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Isolation and Properties of Cytoplasmic α-Glycerol 3-Phosphate Dehydrogenase from the Pectoral Muscle of the Fruit Bat, *Eidolon helvum*

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Cytoplasmic α-glycerol-3-phosphate dehydrogenase from fruit-bat-breast muscle was purified by ion-exchange and affinity chromatography. The specific activity of the purified enzyme was approximately 120 units/mg of protein. The apparent molecular weight of the native enzyme, as determined by gel filtration on Sephadex G-100 was 59,500 ± 650 daltons; its subunit size was estimated to be 35,700 ± 140 by SDS-polyacrylamide gel electrophoresis. The true Michaelis-Menten constants for all substrates at pH 7.5 were 3.9 ± 0.7 mM, 0.65 ± 0.05 mM, 0.26 ± 0.06 mM, and 0.005 ± 0.0004 mM for L-glycerol-3-phosphate, NAD^+, DHAP, and NADH, respectively. The true Michaelis-Menten constants at pH 10.0 were 2.30 ± 0.21 mM and 0.20 ± 0.01 mM for L-glycerol-3-phosphate and NAD^+, respectively. The turnover number, k_{cat}, of the forward reaction was $1.9 \pm 0.2 \times 10^4$ s^{-1}. The treatment of the enzyme with 5,5'-dithiobis-2-nitrobenzoic acid (DTNB) under denaturing conditions indicated that there were a total of eight cysteine residues, while only two of these residues were reactive towards DTNB in the native enzyme. The overall results of the *in vitro* experiments suggest that α-glycerol-3-phosphate dehydrogenase of the fruit bat preferentially catalyses the reduction of dihydroxyacetone phosphate to glycerol-3-phosphate.

Keywords: Animal/bat flight, Fruit bat, Glycerol-3-phosphate dehydrogenase, Pectoral/breast muscle

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Introduction

There are two distinct forms of alpha-glycerophosphate dehydrogenase. There is a mitochondrial (particulate) flavin-linked glycerol-3-phosphate dehydrogenase (m-α-GDH, EC 1.1.1.99) and a cytoplasmic (soluble) NAD^+-linked glycerol-3-phosphate dehydrogenase (sn-glycerol-3-phosphate: NAD^+ oxidoreductase, α-GDH, EC 1.1.1.18) (Green, 1936; von Euler *et al*., 1937), which are capable of reducing dihydroxyacetone phosphate (DHAP) to L-glycerol-3-phosphate (α-GP). The cytosolic α-GDH, as well as the mitochondrial α-GDH, is of interest because it catalyses a reaction in the metabolic pathway that links glycolysis with lipid biosynthesis and breakdown. It is not surprising that the enzyme is reported to be present in many animal tissues and microorganisms.

The energy source for the long flight of bats, the only mammal capable of flight, is of particular interest from both an evolutionary and physiological viewpoints. The sustained flights of bats, such as in nocturnal foragings and migratory movement, may require energy that is derived from lipids from the fat deposit in the breast muscle in a similar way to that which occurs in insect muscles during flight (George and Jyoti, 1955; Bennakkers, 1969). Fruit bats probably satisfy their fat requirement through the endogenous conversion of carbohydrates, which form the majority of their diet (Okon *et al*., 1978). Fatty acid oxidation has long been recognized as the source of energy for migratory animals, such as insects (Gilbert, 1967; Bennakkers, 1969). Furthermore, the importance of α-GDH in the flight muscle of insects that use carbohydrate as fuel, in relation to the energy need during flight, has also been extensively studied (Chance and Sacktor, 1958; Sacktor, 1965). In these insects, the enzyme appears to be kinetically suited to the function in the glycerophosphate shunt. There is no information, to the best of our knowledge, on α-glycerol phosphate dehydrogenase from any bat tissue or organ.

In this study, we purified α-glycerol-3-phosphate dehydrogenase from the breast muscle of the fruit bat, *Eidolon helvum*, and evaluated some of its physical and catalytic characteristics. Our investigations suggest that the enzyme is kinetically favored towards the production of α-glycerol phosphate. Presently, it is impossible to confirm that cytosolic α-GDH of the fruit bat essentially catalyses the reduction of DHAP under physiological conditions. Further studies (such as the determination of the physiological pH, level of the substrates, and other equilibrium parameters) will hopefully help to elucidate the proper role of α-GDH in the bat muscle.

Materials and Methods

Materials DEAE-Sephacel, Reactive Blue 2-crosslinked agarose, dihydroxyacetone phosphate, DL-α-glycerol phosphate, D-fructose-1,6-diphospate, alcohol dehydrogenase (from Baker's yeast), aldolase (from rabbit muscle), α-nicotinamide adenine dinucleotide (oxidized and reduced forms), and creatine phosphate were purchased from the Sigma Chemical Company, St. Louis, USA. The other reagents that were obtained from Sigma Chemical Company are 5,5'-dithiobis-2-nitrobenzoic acid, adenosine-5'-triphosphate, and the gel filtration marker proteins. Sephadex G-100 was purchased from Pharmacia AB, Stockholms, Sweden. All the other reagents that were used were of analytical grade and obtained from either Sigma or BDH. The bats were collected at the International Institute for Tropical Agriculture (IITA), Ibadan, Nigeria.

Purification of cystolic α-glycerol-3-phosphate dehydrogenase from fruit-bat-breast muscle All the procedures were carried out at temperatures between 0-4°C. All the buffers contained 1 mM EDTA and 5 mM 2-mercaptoethanol. The fruit bats were collected from farms and brought into the laboratory in cages. Typically, a fruit bat was rendered unconscious by a blow on the back of the head. The breast muscle was quickly excised, blotted with filter paper, weighed, and placed in ice. Approximately $1,500 \times g$ (wet weight) of tissue (collected from 25 bats) was used for this preparation. A 50% (w/v) homogenate of the muscle was prepared in a 0.1 M sodium phosphate buffer, pH 7.2 (buffer A) using a Warring Blender. The homogenate was occasionally stirred for 1 h, then centrifuged at $12,400 \times g$ for 30 min at 4°C. The supernatant was filtered through a loose plug of glass wool and saved. The debris was resuspended and homogenized in one volume of the same buffer and recentrifuged at the same speed. The resulting supernatant solutions from the two homogenization steps were combined and the total volume was subjected to ammonium sulphate precipitation. The precipitate, obtained between a 40-70% $(NH_4)_2SO_4$ saturation, was collected after centrifugation at $30,000 \times g$ for 30 min and resuspended in a small amount of 5 mM Tris-HCl, pH 7.6 (buffer B). The suspension was dialyzed against two changes of buffer B.

The dialysate that was obtained was subjected to heating at 50°C for 3-5 min and was cooled rapidly in an ice-bath. The heat-inactivated precipitate was later removed by centrifugation at $30,000 \times g$. The supernatant was stirred into 200 ml of Reactive Blue 2-crosslinked agarose that was previously washed with several volumes of distilled water and equilibrated in buffer B. The slurry was packed into a 5×30 cm column and eluted with 800 ml of 0.5 M KCl in buffer B. The eluate was brought to 60% $(NH_4)_2SO_4$ saturation by the addition of a saturated-ammonium-sulphate solution (761.4 g/litre) in buffer B. The precipitate that formed after 10 h was collected at $30,000 \times g$ for 30 min, and was suspended in a minimal amount of the same buffer. The suspension was dialyzed for 12 h, with four changes against buffer B.

The dialysate, after clarification by centrifugation, was applied onto a DEAE-Sephacel column (2.5×40 cm). The column was first washed with 250 ml of buffer B, followed by elution with linear gradient (0-0.3 M KCl) of 400 ml in buffer B. The flow rate of the column was 30 ml/h. The active fractions (4 ml each) were pooled and concentrated by ultrafiltration in an Amicon PM 10 membrane at 30 psi. The concentrated sample was equilibrated by dialysis with 0.04 M sodium phosphate, pH 6.6 (buffer C), and rechromatographed on a DEAE-Sephacel column (2.5×40 cm) that was previously equilibrated in the same buffer. The column was eluted with buffer C at a flow rate of 30 ml/h until the absorbance at 280 nm of the eluate (in 4 ml fractions) was less than 0.05. The enzyme was not bound, and the active flow-through fractions were pooled and concentrated by ultrafiltration on the PM 30 membrane.

The partially-purified enzyme was further purified by affinity chromatography on a Procion Red P-Sepharose 4B column (1×2.5 cm) that was prepared by Balmforth (1982), then pre-equilibrated with buffer B. The column was first washed with 100 ml of buffer B before the elution of α-GDH with a 200 ml linear gradient of 0-0.1 M DL-α-glycerol-3-phosphate, according to McGinnis and Vellis (1974). The flow rate was kept at 60 ml/hr, and fractions of 4 ml were collected. The active fractions were pooled. The pool was first dialyzed against buffer C in order to remove DL-α-GP, then concentrated by dialysis in 50% glycerol in the same buffer. The final purification step on the Sephadex G-100 column (2.5×100 cm) was according to White and Kaplan (1969). The active fractions were pooled and dialyzed against 50% glycerol in 0.1 M sodium phosphate, pH 7.2.

Protein assay The protein concentration was measured during the purification procedures by the method of Warburg and Christian (1942). The protein concentration of the pure enzyme was determined by the method of Lowry *et al*. (1951) using bovine serum albumin as the standard.

Enzyme assay The α-GDH activity was measured spectrophotometrically at 25°C by following the disappearance of NADH that accompanies the reduction of DHAP (forward assay), or the oxidation of α-GP and formation of NADH (reverse assay), respectively, at 340 nm (Marquart and Brosemer, 1966; Lee and Craine, 1971; Gonzalez-Cerezo and Daziel, 1982). The fructose-1,6-bisphosphate (FBP)-linked assay of Marquart and Brosemer (1966) was used with a slight modification for the determination of activity during the purification procedures. The FBP-linked assay gave the same reaction rate as the forward assay, while the reverse assay was much slower. A typical assay mixture contained, in the final concentration, 50 mM TrisHCl, pH 7.5 (containing 1 mM EDTA and 2 mM 2-mercaptoethanol), 0.5 mM DHAP and 0.1 mM NADH for the forward reaction, and 80 mM glycine-NaOH, pH 10.0 (containing 0.5 mM EDTA), 0.3 mM 2-mercaptoethanol,

0.5 mM NAD^+ for the reverse reaction. The assay mixture may contain up to 20 mM hydrazine (Marquart and Brosemer, 1966; Lee and Craine, 1971; McGinnis and Vellis, 1974; Gonzalez-Cerezo and Daziel, 1982). The FBP-linked assay contained 75 mM Tris-HCl, pH 7.5 (containing 0.5 mM EDTA, 5 mM 2-mercaptoethanol, 0.14 mM NADH, 5 mM FBP, 20 mg of rabbit-muscle-triosephosphate isomerase, and 20 µg of rabbit-muscle-fructosebisphosphate aldolase.) In all cases, the unit of enzyme activity was defined as the amount of enzyme that catalyses the disappearance or formation of 1.0 µmol of NADH per min.

Polyacrylamide gel electrophoresis Polyacrylamide gel electrophoresis in the absence of SDS was performed on 7.5% gels on either the rod or slab apparatus in the Tris-glycine buffer solution, pH 8.9. The slab gel electrophoresis was performed according to LKB manuals for a 2050 Midget Electrophoresis unit, while the rod gel electrophoresis was according to the Pharmacia manual. The proteins were stained with Coomassie Brilliant Blue R, while the gels were stained for enzyme activity according to the methods of Fondy *et al*. (1971), and McGinnis and Vellis (1974).

SDS-PAGE was performed on the 10% rod or slab gel using the Tris-glycine or phosphate buffer system of Weber and Osborn (1975). The apparatus were as mentioned previously. The M_r standards that were used were obtained from Sigma (SDS-Dalton Mark VII-L kit, MW range 14,000-70,000).

Molecular weight determination The molecular weight of the native enzyme was estimated by gel filtration on a Sephadex G-100 column (2.5 × 85 cm). The column was equilibrated and eluted with 0.1 M sodium phosphate buffer, pH 7.2, at a flow rate of 14 ml/h. The void volume was determined with bovine thyroglobulin (M 669,000). The standard proteins that were used in calibrating the column were baker's yeast alcohol dehydrogenase (M 141,000), bovine serum albumin (M 66,000), egg albumin (M 43,000), bovine erythrocyte carbonic anhydrase (M 29,000), bovine pancreas chymotrypsinogen A (M 25,000), and horse-heart myoglobin (M, 17,000).

Amino acid analysis The amino acid composition of the purified enzyme was determined by an automatic LKB-alpha amino acid analyzer after a 24-h hydrolysis in 6 N HCl at 110°C at the University of Edinburgh, Scotland, through the kind assistance of Mr. Akindele Famurewa.

Kinetic studies The kinetic parameters were evaluated from the secondary plots according to the method of Florini and Vestling (1951). The determination of the kinetic parameters for the forward reaction that involved the reduction of dihydroxyacetone phosphate (and oxidation of NADH) was only performed at pH 7.5. The effect of glycerol-3-phosphate and NAD^+ concentrations on the velocity of the reaction in the reverse direction were measured at both pH 7.5 (in a 50 mM Tris-HCl buffer) and pH 10.0 (in a 80 mM glycine-NaOH buffer).

The effects of the added glycerol-3-phosphate on the rate of the forward reaction were studied at pH 7.5 by varying the concentration of glycerol-3-phosphate at two fixed but different concentrations of the DHAP, according to the method of Dixon (1953) as modified by Lee and Craine (1971).

Effect of creatine phosphate and ATP The sensitivity of the α-GDH activity to these metabolites was investigated, because of the possible roles of the compounds in the regulation of this enzyme (Guppy and Hochachka, 1974 a,b). The inhibitory effect of the creatine phosphate (CP) and ATP was studied at pH 7.5 at various concentrations of creatine phosphate between 5 and 20 mM while that of ATP ranged from 0.4 to 8.0 mM.

Reaction with 5,5'-dithiobis-2-nitrobenzoic acid (DTNB) The reaction of bat α-GDH with DTNB was carried out according to the method of Afolayan (1989). The modification was performed in a reaction mixture that contained 1.79 µM enzyme and 0.5 mM DTNB in a 0.05 M sodium phosphate buffer, pH 8.0 in the dark. The process was monitored by reading the absorbance at 412 nm and assaying for residual activity using the FBP-linked assay method. The number of reactive sulphydryl groups was calculated using the extinction coefficient of 1.36×10^4 M^{-1} cm^{-1} and 1.39×10^4 M^{-1} cm^{-1} in the presence or absence of 6 M guanidinium hydrochloride (Gdn-HCl) for the thionitrobenzoate (TNB) ion. The total number of the available sulphydryl (SH) groups was determined by carrying out the reaction in the presence of 0.2% SDS or 6.4 M Gdn-HCl.

Results

Enzyme purification The results of the purification procedure are summarized in Table 1. The 40-70% $(NH_4)_2SO_4$ precipitate contained more than 70% of the active enzyme in the crude homogenate. The procedure yielded a homogeneous α-GDH with a specific activity of about 120 units/mg of protein, representing an increase in purification of about 850-fold. All of the experiments that were presented in this report were carried out with this enzyme preparation. The enzyme was considered pure by the presence of a single band of protein after a polyacrylamide gel electrophoresis both in the presence and absence of SDS (figures not shown). Only one band each was obtained when the pure enzyme and crude extract were stained for α-GDH activity after the polyacrylamide gel electrophoresis under non-denaturing conditions (figures not shown).

Molecular weight determination The molecular weight of the native enzyme was estimated to be 59,500 ± 650 Da by gel filtration on Sephadex G-100. The subunit molecular weight estimated by SDS-PAGE was 35,700 ± 140 Da. This suggests that the fruit bat α-GDH is a dimer. The minimum subunit molecular weight that was estimated from the amino acid composition was 30,047 Da, which corresponds to a molecular weight of about 60,000 Da for the dimeric enzyme.

Kinetic studies Figure 1 shows the typical Lineweaver-Burk plot of the initial reaction velocity against the DHAP concentrations at fixed NADH concentrations at pH 7.5, as well the secondary slope and intercept plots. Table 2 presents a summary of the K_m and the V_{max} values, estimated from the secondary plots. Figure 2 shows the Dixon plot of the product

Table 1. Purification of fruit bat pectoral muscle α-glycerol-3-phosphate dehydrogenase

Step/Fraction	Volume (ml)	Total units	Total protein (mg)	Specific activity (units /mg)	Purification fold
Crude extract	3,600	11,600	85,300	0.14	1.00
40-70% ammonium sulphate precipitate	520	8,860	32,700	0.27	1.90
Heat treatment	505	7,750	11,800	0.66	4.71
Reactive Blue-Agarose batch elution	800	7,690	3,380	2.28	16.30
DEAE-Sephacel ion-exchange followed by ultrafiltration on PM 10 membrane	95	6,900	977	6.92	49.40
Rechromatography on DEAE-Sephacel followed by ultrafiltration on PM 30 membrane	30	5,990	482	12.40	88.80
Procion red P-4B coupled to Sepharose 4B	45	3,510	79.2	44.30	316
Gel filtration on Sephadex G-100 followed by concentration by dialysis in 50% glycerol	75	1,450	12.3	119	848

Each step was carried out as described in the text. Activity was measured by using the fructose-1,6-bisphosphate-linked assay that involved aldolase and triosephosphate isomerase. Protein was determined by the method of Warburg and Christian [13]. One unit of activity is the amount that will catalyzed the oxidation of 1.0 µmole of NADH/min. Other workers [12, 30] had defined one unit of their preparation as the amount of enzyme required to catalyze a 1.00 optical density change per min and this is equivalent to 0.48 µmole NADH/min.

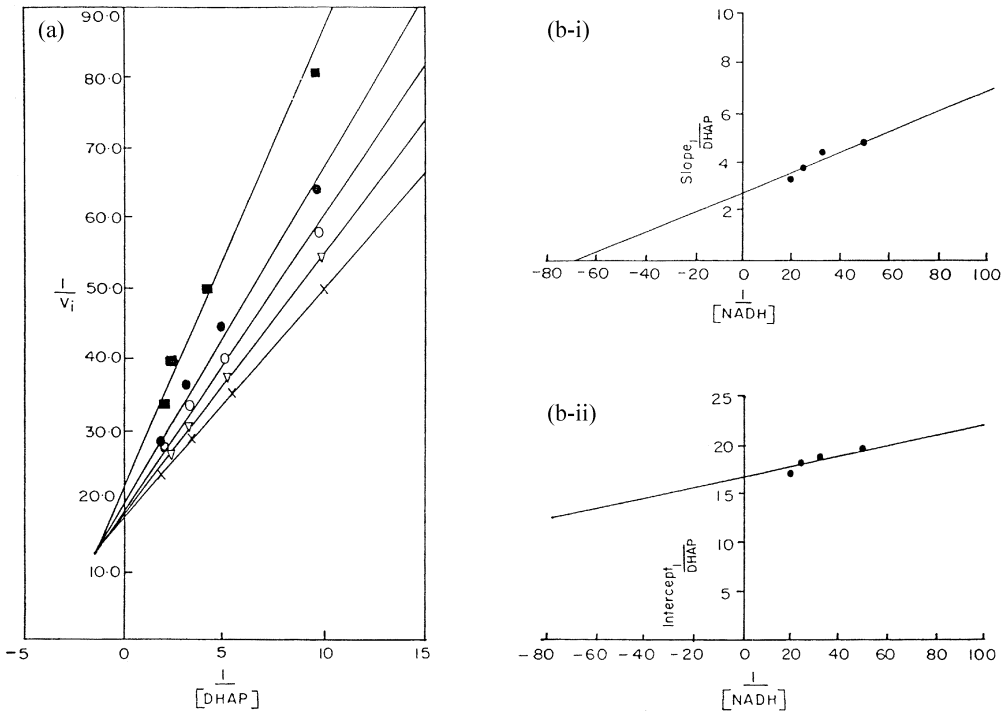

Fig. 1. Effect of DHAP concentration on the activity of fruit bat pectoral muscle α-glycerophosphate dehydrogenase. (a) The Lineweaver-Burk plot showing the effect of varying the concentraion of DHAP at different fixed NADH concentrations. The reaction mixture of 1 ml contained 50 mM Tris-HCl, pH 7.5, 10 µl of the enzyme (0.015 mg/ml), different DHAP concentrations (varied between 0.1 and 0.5 mM), and indicated amount of NADH: *viz* 0.01 mM (■), 0.02 mM (●), 0.03 mM (○), 0.04 mM (▽), and 0.05 mM (×). The initial velocity, V_i, of the reaction was recorded as change decrease in absorbance at 340 nm per min. (b) Secondary plots of data from the Lineweaver-Burk plots showing the effect of varying NADH concentrations. (i) $slope_{1/DHAP}$ replot (ii) $1/v_i$-axis intercept replot.

inhibition of the forward reaction (DHAP + NADH → G3P + NAD^+) by added glycerol-3-phosphate. The inhibition constant, K_i, for glycerol-3-phosphate was estimated to be 9.4 ± 2.9 mM. The nature of the inhibition is non-competitive with respect to DHAP.

Table 2. Kinetic parameters of fruit bat pectoral muscle α-glycerophosphate dehydrogenase

Parameter	Substrate							
	DHAP	NADH	GP			NAD^+		
pH	7.5[a,b]	7.5[a,d]	7.5[a,e]	10[b,e]	10[c,e]	7.5[a,d]	10[b,e]	10[c,e]
K_m (mM)	0.26 ± 0.05	0.005 ± 0.0004	3.9 ± 0.67	2.3 ± 0.21	2.6 ± 0.06	0.65 ± 0.06	0.20 ± 0.01	0.35 ± 0.05
V_{max} (units/ml)	20 ± 2.2	20 ± 2.2	1.1 ± 0.27	1.7 ± 0.36	1.9 ± 0.37	1.1 ± 0.27	1.7 ± 0.36	1.9 ± 0.37
V_{max} (μM/s)	330 ± 36	330 ± 36	18 ± 4.5	28 ± 5.9	31 ± 6.1	18 ± 4.5	28 ± 5.9	31 ± 5.9
k_{cat} (s^{-1})	19000 ± 2000	19000 ± 2000	51 ± 13	78 ± 17	86 ± 17	51 ± 13	78 ± 17	86 ± 16
k_{cat}/K_m x 10^{-5} (M^{-1} s^{-1})	870 ± 190	3300 ± 2700	0.14 ± 0.02	0.35 ± 0.03	0.38 ± 0.08	0.80 ± 0.07	4.1 ± 0.03	2.3 ± 0.68

All K_m and V_{max} values are means of five determinations. Assays were carried out in the following buffers: 50 mM Tris-HCl containing 1 mM EDTA and 1 mM 2-mercaptoethanol (a), 80 mM glycine-NaOH containing 0.8 mM EDTA and 0.3 mM 2-mercaptoethanol (b), and 80 mM glycine-NaOH containing 0.8 mM EDTA and 20 mM hydrazine sulphate (c). Values are for the forward reaction (d) and backward reaction (e). These kinetic parameters were evaluated from the secondary plots according to the method of Florini and Vestling (1951) following an ordered Bi Bi systesm for NAD^+-dependent dehydrogenase. One unit of activity is as defined in the text. The maximum velocity in mM/s is expressed as micromolar NADH concentration that appeared or disappeared per second. The k_{cat} (catalytic efficiency) was estimated from the expression, $V_{max} = E_T\ k_{cat}$, where E_T is the enzyme concentration in the assay mixture. k_{cat}/K_m is known as the specificity constant.

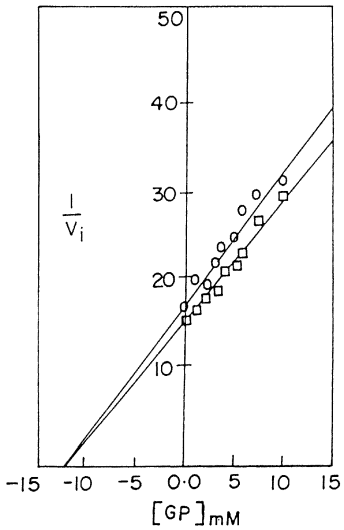

Fig. 2. Dixon plot of the inhibition of the forward reaction by glycerol-3-phosphate. The concentration of the inhibitor, α-GP was varied between 1 and 10 mM while the concentrations of DHAP were fixed at 0.4 mM (○) and 0.6 mM (□), respectively. For the details of the assay, see the text.

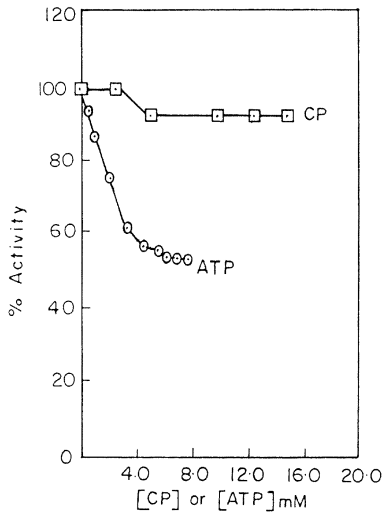

Fig 3. Effect of ATP and creatine phosphate on fruit bat pectoral muscle α-glycerophosphate dehydrogenase. Assays were carried out in 50 mM Tris-HCl, pH 7.5, and 0.5 mM NADH. The concentration of ATP was varied between 0.4 and 8.0 mM while that of creatine phosphate was between 5 and 20 mM.

Effects of creatine phosphate and ATP Creatine phosphate caused no significant inhibition of the fruit-bat-muscle α-GDH, with an inhibition that was less than 10% at 16 mM (Fig. 3). The enzyme was inhibited by ATP with a 35% inhibition at 3 mM ATP and almost 50% around 8 mM (Fig. 3).

Reaction with DTNB DTNB rapidly inactivated the enzyme at pH 8.0. This inactivation process increased with increasing concentrations of the reagent. After one hour, less than 20% of the activity remained in all of the reagent concentrations that were tested. The enzyme treatment with 0.5 mM DTNB at pH 8 released an average of 1.68 mole of TNB per mol of enzyme after one hour with consequent loss (over 93.4%) of the enzyme activity. The loss of activity followed a pseudo first-order kinetics with two phases (Fig. 4). The analysis showed an average of 0.82 SH groups that were modified per mole of the enzyme in the initial fast phase with a half-life of 4.48 min and a rate constant of 2.55×10^{-1} min^{-1}. In the slow phase, 0.8 SH was modified with a half-life of 37.96 min and a rate constant of 1.83×10^{-2} min^{-1}. When the enzyme was treated with 0.5 mM DTNB in the presence of SDS, about 8 moles of SH groups per mol of enzyme were modified. This represents the total number of sulphydryl groups in enzyme.

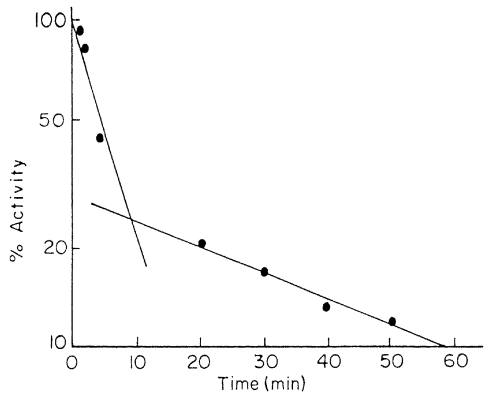

Fig. 4. Semilog plot of the rate of inactivation (loss of activity) during modification with DTNB. The enzyme solution (1.79 mM) was incubated with 0.5 mM DTNB in 0.05 mM sodium phosphate buffer, pH 8.0, in the dark at 25°C. Aliquots were removed at indicated times and assayed for the residual activity by the standard FDP linked method.

Amino acid composition Table 3 shows the amino acid composition of the enzyme. Cysteine and trytophan could not be quantitatively estimated after acid hydrolysis. Since the analysis was only obtained from a 24-h hydrolyzate, then the amino acids contents that were determined may be slightly underestimated.

Table 3. Amino acid composition of fruit bat pectoral muscle α-glycerophosphate dehydrogenase

Amino acid	Number of residue
Asp/Asn	24
Glu/Gln	11
Ser	13
His	12
Gly	16
Thr	16
Arg	10
Ala	41
Try	3
Met	11
Val	17
Phe	17
Ile	22
Leu	30
Lys	31
Trp	n.d
Pro	16
Cys[a]	8
Total residue	278
Total residue molecular weight	30,909
Total wt. of primary sequential unit[b]	30,927
Molecular weight of dimmer	61,854

The values for fruit bat α-GDH were obtained from the 24 h-hydrolyzate analysed by the LKB-alpha amino acid analyser. (a) Total weight of primary sequential unit represents the calculated molecular weight plus 18 due to the water associated with the terminal amino acid. (b) the total cysteine residue was determined by reaction with DTNB.

Discussion

Cytoplasmic α-glycerol-3-phosphate dehydrogenase from the pectoral (breast) muscle of the fruit bat, *Eidolon helvum* Kerr, was purified to homogeneity using a procedure that involved ion-exchange chromatography (DEAE-Sephacel) and affinity chromatographies on Reactive Blue 2-Agarose and Procion Red P-Sepharose 4B. The purified enzyme was stored in 50% glycerol or sucrose, since it was inactivated when stored in ammonium sulphate (Sellinger and Miller, 1959; Fondy *et al*., 1968). Although affinity chromatography was previously used for the purification of α-GDH by other workers (Kornbluth *et al*., 1974; McGinnis and Vellis, 1974; Ostro and Fondy, 1977; Koekmoer *et al*., 1995), the use of triazine dye as a ligand is novel. The affinity of various reactive chlorotriazinyl dyes for a wide range of proteins, and its application in enzyme purification and as site-specific inhibitors, has been discussed (Turner, 1981). The specific activity of α-GDH (120 units per mg of protein, pH 7.5) is, however, slightly lower than the ones that were obtained for the same enzyme from honeybee-flight and rabbit muscles (Marquart and Brosemer, 1966; Fondy *et al*., 1971)

The molecular weight of the purified enzyme is estimated to be about 60,000 with a subunit size of 36,000. The enzyme is, therefore, a dimer like the α-GDHs from honeybee (Brosemer and Marquart, 1966; Marquart and Brosemer, 1966), rat tissues (Fondy *et al*., 1968; 1971), rabbit muscle (Fondy *et al*., 1969), and chicken tissues (White and Kaplan, 1969), whose molecular weights range from 60,000 to 67,000.

The inactivation of the fruit-bat-muscle α-GDH by DTNB indicates that the two cysteine residues are freely reactive towards DTNB, out of the total number of eight that are present in the enzyme. The remaining cysteine residues are probably buried in the interior of the enzyme molecule. The biphasic nature of the inactivation process suggests an asymmetrical association of the subunits in which each subunit cysteine residue reacted differently with the inactivating reagent (Degani and Degani, 1979).

The results of the kinetic experiments indicate that the enzyme preferably catalyses the reaction that involves the reduction of DHAP and oxidation of NADH *in vitro*, because of the low K_m and high specificity constant (k_{cat}/K_m) values for both DHAP and NADH (Table 2). These kinetic parameters are comparable with data that was obtained for α-GDH from chicken skeletal muscle (White and Kaplan, 1969). It can, therefore, be suggested that the enzymes from the breast muscle of both the fruit bat and chicken will probably perform

similar functions. It has been suggested that chicken-breast-muscle α-GDH is involved in the regeneration of NAD^+ for maintaining maximum glycolytic activity for the anaerobic work (White and Kaplan, 1969). Generally, the formation of α-glycerol-3-phosphate and NADH oxidation would lead to either the generation of α-GP for phospholipid and triglyceride syntheses, the maintenance of the cytosolic NAD^+ level during anaerobic glycolysis, or the production of reducing equivalents in the form of α-GP for transport across the mitochondrial membrane, as elucidated in the glycerophosphate cycle (Ostro and Fondy, 1977). Alternatively, under aerobic conditions, as in insect flight muscles (with an abundant supply of tracheoles and densely-packed mitochondria) (Bennakker, 1969) and the oxygen-dependent squid mantle muscle (Guppy and Hochachka, 1978a), which possess significant carbohydrate-based aerobic potential, the α-GDH role is the reoxidation of NADH during carbohydrate degradation. Warkentin and Fondy (1973) suggested that α-GDH in rabbit liver is involved in the conversion of glycerol-3-phosphate to DHAP from glycerol during gluconeogenesis. It should be noted that α-GDH catalyses near equilibrium reaction in the cell; therefore, it is not considered a regulatory enzyme. The path of carbon flow from mainline glycolysis will depend on the level of NADH, α-GP, and creatine phosphate (Guppy and Hochachka, 1978a).

The results of the product inhibition experiment showed that glycerol-3-phosphate was a non-competitive inhibitor of the reduction of DHAP (Fig. 2). The sensitivity of α-GDH to feedback inhibition by α-GP determines its role(s) in the tissue of origin. Fruit bat α-GDH sensitivity to product inhibition by α-GP is low, because of its high K_i value. In almost all of the tissues that were studied, glycerol-3-phosphate is not only one of the substrates, but also one of the modifiers of the NAD-linked glycerol-3-phosphate dehydrogenase (Lee and Craine, 1971; Guppy and Hochachka, 1978 a,b; Brosemer and Marquart, 1966; Kito and Pizer, 1969).

Creatine phosphate levels did not significantly affect the activity of fruit bat α-GDH (Fig. 3). It is possible that the inhibitory effect that was observed at high concentrations of creatine phosphate may be the result of the high ionic strength that was introduced by the addition of this reagent (Sellinger and Miller, 1959). Creatine phosphate has been suggested as an alternative source of energy, at least for the initiation of flight in this bat (Afolayan and Daini, 1986). ATP inhibited the enzyme only about 35% at 3 mM. This probably means that if the ATP levels do not drop below 3 mM, then ATP may not be an important regulator of this enzyme. ATP has been reported to be a competitive inhibitor with respect to NADH of dehydrogenases (Holbrook *et al*., 1975).

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