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PURIFICATION AND BIOCHEMICAL PROPERTIES OF ALLELIC FORMS OF CYTOPLASMIC GLYCEROL-3-PHOSPHATE DEHYDROGENASE FROM *DROSOPHILA VIRILIS*

SUMIKO NARISE

Department of Chemistry, Josai University, Sakado, Saitama 350-02 (Japan)

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Summary

Three homozygous allelic forms (αGPDH^f, αGPDH^m and αGPDH^s) of NAD$^+$-dependent glycerol-3-phosphate dehydrogenase (*sn*-glycerol-3-phosphate:NAD$^+$ 2-oxidoreductase, EC 1.1.1.8) of *Drosophila virilis* were purified to homogeneity and their biochemical properties were compared.

Although these three forms were mutually distinguishable by electrophoresis, no significant differences were found with respect to pH optima for both forward and reverse reactions (pH 6.0—6.5 for dihydroxyacetone phosphate reduction; pH 10.0—10.5 for glycerol 3-phosphate oxidation), native and subunit molecular weights (65 000 for native form; 35 000—37 000 for subunit) and Michaelis constants for NADH, glycerol 3-phosphate and NAD$^+$ (5.3—6.0 μM for NADH; 1.8—1.9 mM for glycerol 3-phosphate; 100—110 μM for NAD$^+$). Significant differences among three forms were observed in thermostability at 35°C and inhibition by excess of dihydroxyacetone phosphate. The αGPDH^f form was found to be most thermolabile and the αGPDH^s form most susceptible to the inhibition.

Introduction

Cytoplasmic glycerol-3-phosphate dehydrogenase (*sn*-glycerol-3-phosphate: NAD$^+$ 2-oxidoreductase, EC 1.1.1.8) carries out a number of diverse functions in carbohydrate and lipid metabolism. The enzyme catalyzes the reduction of dihydroxyacetone phosphate to glycerol 3-phosphate, on the one hand to maintain the cytoplasmic NAD$^+$ level during anaerobic glycolysis [1—3], and on the other hand as a counterpart of α-glycerophosphate cycle through which

glycolysis is coupled to oxidative phosphorylation [4—6]. The enzyme also plays a role in generation of cytoplasmic glycerol 3-phosphate for triglyceride and phospholipid synthesis [7—9].

The enzyme has been shown to exist as multiple forms (isozymes) in many kinds of animals [10—15]. These isozymes generally occur in different tissues or at different developmental stages of an organism. The organ-specific isozymes seem to be the product of separate genes, because of marked differences in their amino acid compositions and seem to have different physiological roles in vivo, because of striking differences in their catalytic properties [10,16].

Since alleles of the structural gene for this enzyme, which specify electrophoretic variants, allozymes, were identified in *Drosophila melanogaster* by Grell [17], the allelic variants have been found in several species including *Drosophila* [17—24]. Kozak [25] demonstrated that the purified allelic forms from mice are essentially indistinguishable with respect to several biochemical properties, whereas Miller et al. [26] reported, using partially purified enzymes, that the two allozymes from *D. melanogaster* are quite different in temperature-dependency of K_m values for dihydroxyacetone phosphate. Kinetical comparisons of the purified allozymes of the enzyme from *Drosophila* have so far not been reported. Furthermore, as earlier comparative studies on allelic forms of other enzymes [27—31] have revealed small differences, if any, in some biochemical properties, detailed comparisons are required.

Electrophoretic variants of the enzyme are present in natural populations of *D. virilis*. As shown by Ohba [23], these enzyme variants are products of the three alleles at the enzyme locus (αGDH^f, $\alpha GPDH^m$ and $\alpha GPDH^s$) which occur with the mean frequency of 0.0036, 0.9875 and 0.0089, respectively, in Japanese populations. This report deals with purification of three allelic forms (αGPDH^f, αGPDH^m and αGPDH^s) of glycerol-3-phosphate dehydrogenase from the homozygous strains for the alleles of *D. virilis*, and a biochemical characterization and comparison of the three forms.

Materials and Methods

Chemicals. NAD^+, NADH, glycerol 3-phosphate and *E. coli* alkaline phosphatase were purchased from Sigma Chemical Co. Sephadex G-100 was obtained from Pharmacia Fine Chemical Inc. DE-52 and DEAE-cellulose were from Whatman Ltd. and Brown Co., respectively. Hydroxyapatite gel was from Nihon Chemical Co. Dihydroxyacetone phosphate was prepared from dimethylketal dicyclohexylamine salt obtained from Sigma Chemical Co. Marker proteins for molecular determination (cytochrome *c*, horse heart myoglobin; whale sperm chymotrypsinogen A; beef pancreas, ovalbumin and bovine serum albumin) were obtained from Schwarz/Mann.

Stocks and enzyme sources. D. virilis stocks, homozygous for three different alleles ($\alpha GPDH^f$, $\alpha GPDH^m$ and $\alpha GPDH^s$) at cytoplasmic NAD^+-dependent glycerol-3-phosphate dehydrogenase locus, were isolated from isofemale lines collected at Omaezaki in Japan, 1978. Flies were grown on a sucrose-yeast medium in vials and 1- to 2-day-old adults were stored at −70°C until used for enzyme preparation.

Assays. Glycerol-3-phosphate dehydrogenase was routinely assayed by an

increase in absorbance at 340 nm in 0.1 M glycine-NaOH, pH 10.0, containing 30 mM glycerol 3-phosphate and 0.1 mM NAD^+. The activity for dihydroxyacetone phosphate reduction was followed by a decrease in absorbance in 0.1 M Tris-acetate, pH 6.75, in the presence of 0.1 mM dihydroxyacetone phosphate and 0.1 mM NADH. Assays were run at 25°C, unless otherwise stated. 1 unit of activity was defined as 1 μmol NAD^+ or NADH produced per min.

Protein concentration was determined either by the phenol reagent method [32] with bovine serum albumin as the standard or by the 280- to 260-nm absorbance method according to Kalckar [33].

Enzyme preparations. Enzyme preparations were carried out at 0—4°C.

60 g frozen flies were homogenized in 300 ml 0.1 M potassium phosphate buffer, pH 7.2. After centrifugation at 20 000 × *g* for 30 min, the supernatant is referred to as the crude extract. 1% protamine sulfate (0.20 volume of the extract) was added to the extract and the precipitate was removed by centrifugation at 20 000 × *g* for 15 min. Solid $(NH_4)_2SO_4$ was added to the supernatant to bring it to 80% saturation. After another centrifugation at 20 000 × *g* for 15 min, the pellet was saved, dissolved in 50 ml of 0.1 M potassium phosphate buffer, pH 7.2, and dialyzed overnight against 2 l of the same buffer. The resulting precipitate was discarded by centrifugation. About 20 g DEAE-cellulose (wet weight) equilibrated with 0.1 M potassium phosphate buffer, pH 7.2, were suspended in the supernatant fraction. After 1 h, the DEAE-cellulose suspension was filtered and then washed twice with equilibration buffer with the use of suction. The pooled filtrates were dialyzed overnight against 2 l of 10 mM Tris-HCl, pH 7.6. After elimination of precipitate by centrifugation, the supernatant was applied to a DEAE-cellulose column (4.4 × 20 cm) equilibrated with 10 mM Tris-HCl, pH 7.6, containing 1 mM $EDTANa_2$. The column was washed with 2 column vols. Tris-HCl buffer and the enzyme was eluted with 1 l linear gradient of NaCl (0—0.2 M) prepared in the same buffer. Enzyme fractions from DEAE-cellulose were pooled, concentrated to 10 ml by $(NH_4)_2SO_4$ precipitation, added to a Sephadex G-100 column (5 × 50 cm) equilibrated with 5 mM potassium phosphate buffer, pH 7.2, and subsequently run in the same buffer. In the purification of $\alpha GPDH^m$ and $\alpha GPDH^s$, the most active fractions from Sephadex G-100 were collected, and applied to a hydroxyapatite column (2.1 × 15 cm) equilibrated with the same buffer. The column was washed with 5 column vols 5 mM phosphate buffer and then elution was performed with 500 ml linear gradient of potassium phosphate, pH 7.2, from 5 to 200 mM. These enzymes were eluted at phosphate concentrations from 30 to 60 mM. The enzyme fractions, after dialysis overnight against 10 mM Tris-HCl, pH 7.6, containing 1 mM $EDTANa_2$ were loaded on a DE-52 column (4.4 × 20 cm) equilibrated with Tris-HCl buffer. After washing the column with 2 column vols. equilibration buffer, elution was performed with 500 ml of 0—0.2 M NaCl linear gradient. Since $\alpha GPDH^f$ was not adsorbed to the hydroxyapatite gel under these conditions, the enzyme fractions from Sephadex G-100 were loaded to a DE-52 column equilibrated with 5 mM potassium phosphate buffer, pH 7.2, after dialyzation against the same buffer. The activity of each form of the enzyme was found in NaCl fractions ranging from 40 to 60 mM. The active fractions from the DE-52 column were collected and the procedure for the previous DE-52 step was repeated, using 1000 ml NaCl

gradient instead of 500 ml. The purified enzyme was dialyzed against 5 mM Tris-acetate, pH 7.2, and concentrated in an Amicon Diaflo Ultrafiltration Unit using a UM 2 membrane.

Electrophoresis. Starch-gel electrophoresis was performed by the procedures of Shaw and Prasad [34]. Polyacrylamide gel electrophoresis was used to determine enzyme purity. Gels were stained for glycerol-3-phosphate dehydrogenase with 50 mM glycerol 3-phosphate, 5 mM NAD^+, 3 mM Nitrotetrazolium blue and 0.05 mM phenazine methosulfate in 0.05 M Tris-HCl, pH 9.0. Protein was stained by 0.1% Coomassie brilliant blue.

Molecular weight determination. An estimate of molecular weight was obtained by gel filtration on a Sephadex G-100 column [35] calibrated with cytochrome *c* (12 400), myoglobin (17 800), chymotrypsinogen A (25 000), ovalbumin (45 000), bovine serum albumin (68 000) and alkaline phosphatase from *E. coli* (89 000).

Subunit molecular weight was determined by SDS-polyacrylamide gel electrophoresis according to Weber and Osborn [36]. Cytochrome *c*, chymotrypsinogen A, ovalbumin and bovine serum albumin were used as standard for calibrations.

K_m determination. K_m values were determined with non-inhibitory ranges of substrate concentration: dihydroxyacetone phosphate, 0.02—0.35 mM; NADH, 0.003—0.08 mM; glycerol 3-phosphate, 0.5—30 mM and NAD^+, 0.077—1.5 mM. Slopes in the Lineweaver-Burk plots were determined by the least-squares method. Each K_m value was obtained from three to five replications.

Thermal inactivation. Thermal inactivation comparisons of the three allelic forms were performed at 35°C, using similar protein concentrations. Samples of the enzyme preparation were diluted into 0.1 M Tris-acetate buffer, pH 7.0, and heated in a shaking incubator. 0.1-ml aliquots were withdrawn at various periods of time, chilled in an ice water bath and assayed for enzyme activity.

Results

Purification

Starch-gel electrophoretic patterns for glycerol-3-phosphate dehydrogenase obtained from single-fly homogenates are shown in Fig. 1. The homozygotes for αGPDH alleles exhibit a major band and the heterozygotes, three bands. These observations imply a dimeric structure for the enzyme. The major band of homozygotes is sometimes accompanied by a faint, more electropositive band, which has the same mobility as that of the larval enzymes, as Wright and Shaw [14] described of *D. melanogaster*. Starch-gel electrophoresis revealed that the larval band was removed from the enzyme preparation with the step-5-procedure in Table I, which shows the results of one purification. Generally, 300—400-fold purification of the activity for each form was obtained by the procedures and overall yield of the enzyme ranged 20—25%. Similar results were obtained during the purification of the other two forms. Disc electrophoresis of the purified enzymes shows single protein bands coincided to enzyme activity (Fig. 2). Affinity chromatographic procedure applied to purification of this enzyme from *D. melanogaster* [37] was not useful for *D. virilis*, for inacti-

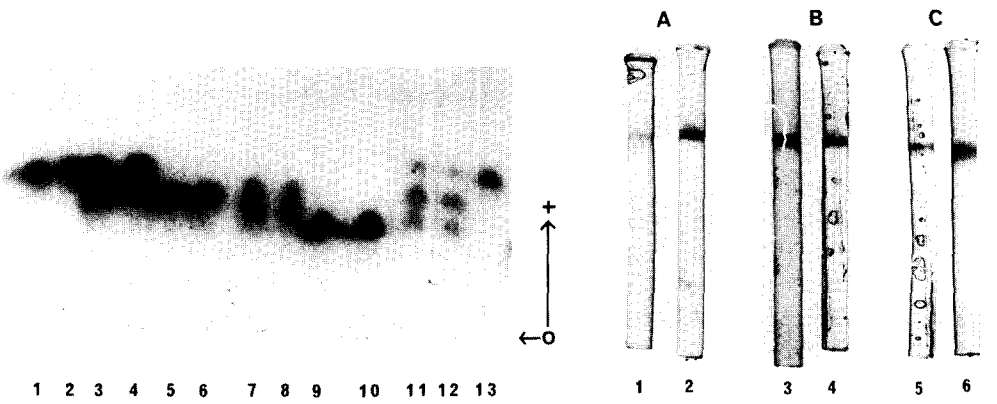

Fig. 1. Electrophoretic patterns for the glycerol-3-phosphate dehydrogenase in homogenates of single flies of *D. virilis*. From left, 1, 2 and 13, the homozygote for αGPDH^f; 3 and 4, the heterozygote for αGPDH^f and αGPDH^m; 5 and 6, the homozygote for αGPDH^m; 7 and 8, the heterozygote for αGPDH^m and αGPDH^s; 9 and 10, the homozygote for αGPDH^s; 11 and 12, the heterozygote for αGPDH^f and αGPDH^s.

Fig. 2. Polyacrylamide gel electrophoresis of the purified allelic forms of glycerol-3-phosphate dehydrogenase. Gels 1, 3 and 5 were stained for enzyme activity and gels 2, 4 and 6 stained with Coomassie brilliant blue. A, αGPDH^s; B, αGPDH^m; C, αGPDH^f.

vation and conformational change of the enzyme occurred when it was maintained at pH 6.5, at which the enzyme was adsorbed to the affinity gel.

Molecular weight

The molecular weight of glycerol-3-phosphate dehydrogenase by means of SDS-polyacrylamide gel electrophoresis was estimated as 35 000–37 000 and by Sephadex G-100 chromatography as approx. 65 000 with three allelic forms. These results show that this enzyme consisted of two similar subunits. These data are also consistent with glycerol-3-phosphate dehydrogenase from other animals sources [10,15,37–41].

TABLE I

PURIFICATION OF GLYCEROL-3-PHOSPHATE DEHYDROGENASE (αGPDH^s) FROM *DROSOPHILA VIRILIS*

Enzyme activity was measured using glycerol 3-phosphate as substrate.

Steps	Total protein (mg)	Total activity (unit)	Specific activity (unit/mg)	Yield (%)	Purification
(1) Crude extract	4622	944.6	0.20	100	1
(2) Protamine sulfate treatment	2453	940.5	0.38	99	1.90
(3) $(NH_4)_2SO_4$ precipitation	1864	861.0	0.46	91	2.30
(4) DEAE-cellulose treatment	1414	827.4	0.59	88	2.95
(5) DEAE-cellulose column	243	655.8	2.70	69	13.5
(6) Sephadex G-100 column	37	395.1	10.68	42	53.4
(7) Hydroxyapatite column	12	305.1	25.43	32	127
(8) DE-52 column I	8	245.7	30.71	26	154
(9) DE-52 column II	3	186.6	71.77	20	359

pH optima

The maximal activities of the three allelic forms were obtained within a pH range of 10.0—10.5 for glycerol 3-phosphate oxidation and 6.0—6.5 for dihydroxyacetone phosphate reduction.

Effects of temperature

Fig. 3 shows the effect of reaction temperature on the activity of the allelic forms. In the glycerol 3-phosphate oxidation at pH 10.0, αGPDH^f activity falls off at temperatures above 30°C, while both αGPDH^m and αGPDH^s activities partly decrease. In the dihydroxyacetone phosphate reduction at pH 6.75, similar tendency is observed. This fact indicates that these enzymes are sensitive to temperature and the αGPDH^f form is most thermolabile. So, thermal inactivation of the three forms was compared. As shown in Fig. 4, the activity of αGPDH^f is reduced to a greater extent than those of αGPDH^m and αGPDH^s by the treatment at 35° C. Moreover, the thermal inactivation is affected by incubation pH (Table II). When pH was lowered below 7.0, the enzyme inactivation increased and αGPDH^f, among the three forms, lost its activity.

Effects of substrate concentration

The apparent K_m values for the three allelic forms and their standard deviations are shown in Table III. K_m values for NAD^+, glycerol 3-phosphate and NADH are very similar among the three forms, but that for dihydroxyacetone

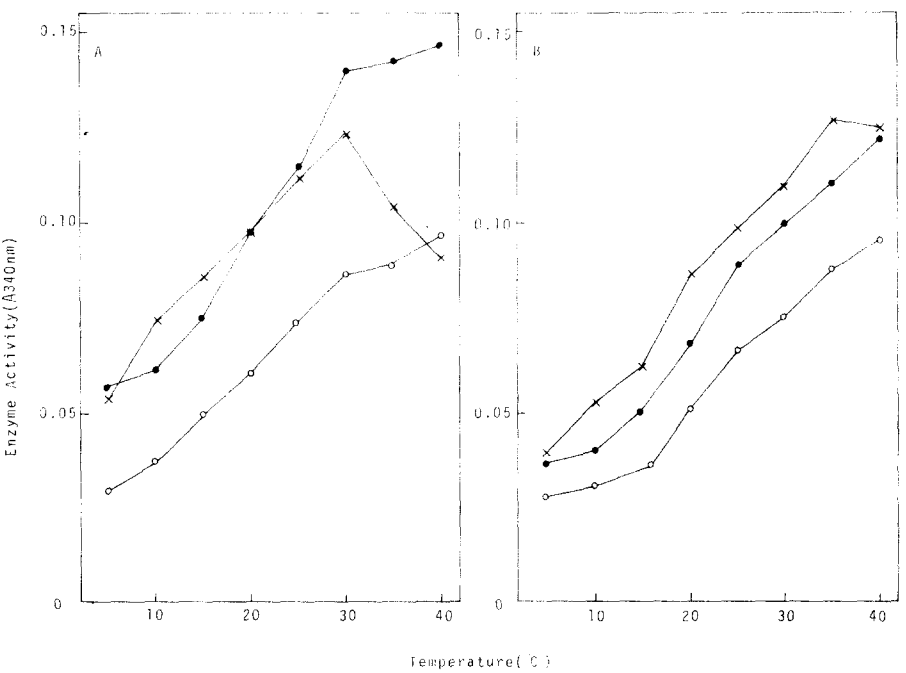

Fig. 3. The effect of reaction temperature on the activity of the allelic forms of glycerol-3-phosphate dehydrogenase. Enzyme activities were determined with glycine-NaOH, pH 10.0, for glycerol 3-phosphate oxidation (A), and with Tris-acetate, pH 6.75, for dihydroxyacetone phosphate reduction (B). X———X, αGPDH^f; ●———●, αGPDH^m; ○———○, αGPDH^s.

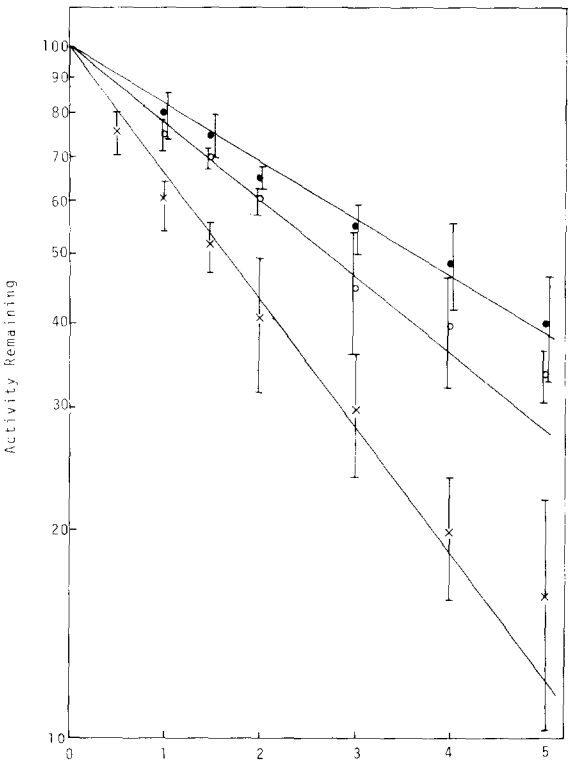

Fig. 4. Thermal inactivation of the allelic forms of glycerol-3-phosphate dehydrogenase at 35°C. Enzyme activities were measured with dihydroxyacetone phosphate as substrate. Bars around representative points indicate ± S.D. of the mean. X———X, αGPDH^{f}; ●———●, αGPDH^{m}; ○———○, αGPDH^{s}.

phosphate with αGPDH^{s} seems lower than those with αGPDH^{m} and αGPDH^{f}. Although the K_m values for dihydroxyacetone phosphate determined at 35°C were somewhat lower than the values presented in this table, no significant

TABLE II

EFFECT OF pH ON THERMAL INACTIVATION OF THE ALLELIC FORMS OF GLYCEROL-3-PHOSPHATE DEHYDROGENASE AT 35°C

Activity was measured at pH 6.75 with dihydroxyacetone phosphate as substrate. Values presented show the mean percentages of activity remaining in treated samples for that of the corresponding untreated samples. Activity of the enzymes retained 90—100% of initial activity, when the enzymes were maintained in ice-cold buffer for 2 h. Values ± show S.D.

pH	Activity remaining (%)		
	αGPDH^{s}	αGPDH^{m}	αGPDH^{f}
6.0	21.3 ± 18.0 *	5.1 ± 7.9 **	0 **
6.5	42.3 ± 5.5 **	35.3 ± 1.5 **	0 **
7.0	60.8 ± 3.3	65.3 ± 2.5	41.3 ± 8.9
8.0	61.1 ± 14.6	66.3 ± 7.1	42.7 ± 2.0
8.5	50.3 ± 15.6	40.3 ± 3.8 **	27.0 ± 4.0
9.0	24.7 ± 7.7 **	14.1 ± 6.2 **	11.2 ± 2.3 **

* Differ from the corresponding values at pH 7.0 at the 0.05 probability level.
** Differ from the corresponding values at pH 7.0 at the 0.01 probability level.

TABLE III

APPARENT MICHAELIS CONSTANTS FOR THE ALLELIC FORMS OF GLYCEROL-3-PHOSPHATE DEHYDROGENASE

The pH of the assay medium was 6.5 for NADH and dihydroxyacetone phosphate as substrate and 10.0 for NAD^+ and glycerol 3-phosphate. Values ± S.D.

Substrates	rep.	αGPDH^f	αGPDH^m	αGPDH^s
NADH (μM)	5	6.03 ± 1.05	6.54 ± 2.03	5.26 ± 0.05
Dihydroxyacetone phosphate (μM)	3	303.8 ± 55.3	309.0 ± 76.8	214.4 ± 21.5 *
NAD^+ (μM)	3	110.4 ± 13.0	107.4 ± 12.7	100.3 ± 4.1
Glycerol 3-phosphate (mM)	3	1.89 ± 1.17	1.77 ± 0.17	1.89 ± 0.25

* Differ from αGPDH^f and αGPDH^m at the 0.01 and 0.05 probability level, respectively.

differences were noticed.

The enzymes were not inhibited with 10-times concentrations of the K_m values for glycerol 3-phosphate, NAD^+ and NADH, but they were susceptible to inhibition by dihydroxyacetone phosphate. Fig. 5 shows that αGPDH^s activity is more susceptible than those of αGPDH^m and αGPDH^f.

Discussion

The three allelic forms of glycerol-3-phosphate dehydrogenase were purified to homogeneity from the homozygous strains of *D. virilis*. These allozymes are characterized by thermolability at 35°C and susceptibility to dihydroxyacetone phosphate inhibition. Although each of the purified forms showed a distinct behavior on both starch and polyacrylamide gel electrophoresis, the biochemical comparison revealed a number of similarities.

The one significant difference was observed in thermal stability (Fig. 3, 4

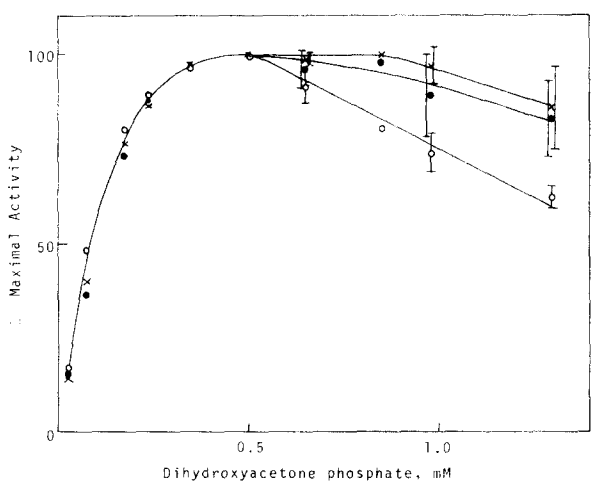

Fig. 5. The effect of the concentration of dihydroxyacetone phosphate on the activity of the allelic forms of glycerol-3-phosphate dehydrogenase. The vertical bars represent ± S.D. X———X, αGPDH^f; ●———●, α-GPDH^m; ○———○, αGPDH^s.

and Table II). The αGPDH^f form is more thermolabile than the other two forms at 35°C, pH 6.5. This indicates that the αGPDH^f enzyme is more unfavorable than αGPDH^m and αGPDH^s whenever cellular temperature and pH fluctuate within a physiological range. A comparison between two allelic forms of the enzyme from *D. melanogaster* has shown that αGPDH^f is more thermolabile than αGPDH^s at 39°C [42]. We found that electrophoretic mobilities of αGPDH^f and αGPDH^m from *D. virilis* correspond to those of αGPDH^f and αGPDH^s from *D. melanogaster*, respectively.

The other difference is the inhibition by dihydroxyacteone phosphate. Activities of the allozymes are inhibited with the concentrations slightly greater than their K_m values for dihydroxyacetone phosphate. αGPDH^s is more sensitive to this inhibition than the other two. Since the cellular concentration of dihydroxyacetone phosphate in insect is very close to the K_m value [43], this differential inhibition seems significant.

In the present study, the two forms specified by the rare alleles in *D. virilis* populations have been found to be inferior under some possible physiological conditions, but it remains to be demonstrated whether such a biochemical disadvantage is directly related to physiological deficiency in vivo.

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