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PURIFICATION OF α-GLYCEROPHOSPHATE DEHYDROGENASE FROM *DROSOPHILA MELANOGASTER*

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Summary

A simple procedure has been devised for the purification of α-glycerophosphate dehydrogenase (EC 1.1.1.8) from *Drosophila melanogaster*. The method involves substrate elution of the enzyme from a carboxymethyl cellulose column, followed by salt elution from agarose-hexane-AMP and DEAE columns. The procedure requires only 3 days to complete, results in high yield, and preparations that appear homogeneous by several criteria. A subunit molecular weight of 31 700 was obtained by sodium dodecyl sulphate electrophoresis in 10% acrylamide gels. This value is half that published for the native enzyme, confirming the homodimeric structure of this enzyme suggested by genetic evidence.

Introduction

α-Glycerophosphate dehydrogenase (L-glycerol-3-phosphate: NAD^+ oxidoreductase, EC 1.1.1.8) plays a central role in the production of energy in insect tissues, particularly the flight muscles [1]. It participates in the so-called α-glycerophosphate cycle, which is composed of two enzymes. One is the cytoplasmic, soluble, NAD^+-dependent dehydrogenase which reduces dihydroxyacetone phosphate to α-glycerophosphate, which readily diffuses into insect mitochondria. In the mitochondria, an NAD^+-independent, particle-bound flavoprotein, α-glycerophosphate oxidase converts α-glycerophosphate back to dihydroxyacetone phosphate. The electrons liberated by this reaction are passed down the electron transport chain with the concomitant production of ATP. This is the most important way in which glycolysis is coupled to oxidative phosphorylation in insect flight muscle.

The α-glycerophosphate cycle has recently been studied in *Drosophila*

melanogaster [2]. It was found that the cycle in this species essentially operates the same as in other insects. The soluble enzyme had previously been localized in the thoraces of adult flies [3]. The importance of the soluble enzyme to flight energetics was demonstrated by the fact that flies which lack α-glycerophosphate dehydrogenase activity ("null" mutants) cannot fly [4].

In *Drosophila*, this enzyme exists as different isozymes that are present in different proportions in larval and adult tissues [5]. Although these forms differ in kinetic parameters and heat stability, they are derived from the same structural gene [6]. However, the nature of the epigenetic modification that is responsible for these differences is not known. These multiple forms in *Drosophila* have been discussed with regard to the several metabolic involvements of α-glycerophosphate dehydrogenase [2].

Additionally, alleles of the structural gene for α-glycerophosphate dehydrogenase in *D. melanogaster* which specify "fast" (A/A) and "slow" (B/B) electrophoretic variants or allozymes, have been identified [5] and implicated in temporal changes in gene frequencies in natural populations [7]. The alleles are designated α-glycerophosphate dehydrogenase-1^A and α-glycerophosphate dehydrogenase-1^B, respectively.

So that the molecular basis of these phenomena can be studied, and so that the evolutionary changes in the enzyme can be precisely documented, a rapid and efficient procedure for the purification of this enzyme has been developed.

Materials and Methods

Culture of stocks. Stocks of *D. melanogaster* monomorphic for the A/A and B/B allelic forms of the enzyme were established from wild flies collected near Ithaca, N.Y. Flies were reared on standard cornmeal-molasses-agar food in pint bottles. Adults were stored frozen at −20°C until needed.

Assays. Activity was measured spectrophotometrically by following the production of NADH at 340 nm. The final concentrations of the reaction mixture were 1.5 mM NAD^+ and 2.6 mM α-glycerophosphate in 2.3 ml of 0.05 M glycine buffer, pH 9.6, plus 0.2 ml enzyme. One unit of activity was defined as 1 μmol of NAD^+ reduced/min per ml. All assays were performed at 25°C.

Protein determinations were made following the method of Lowry et al. [8].

Reagents. Carboxymethyl cellulose (CMC, Whatman CM-52) and diethylaminoethyl cellulose (DEAE, Whatman DE-52) were obtained from Reeve Angel, Clifton, N.J. Agarose-hexane-AMP (Ag-AMP, type II) was obtained from Pabst Laboratories, Milwaukee, Wisc. NAD^+ (Grade III), α-glycerophosphate (Grade X), dithiothreitol, bovine serum albumin (Fraction V), hemoglobin, lysozyme (Grade 1), and cytochrome *c* (type III) were obtained from Sigma Chemical Co., St. Louis, Mo. Ovalbumin, trypsin, and pepsin were purchased from Worthington Biochem. Corp. Freehold, N.J.

Columns. All columns were run in a cold room (4°C) at a flow rate of 45 ml/h. Further conditions of chromatography are given under Purification procedure.

Electrophoresis. Acrylamide disc gels were prepared according to Davis [9]. Sodium dodecyl sulphate acrylamide gels were prepared according to the

procedure of Laemmli [10]. Cellulose acetate electrophoresis was performed as previously described [11]. The procedures followed for immunoelectrophoresis are described in the Beckman Instruction Manual RM-TB-003B.

Antisera. Rabbit antisera to a crude *D. melanogaster* extract was a gift of Dr. Norman Arnheim.

Purification procedure

Homogenization. Frozen, adult flies were homogenized for 10 min in a Virtis homogenizer in 0.1 M sodium phosphate buffer, pH 7.1, that was 10^{-6} M NAD, 0.5 mM dithiothreitol and 1 mM EDTA. These additives were found to enhance the stability of the enzyme. The ratio of flies to buffer was 1 : 3 (g, wet weight : ml). The homogenate was centrifuged at 27 000 × *g* for 20 min and the supernatant filtered through glass wool.

Heat treatment. The filtered, supernatant was immersed in a 50°C water bath for 20 min, cooled on ice for 5 min, and centrifuged again at 27 000 × *g* for 20 min.

Concentration. If the extract was large (>100 ml), it was concentrated to approx. 50 ml in an Amicon Diaflo Ultrafiltration Unit (Model 202) using a UM10 membrane.

Dialysis. The heat-treated extract was titrated to pH 5.6 with 1 M acetic acid, dialyzed overnight (15 h) against 0.05 M sodium acetate buffer, pH 5.6, that was 10^{-6} M NAD, 0.5 mM dithiothreitol, and 1 mM EDTA. This was followed by dialysis against a 1 : 500 dilution of the same buffer.

CM-cellulose column chromatography. The dialyzed extract was then applied to a 2 × 50 cm column of Whatman CM-52 that had been equilibrated with 0.05 M sodium acetate buffer, pH 5.6. After absorption of the extract, the column was washed first with one column volume of equilibration buffer, then with one half column volume of equilibration buffer containing low concentrations of NaCl (0.09 M for the A/A form of the enzyme and 0.12 M for the B/B form), and finally with 1—2 column volumes of equilibration buffer alone. Equilibration buffer containing 0.01 M α-glycerophosphate and 0.02 M NaCl was passed through the column until α-glycerophosphate dehydrogenase was eluted. The pooled peak of activity was dialyzed against the acetate buffer to remove the α-glycerophosphate and NaCl.

Ag-AMP column chromatography. The dialyzed, pooled CM-cellulose peak was then applied to a 0.75 × 20 cm column of agarose-hexane-5′-AMP prepared in 0.05 M sodium acetate buffer, pH 5.6. This column was washed with two column volumes of buffer and the enzyme eluted with a 60-ml linear gradient of NaCl from 0 to 0.5 M. The pooled peak of activity was titrated to pH 7.5 with 1 M NaOH. It was dialyzed first against 0.1 M sodium phosphate buffer, pH 7.5, that was 10^{-6} M NADH, 0.5 mM dithiothreitol, and 1 mM EDTA, secondly against a 1 : 500 dilution of this buffer, and finally against distilled water until the conductivity was less than the equilibration buffer for the DEAE column.

DEAE column chromatography. The dialyzed, pooled peak from the Ag-AMP column was applied to a 0.75 × 30 cm column of Whatman DE-52 equilibrated with 10 mM sodium phosphate buffer, pH 7.5, that was 10^{-6} M NADH, 0.5 mM dithiothreitol, and 1 mM EDTA. The column was washed with two

column volumes of equilibration buffer and the enzyme was eluted with a 60 ml linear gradient of NaCl from 0 to 0.2 M.

Results and Discussion

Bewley et al. [6] have estimated that the larval form of *Drosophila* α-glycerophosphate dehydrogenase contributes approx. 25% of the total activity in crude extracts from *Drosophila* adults. These authors found that the larval form was heat labile and could be eliminated by heating the extract to 50°C for 20 min. This step was included early in the purification procedure to insure that only the adult form of the enzyme would be purified. Additionally, this heat step eliminated one quarter to one half of the total protein in the crude extract.

The enzyme was eluted from the CM-52 column with substrate rather than by the usual means of increasing ionic strength [12]. The column was first washed with concentrations of NaCl just below that required to elute the enzyme. These values were empirically determined for each of the two electrophoretic forms of the enzyme. α-Glycerophosphate dehydrogenase was then eluted with a mixture of substrate and salt that had a lower ionic strength than the salt wash itself (Fig. 1). The elution, therefore, was presumably due to the specific interaction of the enzyme with its substrate. This step resulted in an average of 30—40-fold purification across the peak. This is approximately five times the purification effected by a linear salt gradient. Peak tubes were as much as 85-fold pure. Although gradients of α-glycerophosphate have been used in a previous purification scheme for rat brain α-glycerophosphate dehydrogenase [13], this is the first time α-glycerophosphate has been used for the specific elution of this enzyme from an ion-exchange column.

There are a number of ways to elute the enzyme from the agarose-hexane-Ag-AMP column [14,15], a simpler linear salt gradient was chosen as being the easiest and most reproducible method (Fig. 2).

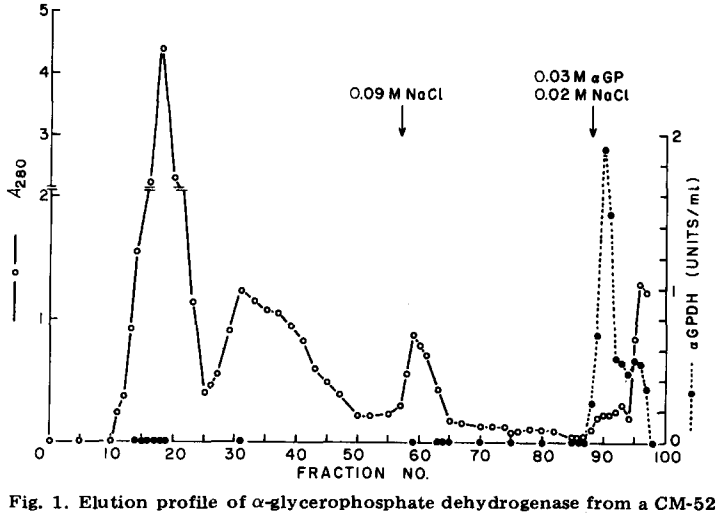

Fig. 1. Elution profile of α-glycerophosphate dehydrogenase from a CM-52 column. Details of the elution are given under Materials and Methods. Fractions of 6 ml were collected.

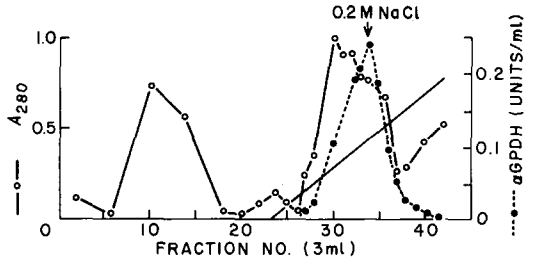

Fig. 2. Ag-AMP elution profile of α-glycerophosphate dehydrogenase. Details of elution are given under Materials and Methods. The salt gradient is indicated by the slanting straight line. The concentration of NaCl is indicated for the peak tube of activity.

The results of one purification are presented in Table I. Although these data are for the B/B form of the enzyme, similar values were obtained during the purification of the A/A form. Although the yield as presented is good, it actually represents a yield of well over 50% of the desired adult form of the enzyme, since the larval form was purposely eliminated in the heat step. Although a final purification of 500-fold may seem quite low if compared to purified bacterial enzymes, it is similar to values reported for other *Drosophila* enzymes [16,17]. The final purification of preparations from synchronous, aged adults has been even lower. Considering the importance of the enzyme to flight energetics, it is not surprising that the enzyme is relatively abundant in crude extracts.

The final product was judged pure by disc gel electrophoresis (Fig. 4), sodium dodecyl sulphate-acrylamide and 8 M urea-acrylamide electrophoresis (Fig. 4), and by cellulose acetate immunoelectrophoresis. After immunoelectrophoresis, only one precipitin arc was seen for the purified preparations while many precipitin arcs were seen for crude extracts. In Fig. 4, gel 7 shows the purified preparation of α-glycerophosphate dehydrogenase-1^A. Comparison with gel 8 demonstrates the coincidence of the activity and protein bands. Gel 9 is of a purified preparation of α-glycerophosphate dehydrogenase-1^B. It is included to show that the method can be used to obtain pure preparations of both allelic forms of α-glycerophosphate dehydrogenase in *D. melanogaster*.

TABLE I

PURIFICATION OF α-GLYCEROPHOSPHATE DEHYDROGENASE-1^B FROM *D. MELANOGASTER*

Step	Total units	Total protein (mg)	Specific activity (units/mg)	Fold purification	Yield (%)
Crude extract	406	1997	0.203	—	
Post heat	317	1425	0.223	1.10	78
Post concentration and dialysis	311	733	0.424	2.09	77
CM-cellulose peak	276	33	8.36	41.0	68
Ag-AMP peak	220	10	22.0	108	54
DEAE peak	176	1.71	103.5	510	44

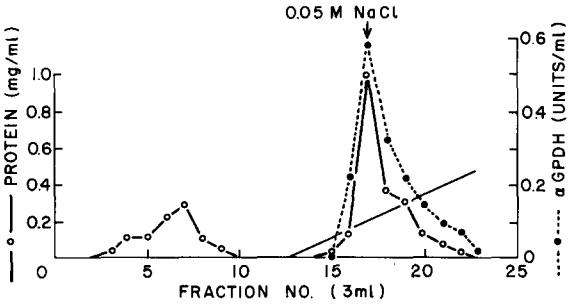

Fig. 3. Elution profile of α-glycerophosphate dehydrogenase from DE-52 column. Details of elution are given under Materials and Methods. The slanting straight line indicates the salt gradient with the concentration of NaCl indicated for the peak tube of activity.

The contaminants present in the Ag-AMP peak that are visible in acrylamide gels, were not detectable after electrophoresis on cellulose acetate membranes.

A subunit molecular weight estimate of 31 700 was obtained by sodium dodecyl sulphate electrophoresis in 10% acrylamide gels (Fig. 5), indicating that the native enzyme is composed of equal sized subunits. Gel filtration on a

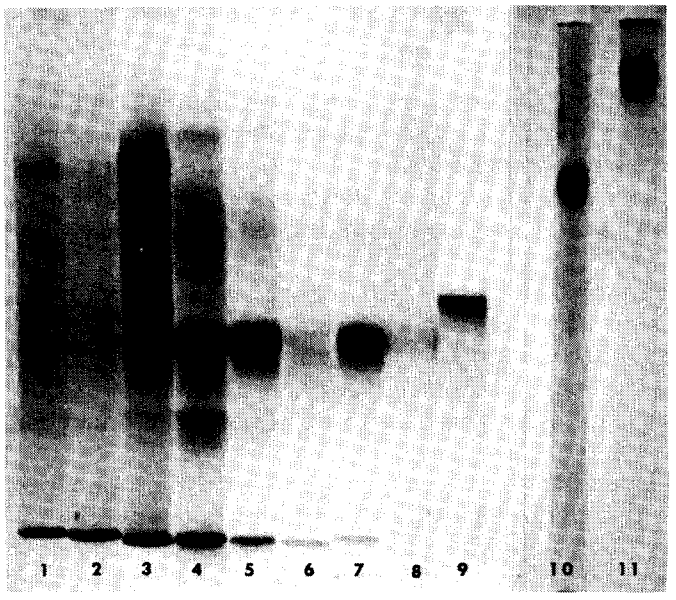

Fig. 4. Disc acrylamide gels containing 0.2-ml aliquots from each step in the purification procedure for the A/A form of α-glycerophosphate dehydrogenase. All gels, except No. 8, were stained with Coomassie brilliant blue. Gel No. 8 was stained for enzyme activity with a mixture containing 15 mg NAD^+, 3 mg phenazine methosulfate, 5 mg MTT tetrazolium, and 200 μg α-DL-glycerophosphate per 25 ml of 0.2 M Tris · HCl buffer, pH 8.6 [2]. From left to right the gels contain aliquots from the crude extract (1), heat-treated extract (2), concentrated and dialyzed post-heat extract (3), pooled CM-cellulose peak (4), Ag-AMP pooled peak (5), DEAE pooled peak (6), 10-fold concentrate of pooled DEAE peak (7), DEAE pooled peak (8), and a 10-fold concentrate of the pooled DEAE peak from the purification of the B/B form of the enzyme (9). Gel No. 10 is a 7.5% acrylamide-sodium dodecyl sulphate gel. Gel No. 11 is a 7.5% acrylamide-8 M urea gel. Both gels contained approx. 65 μg of purified *Drosophila* α-glycerophosphate dehydrogenase.

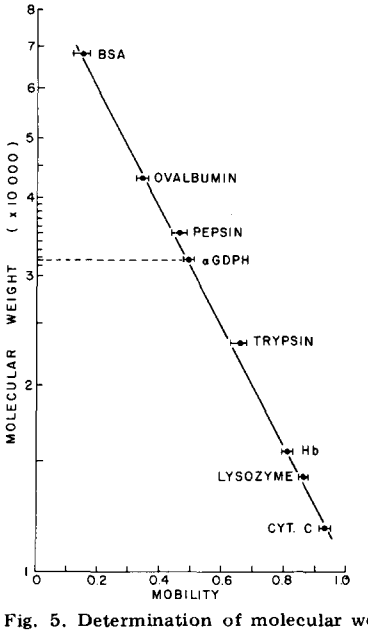

Fig. 5. Determination of molecular weight of *Drosophila* α-glycerophosphate dehydrogenase by sodium dodecyl sulphate-acrylamide electrophoresis. The standards used were bovine serum albumin, ovalbumin, pepsin, trypsin, hemoglobin, lysozyme, and cytochrome *c*. The horizontal bars represent the range of values for four separate determinations.

calibrated Sephadex G-75 column indicates a molecular weight of 67 000. Assuming the native enzyme is a dimer which is suggested by genetic evidence [5], these values are in excellent agreement with 66 000 and 63 000 molecular weight estimates for the native enzyme by Bewley et al. [6] and Rechsteiner [3], respectively, but are at variance with the 81 000 estimate by O'Brien and MacIntyre [2].

A value of 63 400 for the native enzyme is also in good agreement with the molecular weight estimates of the presumably homologous enzymes from honeybee [18], bumble bee [19], rat muscle [20], rabbit muscle [21], and chicken breast muscle. [22]. The estimates for these enzymes range from 58 300 for rat muscle α-glycerophosphate dehydrogenase to 65 400 for the honeybee enzyme.

The *Drosophila* enzyme is similar to the enzymes purified from honeybee and rat brain [13] in that it is inhibited by low concentrations of *p*-chloromercuribenzoate($ClH_9B_2O^-$) (100% inhibition at 50 μM $ClH_9B_2O^-$). The heat stability of the adult form of the *Drosophila* enzyme is also shared by the honeybee enzyme [18].

The method presented here for the purification of *Drosophila* α-glycerophosphate dehydrogenase is rapid (it can be completed in three days), provides a high yield, and is adaptable to different forms of the enzyme. With only minor modifications, it should be possible to use this method to purify this enzyme from most, if not all, species of *Drosophila*. To date, it has been successfully used to purify α-glycerophosphate dehydrogenase from five of six other *Drosophila* species tested. This will facilitate the study of the evolution of the enzyme within this genus. Also if the heat step is omitted and the

initial extracts are made from larvae, it may be possible to purify the larval isozyme, thus making it possible to study the molecular differences between the adult and larval isozymes.

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