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Purification and Properties of the Flavine-Stimulated Anaerobic L-α-Glycerophosphate Dehydrogenase of *Escherichia coli*[1]

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The anaerobic L-α-glycerophosphate (L-α-GP) dehydrogenase of *Escherichia coli* was purified approximately 40-fold. The activity of the dehydrogenase, although not affected by the addition of pyridine nucleotides, was stimulated three- to fourfold by flavine adenine dinucleotide (K_m about 10^{-7} M) and up to 10-fold by flavine mononucleotide (K_m about 10^{-4} M). Maximal activity of the enzyme was found only in the combined presence of saturating concentrations of both flavines (stimulation by a factor of 10 to 15). The dependence of the rate of the reaction on the concentration of L-α-GP was complex in the presence of both flavines, but in the presence of flavine adenine dinucleotide alone the kinetics were of the Michaelis-Menten type with the K_m for L-α-GP being about 10^{-4} M. The product of the reaction was identified as dihydroxyacetone phosphate, and the molecular weight of the dehydrogenase was estimated to be 80,000 ± 10,000. Phenazine methosulfate, menadione and ferricyanide served as artificial acceptors for the dehydrogenase. The enzyme was sensitive to iodoacetate, *p*-chloromercuribenzoate, and *N*-ethymaleimide.

The dissimilation of glycerol and L-α-glycerophosphate (L-α-GP) in *Escherichia coli* K-12 is dependent upon one of two distinct L-α-GP dehydrogenases neither of which is linked to pyridine nucleotides (Fig. 1) (8, 19). The factor determining which of the two enzymes is necessary for growth is apparently the nature of terminal electron acceptors available for the respiratory chain. With oxygen as the acceptor, a membrane-associated dehydrogenase which is not stimulated by added flavines (aerobic L-α-GP dehydrogenase) is required (19, 22). With fumarate (28) as the acceptor, a soluble dehydrogenase which is greatly stimulated by added flavines (anaerobic L-α-GP dehydrogenase) is required (19). When nitrate (29) serves as the acceptor, either the soluble or the particulate dehydrogenase is sufficient to permit growth (19). The present paper reports the partial purification of the anaerobic L-α-GP dehydrogenase and some of the properties of the purified enzyme.

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MATERIALS AND METHODS

Bacteria. Strain 233 is a derivative of *E. coli* K-12 Hfr Cavalli that produces both the aerobic and anaerobic L-α-GP dehydrogenases constitutively (*glp*R$^-$) (8). Strain 13 (*glp*R$^-$, *glp*D$^-$, *mal*A$^-$, *bio*$^-$, *his*$^-$) is a derivative of *E. coli* K-12 Hfr G-6 that contains a deletion including the *glp*R gene and extending into the neighboring structural gene for the aerobic dehydrogenase (*glp*D). This strain was a gift from M. Schwartz.

Media. Cells were grown in a high phosphate mineral salts medium previously described (32). The medium used for the cultivation of strain 13 for isolation of the anaerobic dehydrogenase contained in addition 1% salt-free, vitamin-free casein hydrolysate (Nutritional Biochemical Co., Cleveland, Ohio) and 0.1% yeast extract (Difco). The phosphate and sulfate components of the salts medium were sterilized separately and aseptically added to the remainder of the medium when casein hydrolysate was used.

Enzyme assays. The assay for the anaerobic L-α-GP dehydrogenase was based on the phenazine methosulfate (PMS) mediated reduction of 3(4,5-dimethylthiazolyl-2)-2,5-diphenyl tetrazolium bromide (MTT), which was followed spectrophotometrically at 570 nm (molar absorbancy = 17,000 cm^{-1}). Assays were performed as described previously (19) except that KCN was omitted from determinations of the anaerobic enzyme. Flavine-

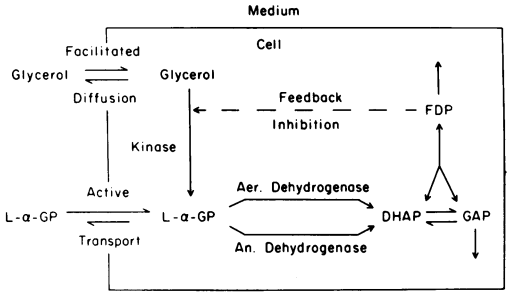

FIG. 1. *Pathway for the dissimilation of glycerol and L-α-GP in E. coli.*

adenine dinucleotide at 10 μM and flavine mononucleotide (FMN) at 1 mM were added to routine assay mixtures. No enzymatic reduction of MTT was observed in the absence of PMS whether or not flavines were added to the assay mixture. The assay was linear with respect to the amount of enzyme added up to at least a velocity corresponding to a change in absorbancy at 570 nm of 0.5 min^{-1} cm^{-1}. The aerobic L-α-GP dehydrogenase was determined in the same way, except that flavines were omitted.

Triosephosphate isomerase was determined by an assay which couples the oxidation of reduced nicotinamide adenine dinucleotide (NADH) to the reduction of dihydroxyacetone phosphate derived from glyceraldehyde-3-phosphate (GAP) (6). The reaction mixtures contained, in a final volume of 1 ml, 14 μmoles of triethanolamine-chloride at *p*H 7.8, 0.2 μmole of NADH, 2 μmoles of GAP, and 10 μg of crystalline rabbit muscle L-α-GP dehydrogenase.

All reactions were monitored with a Gilford model 2000 recording spectrophotometer with the cuvette chamber thermostated at 25 C. Enzyme activity throughout is expressed in units of micromoles per minute per milligram of protein.

Enzymatic determination of compounds. Dihydroxyacetone phosphate (DHAP) was determined stoichiometrically by coupled oxidation of NADH under conditions identical to those specified for the above assay except that the isomerase was absent and GAP was omitted. The reaction in this case was allowed to reach equilibrium. Addition of crystalline triosephosphate isomerase (10 μg) allowed the determination of GAP. L-α-GP was determined by enzymatic coupling to the reduction of nicotinamide adenine dinucleotide (NAD) with rabbit muscle L-α-GP dehydrogenase (14).

Purification of FMN. Chromatography of commercial FMN on Whatman no. 1 paper developed in ascending fashion with 0.2 M Na_2HPO_4 (adjusted to *p*H 7.8 with HCl) (17) revealed, in addition to the major spot of FMN, two minor fluorescent spots when the chromatogram was examined under ultraviolet light. One of these minor components co-chromatographed with authentic riboflavine, whereas the other was likely a diphosphorylated derivative of riboflavine (G. L. Kilgour, *personal communication*). To remove these as well as other possible contaminants, FMN was purified by chromatography on diethylaminoethyl (DEAE)-cellulose by a modification of the procedure of Rao et al. (30). To a column (3 by 14 cm) of DEAE-cellulose previously equilibrated with 0.1 M K_2HPO_4 and rinsed with water was applied 20 mg of FMN dissolved in 5 ml of water. A water wash removed riboflavine. Most of the adsorbed material was then eluted by a linear gradient of 0 to 0.2 M KH_2PO_4 (500 ml). FMN emerged as a symmetrical peak leaving still adsorbed to the column a contaminating yellow band which would be eluted by raising the KH_2PO_4 concentration to 0.3 M. The fractions containing the major peak were pooled and concentrated by evaporation in a rotating flask under vacuum at 45 C. Excess salt in the concentrate was removed by gel exclusion chromatography on Sephadex G-10. Chromatography of the final product on paper as before yielded only one fluorescent spot which corresponded to the R_F expected for FMN.

Protein determination. Protein was measured by the biuret reagent (11) or by the Folin reagent (23) where indicated. Crystalline bovine serum albumin was used as a standard for both methods.

Purification of the anaerobic L-α-dehydrogenase. Strain 13 was grown aerobically on casein hydrolysate and yeast extract in 1 liter of medium incubated in a 2-liter Erlenmeyer flask at 37 C on a rotatory shaker. Although the anaerobic dehydrogenase does not function in the presence of oxygen, it can be synthesized both aerobically and anaerobically (19). Cultures were harvested at the onset of stationary phase (300 to 350 units, Klett-Summerson colorimeter, no. 42 filter). Twelve such cultures were combined and the cells were collected by a refrigerated Sharples centrifuge. Alternatively, cultures were grown as a 12-liter batch in a New Brunswick Microferm sparged with air at a rate of 5 liters/min and stirred at 200 rev/min. The packed cells were immediately suspended as described below for disruption; however, storage of the cells at −20 C led to results comparable to those obtained with fresh cells. All further operations were carried out at 0 to 4 C.

Step 1. The packed cells, about 35 g wet weight, were suspended in 50 ml of 0.02 M potassium phosphate, *p*H 7.5 (buffer A) containing 10 μM FAD, 5 mM dithiothreitol, and 2 mg of deoxyribonuclease. The cell suspension was disrupted by one pass through a French pressure cell (Aminco) operated in a Carver laboratory press at a pressure of 30,000 psi. The broken cell suspension was centrifuged for 20 min at 30,000 × *g* and the supernatant fraction was retained.

Step 2. Removal of gross nucleic acid was effected by adding solid NaCl to the fraction retained from the previous step to make the final concentration 0.2 M and by then passing the extract over a column (3 by 10 cm) of DEAE-cellulose equilibrated with buffer A containing 0.2 M NaCl. Under these conditions, the dehydrogenase was not adsorbed, and the fractions emerging from the column which contained activity were combined.

Step 3. An ammonium sulfate solution, saturated at room temperature, was made 10 μM with respect to FAD and adjusted to *p*H 7.0 with NH_4OH immediately prior to use. Fractionation of the material from the previous step was made by the moderately

rapid addition of the saturated ammonium sulfate solution while constant stirring was maintained magnetically. A 10-min precipitation period was allowed for each fraction which was collected by centrifugation for 10 min at 35,000 × *g*. The fraction precipitated between 30 and 40% saturation was retained and dissolved in a small volume of buffer A containing 10 μM FAD and 1 mM ethylenediaminetetraacetate (EDTA).

Step 4. The material retained from ammonium sulfate fractionation was applied to a column (2.5 by 37 cm) of Sephadex G-75 equilibrated with buffer A containing 20% ethylene glycol, 10 μM FAD, 1 mM EDTA, and 0.05 M NaCl. Elution was performed with the same buffer solution, and the active fractions, which emerged from the column almost coincident with the void volume, were pooled.

Step 5. The material from the preceding procedure was applied to a column (1.5 by 16 cm) of DEAE-cellulose (Whatman DE52) equilibrated with buffer A containing 20% ethylene glycol and 0.05 M NaCl. The column was washed with 50 ml of this equilibration buffer, and the dehydrogenase was then eluted by a linear gradient (500 ml) of 0.05 M to 0.3 M NaCl in the equilibration buffer containing in addition 10 μM FAD and 1 mM EDTA. A flow rate of 30 ml/hr was maintained by a peristaltic pump. The dehydrogenase was eluted at a NaCl concentration of approximately 0.15 M, and the fractions of greatest activity were pooled and stored frozen at −20 C in small portions. A summary of the purification procedure is given in Table 1.

In the presence of FAD, the anaerobic L-α-GP dehydrogenase can be further activated by FMN (see Results). This sensitivity to FMN proved to be a particularly labile character of the enzyme. Best results were obtained if the complete procedure of purification was carried out without storage of the enzyme between steps (Table 1). The anaerobic L-α-GP dehydrogenase was unstable in dilute solutions at all stages of purification unless protected by the presence of ethylene glycol (20%) or FAD (10 μM). Increasing the concentration of ethylene glycol to 40% or substituting glycerol for ethylene glycol did not lead to a greater protective effect. In a trial to determine whether ethylene glycol might be a competitive inhibitor of the dehydrogenase, it was found that at a concentration of 2% in the assay mixture it had no effect, even when the concentration of L-α-GP was reduced to 0.2 mM, the vicinity of the K_m of the dehydrogenase for its substrate. The most stable condition of storage appeared to be at −20 C in the presence of both protective agents. Even under this condition, the purified enzyme stored for 3 months with one thawing and refreezing lost 60% of its activity when assayed in the combined presence of FAD and FMN, although the activity when assayed solely in the presence of FAD was not diminished.

Inhibition of triosephosphate isomerase activity. Two milliliters of the pooled fractions of the DEAE-cellulose eluate containing L-α-GP dehydrogenase were passed over a column (0.56 by 40 cm) of Sephadex G-25 equilibrated with 0.04 M tris(hydroxymethyl)aminomethane (Tris)-chloride, *p*H 7.4, containing 1 mM EDTA, 10 μM FAD, and 20% ethylene glycol. Fractions containing high dehydrogenase activity were combined and sodium DL-glycidol phosphate, a specific and irreversible inhibitor of mammalian triosephosphate isomerase (31), was added to give a final concentration of 0.15 mM. The mixture was kept for 12 hr at 4 C. After finding that measurable isomerase activity remained, additional inhibitor was added to raise its concentration to 0.3 mM and the incubation was continued at 15 C for 6 hr. The activity of triosephosphate isomerase was unmeasurable after this treatment whereas the dehydrogenase activity itself was reduced only by 30%.

Chemicals. PMS, FAD, FMN, NAD, NADH, nicotinamide adenine dinucleotide phosphate (NADP), and deoxyribonuclease (bovine spleen) were obtained from Sigma Chemical Co., St. Louis, Mo. MTT was obtained from Nutritional Biochemicals Corp., Cleveland, Ohio. Resolved L-α-GP was from Calbiochem, Los Angeles, Calif. Ammonium sulfate (ultrapure) was obtained from Mann Research Laboratories, New York, N.Y. Crystalline rabbit muscle triosephosphate isomerase, pig heart malate dehydrogenase, rabbit muscle L-α-GP dehydrogenase, and yeast hexokinase were obtained from Boehringer Mannheim Corp., New York, N.Y., and *E. coli* alkaline phosphatase was obtained from Worthington Biochemical Corp., Freehold, N.J. Crystalline bovine serum albumin was obtained from Pentex, Inc., Kankakee, Ill. Asolectin (purified soy bean phosphatides) was from Associated Concentrates, Woodside, N.Y.

TABLE 1. *Purification of the anaerobic L-α-glycerophosphate (L-α-GP) dehydrogenase from E. coli K-12*

Step	Protein (mg)	Vol (ml)	Total activity[a] (units)	Specific activity[a] (units/mg)	Recovery (%)	Activity with only FAD[b] (% of total)
1	3,050	57	820	0.28	100	18
2	2,670	112	740	0.28	90	19
3	280	7.1	284	1.0	35	16
4	169	25	236	1.4	29	17
5	6.4[c]	26	74	12	9	15

[a] Assay mixture contained 10 μM flavine-adenine dinucleotide (FAD) and 1 mM flavine mononucleotide (FMN).

[b] Assay mixture contained 10 μM FAD but no added FMN.

[c] Determined by Folin reagent.

RESULTS

Effect of added flavines on the anaerobic L-α-dehydrogenase. As reported earlier, the anaerobic and aerobic L-α-GP dehydrogenases in a crude extract can be separated by high-speed centrifugation through a gradient of sucrose (18). The activity of the slowly sedimenting anaerobic dehydrogenase was dependent upon the addition of either FAD or FMN to the assay mixtures (Fig. 2). In contrast, the activity of the largely pelleted aerobic dehydrogenase was insensitive to the addition of flavines.

Subsequent studies using mutants such as strain 13, which lacks the aerobic enzyme (thus allowing unambiguous determination of the anaerobic enzyme in unfractionated extracts), revealed that neither FAD nor FMN could fully activate the anaerobic dehydrogenase, whether in the crude extract or in a purified preparation.

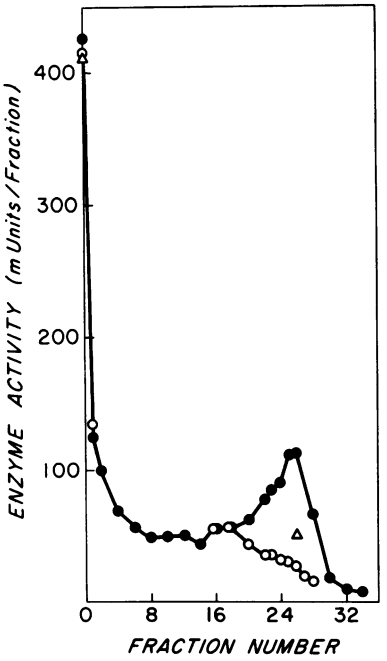

FIG. 2. *Distribution of L-α-GP dehydrogenase activity after sucrose density gradient centrifugation. A sample (0.2 ml containing 7.2 mg of protein) prepared by sonic disruption of cells of strain 233 was centrifuged through a gradient of sucrose as previously described (18) except that FAD was not added to the sucrose solutions. Fractions were assayed for L-α-GP dehydrogenase activity without added flavines (○) or with either 10 μM FAD (△) or 1 mM FMN (●). Direction of sedimentation was from right to left, and the pelleted material was resuspended in buffer and is represented as fraction "0".*

The double dependence of the dehydrogenase activity upon FAD and FMN raised the possibility that two separate proteins were being unwittingly measured. This possibility is rendered unlikely by the following observations. First, the anaerobic L-α-GP dehydrogenase activity measured in the combined presence of the two coenzymes is missing in several independent mutants (19). Second, chromatography on DEAE-cellulose revealed only a single peak of activity whether assayed in the presence of added FAD or in the combined presence of added FAD and FMN (Fig. 3). Third, the combined addition of both flavines resulted in a slightly greater increase in activity than can be accounted for by summation of the individual effects of each coenzyme (Table 2).

To determine the K_m of the dehydrogenase for FAD, the coenzyme normally added as a protective agent was removed by gel filtration. This procedure, however, was always associated with a substantial diminution of the response of the dehydrogenase to FMN. Using such a preparation of the dehydrogenase, the concentration dependence of its response to either FAD or FMN (commercial preparation

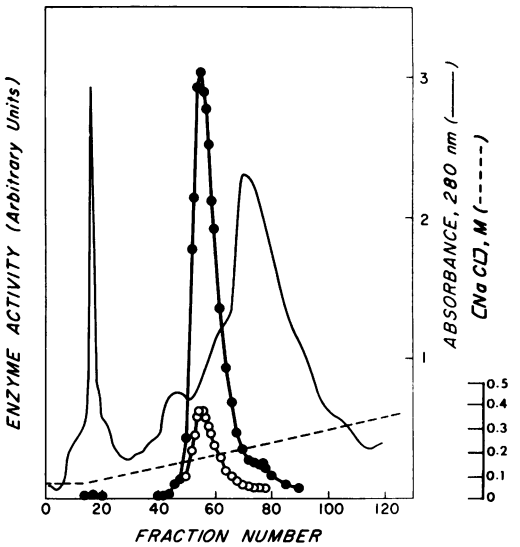

FIG. 3. *Elution profile of L-α-GP dehydrogenase activity from a column of DEAE-cellulose. The material applied to the column was purified through step 4 as described in Materials and Methods except that the material precipitated between 25 and 50% ammonium sulfate was retained in step 3. The column was prepared and developed as described in Materials and Methods. Fractions were assayed for L-α-GP dehydrogenase activity in the presence of 10 μM FAD (○) or in the combined presence of 10 μM FAD and 1 mM FMN (●).*

TABLE 2. *Effect of added flavines on the anaerobic L-α-glycerophosphate (L-α-GP) dehydrogenase*[a]

Flavine mononucleotide concentration (mM)	L-α-GP dehydrogenase activity[b]		
	0[c]	1 μM[c]	1 mM[c]
0	0.07	0.20	0.20
0.2	0.24	0.45	0.23
2.0	0.45	0.63	0.55

[a] A portion of the dehydrogenase preparation from step 5 of the purification procedure described in Materials and Methods was rapidly freed of added flavine-adenine dinucleotide as described in the legend to Fig. 4.

[b] Activity is expressed as change in absorbance at 570 nm/min for 10 μliters of the dehydrogenase preparation.

[c] Flavine-adenine dinucleotide concentration.

without purification) was determined (Fig. 4). A concentration of FAD of approximately 0.1 μM produced a half maximal response. In contrast, the concentration of FMN required for a half maximal effect was over three orders of magnitude greater, approximately 0.4 mM. The latter measurement was repeated with chromatographically purified FMN and a dehydrogenase preparation which had not been freed of added FAD (and therefore fully responsive to FMN). The concentration of FMN required for a half maximal effect in the presence of a constant level of FAD (10 μM) was found to be about 0.1 mM, only slightly lower than found before. Riboflavine, it should be mentioned, was without effect on the dehydrogenase when tested at a concentration of 50 μM either in the presence or absence of FAD.

Removal of free FAD by gel filtration did not result in complete dependence of the enzyme activity upon added flavines (Fig. 4). Indeed, a repetition of the gel filtration treatment did not lead to lower basal activity. An attempt to render the dehydrogenase inactive without added flavines was made by passing a sample of the enzyme preparation over a small column of Florsil, an adsorbant with strong affinity for flavines. This procedure resulted in the total loss of activity even when assayed in the presence of both flavines. In order to prevent direct contact between the dehydrogenase and this adsorbant, a sample of the enzyme was dialyzed against a stirred suspension of Florisil. This procedure, however, neither reduced the basal activity of the dehydrogenase nor altered its dependence upon added flavines.

To determine qualitatively whether FAD and FMN compete with one another in the stimulation of the anaerobic L-α-GP dehydrogenase, a preparation of the purified enzyme which had been quickly freed of added FAD by gel filtration was assayed for the effect of FAD, either 1 μM or 1 mM, in the presence of 0.2 mM or 2 mM FMN. The results presented in Table 2 are compatible with separate sites for the two cofactors, a high affinity site for FAD and a low affinity site for FMN.

Effect of the concentration of L-α-GP on the rate of reaction. When the dehydrogenase was assayed with different concentrations of pure L-α-GP, in the presence of FAD but absence of FMN, a double reciprocal plot typical of Michaelis-Menten kinetics was obtained which yielded a K_m for L-α-GP of 0.1 mM. When the effect of varying the concentration of L-α-GP was determined in the combined presence of FAD and FMN, the double reciprocal plot of the data did not yield a single straight line (Fig. 5). A K_m value of 0.2 mM was indicated at high concentrations of L-α-GP. At low concentrations of the substrate, a K_m value somewhat less than 0.1 mM (0.08 mM) was suggested.

Identity of the product of the dehydrogenase. The purification procedure described in Materials and Methods did not remove the highly active triosephosphate isomerase to a sufficient extent. Therefore, identification of the product of the dehydrogenase was carried out with a preparation in which residual triosephosphate isomerase activity had been eliminated by treatment with a specific, irreversible inhibitor of that enzyme, glycidol phosphate (31). Known amounts of the pure L-isomer of α-GP and of the two possible products of the reaction, DHAP and GAP, were incubated

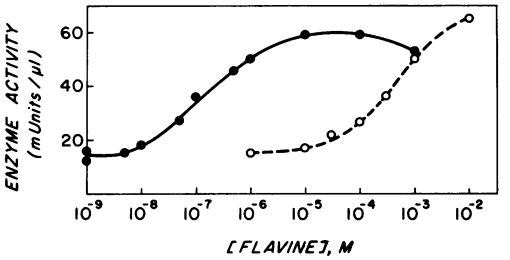

FIG. 4. *Activity of the anaerobic L-α-GP dehydrogenase as a function of the concentration of either added FAD (●) or added FMN (○). A portion of the dehydrogenase preparation from step 5 of the purification procedure described in Materials and Methods was freed of added FAD by passage of a 1-ml portion over a column (40 by 0.56 cm) of Sephadex G-25 (coarse) equilibrated with a buffer solution containing 0.02 M potassium phosphate, pH 7.5, 1 mM EDTA, and 20% ethylene glycol.*

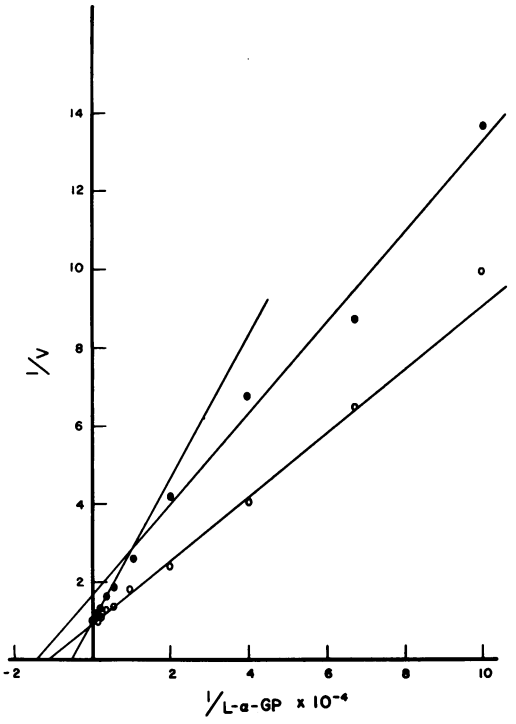

FIG. 5. *Double reciprocal plot of the initial velocity of the anaerobic* L-α-*GP dehydrogenase as a function of the substrate concentration with 10 μM FAD* (○) *or 10 μM FAD and 1 mM FMN* (●). *The enzyme preparation was from step 5 of the purification scheme described in Materials and Methods. Relative velocity was used so that data from the two sets of measurements could be plotted together. For assay with FAD only* (○—○) *a velocity of 1 = 0.13 change in absorbance at 570 nm/min. For assay in the combined presence of FAD and FMN* (●—●) *a velocity of 1 = 0.305 change in absorbance at 570 nm/min.*

separately in the dehydrogenase assay mixture. All three mixtures were incubated at 25 C until no further reduction of MTT was observed in the cuvette containing the L-α-GP (30 min). Each mixture was immediately passed through a column (0.4 by 2.4 cm) of charcoal. The columns were then rinsed with one volume of water (0.4 ml) which was combined with the initial eluate. The resulting solutions, from which all traces of the colored components of the assay mixture had been removed, were assayed enzymatically for the presence of both DHAP and GAP. The results are summarized in Table 3. In the sample originally containing L-α-GP, 84% of the reacted substrate (calculated as MTT reduced) was recovered as DHAP and none was recovered as GAP. In the sample to which GAP was added, recovery of the aldotriose phosphate was 70%. The loss was possibly due to the action of glyceraldehyde phosphate dehydrogenase present in the preparation. Since GAP dehydrogenase is known to contain tightly bound NAD (33), and since PMS rapidly oxidizes NADH, such an explanation would account for the observed GAP dependent reduction of MTT. Control DHAP, on the other hand, was recovered almost quantitatively. Since no interconversion of the two triosephosphates occurred under the experimental condition, the product of the dehydrogenation of L-α-GP is unambiguously identified as dihydroxyacetone phosphate.

Electron acceptors for the anaerobic L-α-GP dehydrogenase. A search was made for other in vitro electron acceptors with which the dehydrogenase could be assayed. Menadione (2-methyl-1,4-naphthoquinone), at its limit of solubility in aqueous solution, was found to give about 25% of the activity of PMS as acceptor and to show comparable dependence upon added flavines for maximum activity (Table 4). Ferricyanide and dichlorophenolindophenol were also tested. The former, which in the absence of MTT was monitored directly at 420 nm, served as an acceptor, but required concentrations sufficiently high to make an optical assay impractical. The latter was ineffective as an acceptor.

NAD or NADP added at 1 mM to the usual PMS-mediated assay had no effect. Furthermore, the dehydrogenase displayed no activity when assayed with L-α-GP and NAD or with DHAP and NADH.

Molecular weight. The molecular weight of the dehydrogenase was estimated by centrifugation through a linear gradient of sucrose (24) with *E. coli* alkaline phosphatase as reference, and by gel filtration (2) on Sephadex G-200 (0.9 by 55 cm column developed with a buffer

TABLE 3. *Identification of the product of the reaction catalyzed by anaerobic* L-α-*glycerophosphate* (L-α-*GP*) *dehydrogenase*[a]

Compound	Micromoles added	Micromoles of MTT reduced	Micromoles recovered as	
			GAP	DHAP
L-α-GP	0.080	0.063	0.000	0.052
GAP	0.12	0.041	0.084	0.002
DHAP	0.10	0.000		0.092

[a] The experiment is described in Results. MTT, 3(4,5-dimethylthiazolyl-2)-2,5-diphenyl tetrazolium bromide; GAP, glyceraldehyde-3-phosphate; DHAP, dihydroxyacetone phosphate.

solution containing 20 mM potassium phosphate at *p*H 7.5, 20% ethylene glycol, 10 μM FAD, and 1 mM EDTA) with pig heart malate dehydrogenase and yeast hexokinase as reference markers. In the first experiment, the activity of the dehydrogenase sedimented coincidently with alkaline phosphatase (molecular weight 80,000) (21), and in the second experiment the dehydrogenase activity emerged between that of hexokinase (molecular weight 100,000) (21) and that of malate dehydrogenase (60,000) (25).

pH optimum of the dehydrogenase. The *p*H optimum for the dehydrogenase lies between 7.5 and 8.0 in 0.1 M potassium phosphate buffer. In this *p*H range, the activity is greater (by some 20 to 40%) in phosphate than in 0.1 M Tris-chloride, imidazole-chloride, or triethanolamine-chloride.

Sensitivity to inhibitors. The sensitivity of the dehydrogenase to sulfhydryl poisons was determined on a stored preparation of the enzyme that was found to have lost responsiveness to FMN, although not to FAD. The enzyme was incubated with the inhibitor to be tested in the standard assay mixture minus L-α-GP, PMS, and flavines for 15 min at 25 C. The reaction was then initiated by the addition of the omitted components. A 98% loss of activity resulted from treatment with 0.1 mM parachloromercuribenzoate; 52% loss with 1 mM *N*-ethylmaleimide, and 36% loss with 1 mM iodoacetate. Under identical conditions, incubation in the presence of 1 mM 1,10 phenanthroline or 5 mM EDTA resulted in no loss of activity. In an attempt to restore the sensitivity of the enzyme to FMN, it was found that incubation with any of the following metal ions at 0.01 mM was ineffective: Cu^{2+}, Fe^{2+}, Fe^{3+}, Mg^{2+}, Mn^{2+}, Ni^{2+}, and Zn^{2+}.

TABLE 4. *Effect of added flavines on the activity of the anaerobic* L-α-*glycerophosphate (*L-α-*GP) dehydrogenase with menadione or PMS as electron acceptor*[a]

Additions	L-α-GP dehydrogenase activity	
	PMS, 0.02%[b]	Menadione, 0.5 mM[b]
None	1[c]	1[d]
FAD, 10 μM	2.6	2.5
FMN, 1 mM	4.2	3.2
FAD + FMN (10 μM + 1 mM)	5.8	4.5

[a] Abbreviations: PMS, phenazine methosulfate; FAD, flavine-adenine dinucleotide; FMN, flavine mononucleotide. The L-α-GP dehydrogenase preparation was taken from step 5 of the purification procedure described in Materials and Methods and was rapidly freed of added FAD by the procedure described in the legend to Fig. 4.

[b] PMS and menadione—acceptors. Menadione replaced PMS in the usual assay mixture, and the reduction of 3(4,5-dimethylthiazolyl-2)-2,5-diphenyl tetrazolium bromide (MTT) was followed as usual. In the absence of either acceptor no reduction of MTT was observed.

[c] 1 = change in absorbance at 570 nm of 0.012/min.

[d] 1 = change in absorbance at 570 nm of 0.0032/min.

DISCUSSION

The available evidence allows no firm conclusion to be drawn on the nature of the cofactor which serves the anaerobic L-α-GP dehydrogenase in vivo, especially in view of the need to employ artificial electron acceptors in our assay system. Further, the enzyme apparently retains a tightly bound endogenous cofactor which is responsible for the basal level of activity in vitro. The high affinity of the dehydrogenase for FAD, however, suggests that it does play a physiological role for the enzyme. The weak affinity of the dehydrogenase for FMN, on the other hand, makes its physiological role questionable. Yet the evidence is not in harmony with the simple possibility that FMN merely substitutes for the FAD.

It is all the more attractive to postulate a special site for FMN in view of the fact that reduced FMN is known to serve in vitro as a hydrogen donor for fumarate reductase, an inducible enzyme in *E. coli* important for the coupling of anaerobic L-α-GP dissimilation with the reduction of exogenous fumarate (13). In this context, it is interesting to note that reduced FAD formed in vitro by a pyridine nucleotide dehydrogenase from *Clostridium kluyveri* would serve as an electron donor for nitrate reduction by a nitrate reductase from *Neurospora crassa* (26, 27). Similarly, an in vitro dismutation of pyruvate was demonstrated using a flavine-dependent pyruvate oxidase from *Lactobacillus delbrueckii* to reduce added riboflavine which in turn served as an electron donor for the reduction of pyruvate to lactate by a flavine-dependent lactate oxidase from the same organism (12).

Other examples of flavodehydrogenases which accept FAD and FMN as cofactors are: swine liver NADPH-cytochrome *c* reductase (15), a quinone reductase from rat or ox brain (10), and a NADH dehydrogenase in the degredative pathway for camphor in *Psuedomonas putida* (34). In the first two, as well as in the

case of the nitrate reductase of *N. crassa* cited earlier, possible additive or cooperative effects of the two flavine species were apparently not examined. In the last case, such effects were looked for but were not found. In some of the studies, however, a possible effect of FMN might have escaped notice because insufficient concentrations were employed.

The only well established case for the participation of both FAD and FMN in the native enzyme is the dehydroorotate reductase isolated from *Zymobacterium oroticum*. Both flavines participate in catalysis (1, 9) and remain associated with the enzyme during purification.

The reactivity of the artificial naphthoquinone, menadione, as an electron acceptor for the anaerobic L-α-GP dehydrogenase in vitro makes it desirable to determine whether the indigenous benzo- or naphthoquinones of *E. coli*, Q8 and K_2C_{45}, respectively (16), play essential roles in linking the dehydrogenation of L-α-GP to certain electron transport chains. Brodie and associates have described an FAD-dependent malate dehydrogenase of *Mycobacterium phlei* which was active with either menadione or a naturally occurring naphthoquinone extracted from *M. phlei* (4). This dehydrogenase would effectively couple the oxidation of malate to the reduction of cytochromes present in membrane particles, presumably by the participation of membrane-associated naphthoquinones (3). The malate enzyme was stimulated by added phospholipids (Asolectin) and inhibited by low concentrations of the nonionic detergent Triton X-100 (4). The anaerobic L-α-GP dehydrogenase, on the other hand, was not found to be stimulated by added Asolectin or inhibited by Triton X-100 which is routinely added to the assay mixtures for the dehydrogenase (19) at a concentration 100-fold greater than that which inhibited the malate enzyme.

The identification of DHAP as the product of the reaction catalyzed by the anaerobic L-α-GP dehydrogenase makes this enzyme the fourth class of protein which acts on the carbon 2 of the three-carbon substrate, the other three examples being the NAD-linked cytoplasmic L-α-GP dehydrogenase of rabbit muscle (serving physiologically as DHAP reductase) (5), the FAD-linked mitochondrial L-α-GP of mammalian muscle (35), and the biosynthetic NADP-dependent L-α-GP dehydrogenase of *E. coli* (serving physiologically for the biosynthesis of L-α-GP) (20). The product of the reaction catalyzed by the membrane-associated L-α-GP dehydrogenase of *E. coli* is yet to be explicitly identified, although indirect evidence also suggests it to be DHAP (7).

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