; ATP, 5; $MgCl_2$, 10; enzyme, 20 μg of protein; for system II: Tris-hydrochloride (pH 7.4) 100; DHA, 5; ATP, 5; $MgCl_2$, 10; enzyme, 70 μg of protein. Incubation was for 15 min at room temperature. Reactions were terminated by 1.0 ml of 0.6 M $HClO_4$, followed by centrifugation. Protein-free supernatants were neutralized with 2 M K_2CO_3.

[b] Components were determined as described in the text, and the amounts are given in micromoles.

pH 6.5 and pH 11.5. No specific buffer effect was observed at several overlapping pH values. (Buffer tested: Tris-maleate, Tris-H_2SO_4, glycine-sodium hydroxide, piperazine-glycyl-glycine.)

Effect of substrate concentration. Double reciprocal plots of velocity against ATP concentration at different levels of glycerol or DHA are shown in Fig. 1. Unlike the glycerokinase from *E. coli* (25) and various other sources (21, 23), for which the response of reaction velocity to ATP concentration resulted in nonlinear double-reciprocal plots, the plots obtained here were linear over the whole range of ATP concentrations tested (0.1 to 5 mM). The apparent K_m values calculated from these plots are 0.45 mM for glycerol, 5 mM for DHA, and 0.5 mM for ATP in both kinase reactions. The K_m for ATP was not affected by the concentrations of glycerol or DHA, and similarly the K_m for either of the two phosphoryl acceptors was independent of ATP concentration. The slopes of the lines obtained from Hill plots (15), calculated from the data in Fig. 1 show that the order of the reaction for the three substrates is 1.0.

Effect of Mg^{2+}. Glycerol and DHA phosphorylation was dependent on the presence of magnesium ions. With 5 or 10 mM ATP and at saturating concentrations of glycerol or DHA, maximal rates were obtained with Mg^{2+} concentrations of 10 mM or more. There was no change in phosphorylating activity when the Mg^{2+} concentration was raised to 20 or 30 mM. Activity was decreased when the [ATP]/[Mg^{2+}] ratio was lower than 1:1. The effect of Mn^{2+} was 30% that of Mg^{2+} on an equimolar basis. Ca^{2+} could not substitute for Mg^{2+}.

Substrate specificity. The ability of the *A. xylinum* enzyme to catalyze the phosphorylation of compounds structurally related to glycerol was assayed by measuring the formation of ADP from ATP. The following compounds, at concentrations up to 20 mM, did not serve as substrates and did not affect activity with either glycerol or DHA: D-glyceraldehyde, DL-glyceraldehyde, ethylene glycol, 1,2-propandiol, glucose, and mannitol. The amount of enzyme used in these experiments was such that a measurable rate would have been obtained if any of the compounds tested would react at a rate of 1% of that of glycerol. ATP was the only effective phosphoryl donor. Guanosine triphosphate, uridine triphosphate, and cytidine triphosphate at 2 to 10 mM concentration could not be shown to donate a phosphoryl residue to glycerol or DHA.

Glycerol and DHA as competitive substrates. The rate of ADP formation was not increased by the addition of glycerol to saturating concentrations of DHA or by the addition of DHA to saturating concentrations of glycerol. Moreover, at nonsaturating concentrations the rates of phosphorylation of glycerol and DHA were found to be competitive (Fig. 2). As ADP inhibits both kinase activities (see below), these assays were carried out in the presence of an ATP generating system composed of pyruvate

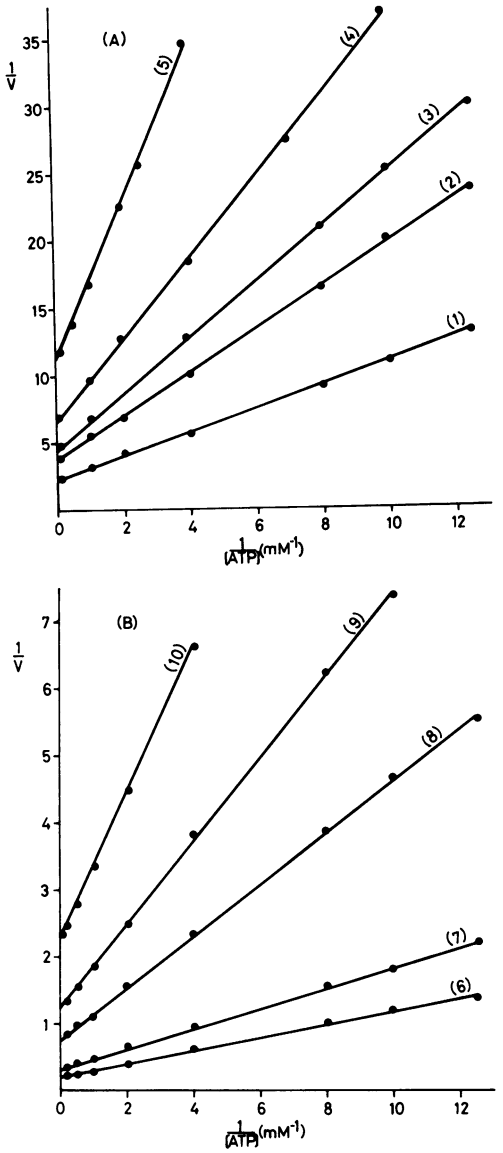

FIG. 1. *Double reciprocal plots of velocity versus ATP concentration at various fixed concentrations of glycerol (A) or DHA (B). The concentrations of glycerol used were: (1) 10 mM; (2) 0.85 mM; (3) 0.5 mM; (4) 0.25 mM; and (5) 0.125 mM. The concentrations of DHA used were: (6) 25 mM; (7) 10 mM; (8) 2.5 mM; (9) 1.25 mM; and (10) 0.75 mM. The amount of enzyme used per assay in (A) and (B) was 0.3 and 5.5 μg of protein, respectively. Velocity is expressed in units of 10^{-2} μmol of substrate phosphorylated per min.*

kinase and phosphoenolpyruvate. The K_i values calculated from these plots are 0.45 M for glycerol and 5 mM for DHA, which are identical with their respective K_m values as substrates.

Effect of reaction products. Glycerol and DHA phosphorylation was inhibited competitively by ADP with respect to ATP (K_i = 0.4 mM), and noncompetitively with respect to glycerol and DHA. AMP, which is not a direct product, acted in a similar manner with a K_i of 0.25 mM (Fig. 3). Guanosine diphosphate, cytidine diphosphate, inosine diphosphate, guanosine monophosphate, cytidine monophosphate, and inosine monophosphate at concentrations up to 3 mM had no effect on enzyme activity. In these experiments Mg^{2+} was kept at a concentration of 10 mM, and the total adenine nucleo-

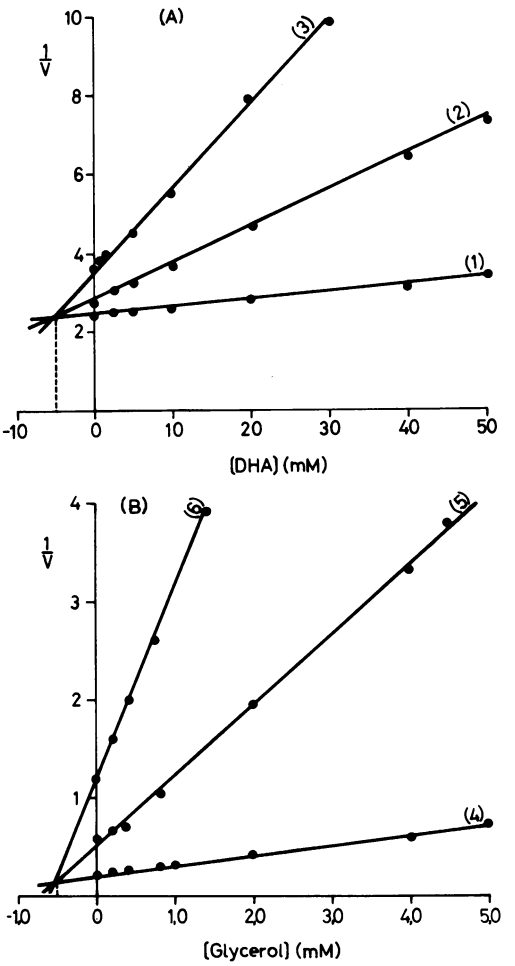

FIG. 2. *Glycerol and DHA as competitive substrates. (A) Plots of 1/v for glycerol phosphorylation versus DHA concentration at glycerol concentrations of 1 mM (1), 0.7 mM (2), and 0.5 mM (3). (B) Plots of 1/v for DHA phosphorylation versus glycerol concentration at DHA concentrations of 25 mM (4), 5 mM (5), and 2.5 mM (6). The amount of enzyme used per assay in (A) and (B) was 0.4 and 7.0 μg of protein, respectively. 1/v is expressed as in Fig. 1.*

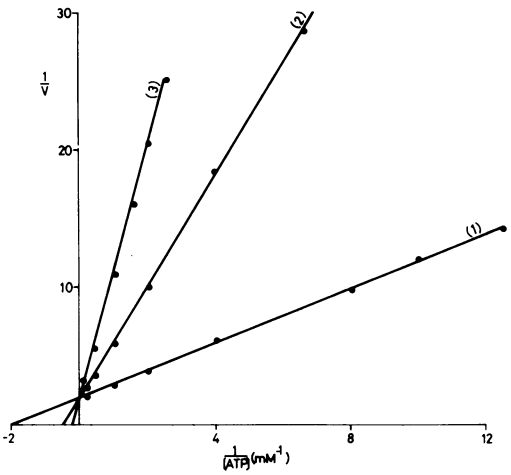

FIG. 3. *Inhibition of glycerol phosphorylation by AMP with ATP as the variable substrate in the constant presence of 5 mM glycerol. The concentrations of AMP used were 0 (1), 0.5 mM (2), and 1.5 mM (3). The amount of enzyme used per assay was 0.4 μg of protein. 1/v is expressed as in Fig. 1.*

tide concentration was 5 mM or less. L-glycerol-3-phosphate and DHA phosphate added up to 10 mM did not affect glycerol and DHA phosphorylation even at concentrations of ATP and the two phosphoryl acceptors close to their respective K_m values.

Effect of FDP. The phosphorylation of glycerol and DHA is strongly inhibited by FDP. Inhibition curves at constant ATP and variable FDP concentrations are given in Fig. 4A. The results show that FDP inhibition is completely competitive with respect to ATP with a K_i of 0.02 mM. Hill plots relating velocity and FDP concentration give a slope of 1.0, which is independent of ATP concentration. Similarly, the slope of the Hill plot for ATP is not altered in the presence of FDP. On the other hand, at a fixed concentration of ATP, a similar analysis for glycerol (Fig. 4B) and DHA (not shown) indicated noncompetitive inhibition with respect to the phosphoryl acceptors.

Effect of metabolites. Other compounds related to carbohydrate metabolism were found to be without any effects on enzyme activity. Assays were made at glycerol, DHA, and ATP concentrations close to their respective K_m values. The compounds tested at concentrations up to 10 mM included fructose, fructose-6-phosphate, fructose-1-phosphate, glucose-1,6-diphosphate, glucose-6-phosphate, glucose-1-phosphate, ribose-5-phosphate, ribulose-1,5-diphosphate, 6-phosphogluconate, glucose, gluconate, 2-phosphoglycerate, 3-phosphoglycerate, glyceraldehyde-3-phosphate, succinate, pyruvate, phosphoenol-pyruvate, citrate, isocitrate, oxaloacetate, malate, phosphate, and pyrophosphate.

Inhibition by *p*-hydroxymercuribenzoate. The phosphorylation of both glycerol and DHA was similarly inhibited by *p*-hydroxymercuribenzoate, 50% inhibition being obtained at a 5 μM inhibitor concentration. An enzyme partially inhibited by this mercurial compound retained its sensitivity to inhibition by FDP. The kinetic pattern of FDP inhibition of such an enzyme did not differ from that of an untreated enzyme.

Intracellular FDP and growth on glycerol. The possible relationship between the intracellular concentration of FDP and the ability of cells to grow on glycerol is shown in Fig. 5. *A. xylinum* cells transferred from a glucose medium to a medium containing glycerol as the

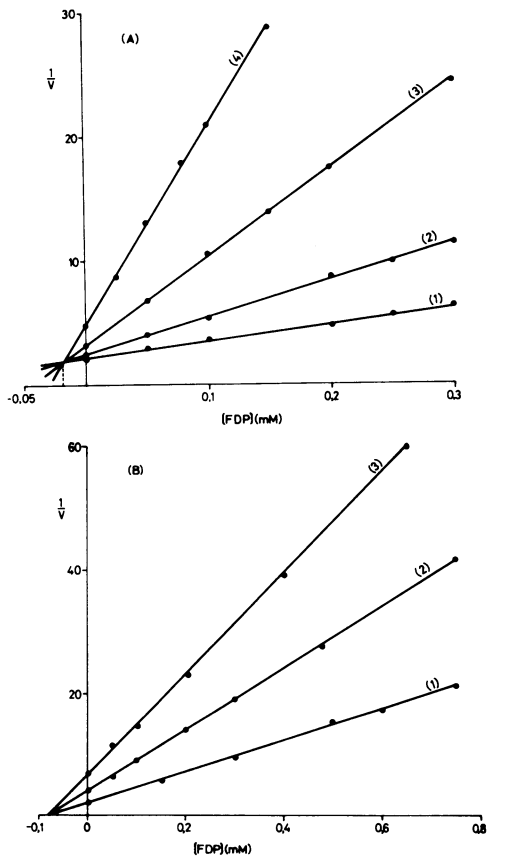

FIG. 4. *Inhibition of glycerol phosphorylation by FDP. Plots of 1/v for glycerol phosphorylation versus FDP concentration (A) at ATP concentrations of 2 mM (1), 1 mM (2), 0.5 mM (3), and 0.25 mM (4) in the presence of 5 mM glycerol; (B) at glycerol concentrations of 5 mM (5), 0.5 mM (6), and 0.25 mM (7) in the presence of 5 mM ATP. The amount of enzymes used per assay was 0.4 μg of protein. 1/v is expressed as in Fig. 1.*

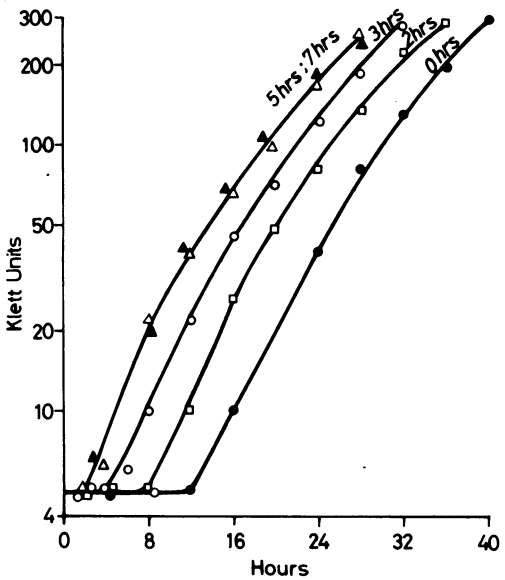

FIG. 5. *Growth on glycerol of previously starved glucose-grown cells. Cells grown on glucose were harvested at their exponentially growing phase, washed twice with cold 0.05 M phosphate buffer (pH 6.0), and resuspended in the same buffer to a cell concentration of 1.2 mg/ml. The cells were starved by incubation at 30°C in a shaking bath. At various time intervals a portion of the starved cells was inoculated into a fresh glycerol medium, and its growth was followed turbidimetrically. Another portion was assayed for intracellular FDP as described in Methods and Materials. Starvation periods (indicated) were 0, 2, 3, 5, and 7 h. Intracellular FDP concentrations at the end of the starvation periods were 0.4, 0.2, 0.1, 0.02, and 0.005 mM, respectively. A control of unstarved glycerol-grown cells inoculated into a fresh glycerol medium (intracellular FDP, 0.01 mM) gave a curve identical with that on the extreme left. No growth was observed during the starvation period.*

sole source of carbon and energy grew rapidly after a relatively long lag period. However, if the cells were starved prior to being transferred into the glycerol medium, the lag period of growth was reduced depending on the length of the starvation period, normal growth on glycerol being achieved after 5 h of starvation. It appears that cells grown on glucose contain an intermediate which severely inhibited glycerol utilization. Determination of the intracellular level of FDP revealed indeed that the concentration of FDP in glucose-grown cells is 0.4 mM, which is sufficient to completely inhibit glycerol-DHA kinase activity. Depletion of cellular FDP by starvation was soon followed by rapid growth on glycerol.

DISCUSSION

The existence of separate kinases for glycerol and DHA has been indicated in some bacterial species which can dissimilate glycerol by two different pathways, one involving glycerol phosphorylation followed by oxidation of the product to DHA phosphate and the other involving direct oxidation of glycerol to DHA and subsequent phosphorylation (12, 19). In *A. xylinum*, which is also capable of utilizing glycerol by these alternate pathways (8, 10), it appears that a single enzyme is responsible for the ATP-dependent phosphorylation of both glycerol and DHA. The ratio of activities with the two substrates remains constant throughout an extensive purification at a value which is the same as that measured in situ in permeabilized cells (24). Both catalytic activities are similarly affected by pH and various reagents (FDP, ADP, AMP, and *p*-hydroxymercuribenzoate). Finally, glycerol and DHA are competitive, their K_i values being equal to their K_m values as substrates. It is noteworthy that the enzyme has no phosphorylating activity toward D- and L-glyceraldehyde, thus differing from other glycerokinases which in addition to DHA could also phosphorylate these aldotrioses (26).

The glycerol-DHA kinase system as the primary step of the pathways metabolizing glycerol in *A. xylinum* would make it a potential control point for regulating glycerol utilization in this organism. This view is consistent with the low total activity of the enzyme in these cells, which is similar to the overall rate of glycerol oxidation in the intact cell (0.02 and 0.08 μmol per mg of cells per min by succinate and glycerol grown cells, respectively [unpublished data]). This view is also consistent with the findings that glycerol readily penetrates bacterial cells and that its transport is probably not a limiting factor in its utilization (11).

A possible mechanism for controlling the activity of the *A. xylinum* glycerokinase is suggested by its sensitivity to inhibition by FDP. The inhibition which is very specific affects the enzyme in its purified form as well as under in situ conditions (24). It was shown to be competitive with respect to ATP, with a very low K_i value of 0.02 mM. This is in contrast to the inhibition by FDP of the *E. coli* glycerokinase, which is noncompetitive with respect to both substrates with a relatively higher K_i value of 1 mM (25). Since in *A. xylinum* FDP is an obligatory intermediate in the gluconeogenic pathway leading from glycerol via hexose phosphate to the pentose phosphate cycle and cellulose synthesis, the FDP-linked control of glycerokinase acting as a negative feedback could prevent glycerol utilization in the presence of a substrate, which is a more efficient source of intermediates of this pathway. Such a mechanism is consistent with the correlation observed between the intracellular concentration of FDP

and the ability of *A. xylinum* cells to grow on glycerol (Fig. 5).

Although glycerol is the preferred substrate for the *A. xylinum* enzyme, its apparent affinity to this substrate is exceptionally low compared to glycerokinases from other sources (26). This may suggest that the activity of glycerokinase may be regulated by the intracellular concentration of glycerol. Optimal conditions for glycerokinase activity in vivo could thus be approached during growth on this compound.

The enzyme is also inhibited by AMP. The inhibition is competitive with respect to ATP, thus differing from AMP inhibition of the glycerokinases of yeast and rat and beef liver, which are noncompetitive with respect to both substrates (9, 18). It is interesting to note that except for the *A. xylinum* enzyme, all other AMP-sensitive glycerokinases are unaffected by FDP. The physiological significance of AMP inhibition of the *A. xylinum* glycerokinase may be to relate glycerol utilization to the energy state of the cell. This in turn could be associated with coupled ATP formation during glycerol oxidation to DHA, which is predominant during the early stages of glycerol utilization in the organism (21).

The results of in vivo experiments on the pattern of glycerol utilization by *A. xylinum* under various physiological conditions which are in accord with the mechanisms proposed here will be reported elsewhere.

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