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Properties of Arginine Kinase from *Drosophila melanogaster*

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1. Starch-gel electrophoresis of extracts of several strains of the genus *Drosophila* reveals neither allelic variants nor isoenzymes of arginine kinase. Arginine kinase of the drosophilid *Zaprionus vittiger* migrates differently.

2. Arginine kinase is non-uniformly distributed in tissues of *Drosophila melanogaster*. The activity in muscle tissue represents about 70% of the total activity. A characteristic fluctuation of the enzyme activity during the development from the egg to the adult stage can be observed.

3. Purification of arginine kinase of *Drosophila melanogaster* has been achieved. Several physico-chemical properties of the enzyme have been determined. The molecular weight in the range of 40000 is comparable to other arthropod arginine kinases and suggests that the active enzyme is a monomer.

There is increasing evidence of close phylogenetic relationships among the various vertebrate and invertebrate phosphagen kinases [1—3]. These enzymes which are found in several types of muscle tissue and in nervous tissue, are known to occur as isoenzymes in vertebrates as well as in some invertebrates.

Arginine kinase is the sole phosphagen kinase found in several major invertebrate groups, including arthropods, echinoderms and molluscs [1]. Arginine kinases from several echinoderms and molluscs have been found to be dimeric molecules, with molecular weights of approximately 80000 [4,5]. In contrast, the arginine kinases of arthropods have molecular weights of about 40000 and are monomeric enzymes [6]. Two electrophoretically distinct forms of monomeric arginine kinase have been observed in extracts of some arthropods; there is evidence that these are the products of different genes [7]. Most recently, arginine kinase has been purified from the housefly [8] and a crystalline preparation has been obtained from the thoraces of the honey bee [9]. We had several reasons for deciding to investigate the arginine kinase in *Drosophila*.

First, we considered arginine kinase, particularly if an electrophoretic variant could be found, a good candidate as a cell-specific marker for studies of cell lineage in the developing organism. With the many strains and mutants of *Drosophila* available, the organism was an obvious choice in a search for electrophoretic variants. An example of the application of the marker enzyme arginine kinase in a study of the development of insect musculature has recently been published from this laboratory [10].

Second, our interest in the comparative biochemistry of creatine kinases from various species [11] prompted us to investigate the related phosphagen kinases from invertebrates. This interest has been stimulated by the recent success of Watts and coworkers [12] in obtaining hybrid molecules between vertebrate creatine kinase and invertebrate arginine kinase.

Third, we chose to work with the *Drosophila* arginine kinase because of the advantages of this organism in studies of biochemical genetics and of genetic regulations of development. The corresponding vertebrate enzyme, creatine kinase, has already been the subject of investigations by those interested in the regulation of enzyme levels in different tissues and at different times during development [3,14].

The work to be described in this paper consists first of a survey of the electrophoretic patterns of arginine kinase of a number of strains and mutants of *Drosophila melanogaster* and related species. Second, we studied the tissue-distribution of arginine kinase and measured the changes in arginine kinase activity during the ontogeny of this organism (strain Oregon-R). Finally, in anticipation of further studies on the regulation of arginine kinase during development, including the need to prepare homogenous antibodies against arginine kinase, we undertook the purification and partial biochemical characterization of the arginine kinase from *Drosophila melanogaster*.

Enzymes. Arginine kinase (EC 2.7.3.3); creatine kinase (EC 2.7.3.2); lactate dehydrogenase (EC 1.1.1.27); pyruvate kinase (EC 2.7.1.40).

MATERIALS AND METHODS

30 wild strains and 7 mutant stocks of *Drosophila melanogaster*, *Drosophila hydei*, *Drosophila simulans*, *Drosophila bifasciata* and *Zaprionus vittiger* were screened.

To obtain material for enzyme purification flies of the strain Berlin$^+$ were grown in mass cultures according to Mitchell and Mitchell [15]. Adult flies were collected into gauze with a vacuum cleaner and stored frozen at −70 °C until used.

Third instar larvae of the strain Adh^{nl} of *D. melanogaster* [20] were dissected and carcass, gut, fat body, brain and salivary gland were prepared for enzyme tests. Several synchronized developmental stages of strain Oregon-R were obtained by timed egg laying.

Arginine kinase activity was assayed spectrophotometrically at 25 °C using the coupled enzyme test slightly modified from Blethen and Kaplan [6]. Our standard conditions were as follows: a mixture of glycine-NaOH buffer pH 8.6, final concn 0.1 M; magnesium acetate, final concn, 5 mM; KCl, final concn, 50 mM; ATP, final concn, 2.5 mM; phospho*enol*pyruvate, final concn, 0.75 mM; lactate dehydrogenase, final concn, 9 IU/ml; pyruvate kinase, final concn, 4 IU/ml; NADH, final concn, 0.45 mM; L-arginine, final concn, 15 mM, was incubated with an appropriate amount of enzyme.

The oxidation of NADH was monitored at 340 nm in a Gilford 2400 Recording Spectrophotometer. Activities are expressed in International Units (IU). The assay was linear with enzyme concentration. Samples with high ATPase activity were preincubated without arginine for 5 min and the reaction then started by adding L-arginine. Protein was determined spectrophotometrically according to Warburg and Chistian [16]. Enzymes and coenzymes were obtained from Boehringer Co., reagent grade substrates from Fluka AG.

Electrophoresis was done in EDTA-Tris-boric acid buffer at pH 8.6 in vertical, 14% starch gels according to Brewer [17], or in 7.5% acrylamide gels according to Davis [18]. Arginine kinase activity was visualized by an overlay technique developed for creatine kinase [19], with the following modifications: the reaction gels were prepared in 0.23 M glycine-NaOH buffer pH 8.6, and contained 1% agar, 100 mM KCl, 10 mM magnesium acetate, 5 mM ATP, 1.5 mM phospho*enol*pyruvate, 1.4 mM NADH, 7 IU/ml lactate dehydrogenase, 3 IU/ml pyruvate kinase and 30 mM L-arginine. Disappearance of NADH fluorescence was monitored with an ultraviolet lamp at 366 nm and recorded on Agfa Lupex LH-1 paper. Protein was visualized by the amidoblack staining technique. Hydrolyzed starch was obtained from Connaught, agarose from Serva. Reagent-grade acrylamide was used.

The molecular weight of arginine kinase was determined by gel-filtration through a Sephadex G-100 column (1.5 × 120 cm). The elution medium consisted of 10 mM Tris-HCl buffer pH 8.3, 0.5 mM dithiothreitol, 1 mM EDTA and 0.5% phenylthiourea. The following protein standards, dissolved in sucrose, were used for calibrating the column: ferritin (*MW* 460000, Fluka), bovine serum albumin dimer (134000, Fluka) pig heart lactate dehydrogenase (109000, Boehringer), bovine serum albumin monomer (67000, Boehringer), egg albumin (45000, Calbiochem), chymotrypsinogen A (25000, Calbiochem), trypsin inhibitor (21000, Calbiochem), myoglobin (17000, Calbiochem) and lysozyme (14000, Sigma). A partially purified preparation of *Drosophila* arginine kinase was used.

RESULTS AND DISCUSSION

Electrophoretic Patterns

Extracts of pupae, larvae and adult flies from *D. melanogaster* stocks were subjected to electrophoresis on starch gels and then stained for arginine kinase activity. Arginine kinase migrated identically in all cases, showing only a single band of activity. So did the arginine kinase from six members of the same genus, *D. hydei*, *D. simulans*, *D. bifasciata*, *D. subobscura*, *D. ambigua* and *D. tristis*. Only the arginine kinase of the drosophilid fly, *Zaprionus vittiger*, migrated differently, moving faster towards the anode. The electrophoretic patterns are shown in Fig. 1. Because successful transplants of imaginal primordia between larvae of *D. melanogaster* and *Z. vittiger* are possible, the electrophoretic variant present in the latter species could be used as a marker in a cell lineage study, as reported elsewhere [10]. No strain studies lacked arginine kinase activity; also, none was found with obviously low activity. Considering the major role of the enzyme in muscle contraction one would expect this result. The absence, in all cases tested, of any isoenzymes is in keeping with observations on arginine kinase from most other arthropods [6,7]. On the other hand, the failure to detect any electrophoretic variants in the *Drosophila* stocks is in contrast to the findings with many other enzymes from *Drosophila*. The evolutionary stability of the genes of phosphagen kinases from other sources has been noted previously [3]. Presumably the greater part of the structure of these enzymes is involved in indispensible interactions with other cellular components, so that the structural changes which can be tolerated are restricted.

Tissue Distribution and Development

The distribution of arginine kinases activity in different tissue of the larval organism is shown in Table 1. Arginine kinase is found primarily in skeletal

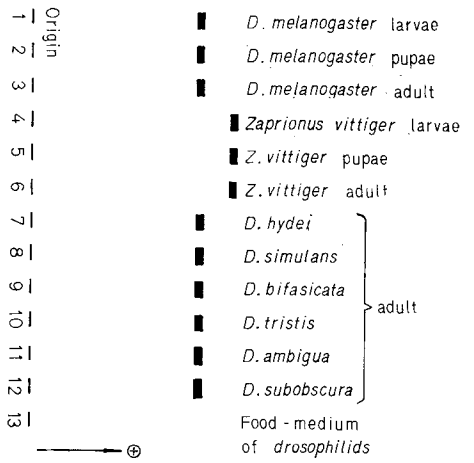

Fig. 1. *Arginine kinase in different drosophilids.* Starch-gel electrophoresis pattern of crude extracts. Conditions as described in Methods

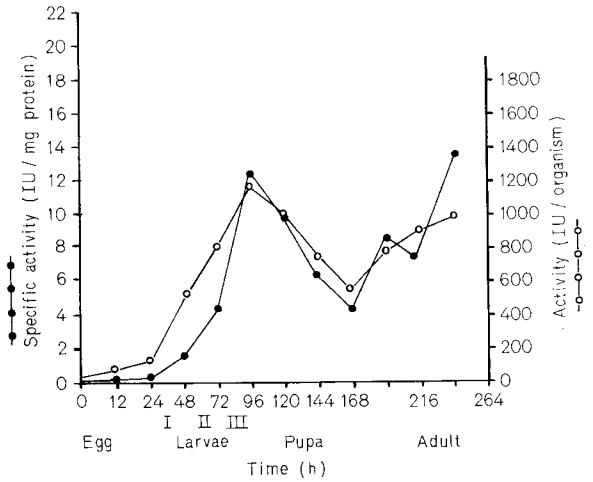

Fig. 2. *Developmental profile of arginine kinase activity in* Drosophila melanogaster

Table 1. *Tissue distribution of arginine kinase in third instar larvae of* Drosophila melanogaster *(Adh^{nt})*

Tissue	Activity
	% total
Muscle	73
Gut	22
Brain	3
Salivary gland	Trace
Fat body	None
Other tissue	2

Table 2. *Partial purification of arginine kinase of* Drosophila melanogaster

Step	Total protein	Total activity	Specific activity	Yield
	mg	IU	IU/mg protein	%
Crude extract	2900	7400	2.55	100
50–95% ammonium sulfate cut, after filtration through Sephadex G-25	760	5700	7.5	77
DEAE-cellulose	29	2800	97	38
Sephadex G-100	6.5	1000	154	12

muscle and gut. The absence of the enzyme in fat body is an advantage in cell-lineage studies because small amounts of fat body material rather frequently adhere to abdominal implants derived, *e.g.* from imaginal discs. From these results it may be concluded that developmental changes of the enzyme activity reflect mainly changes in the musculature where the bulk of arginine kinase is present. The arginine kinase activity of extracts of pooled intact organisms during larval and pupal stages of *Drosophila melanogaster* was determined and the electrophoretic pattern was shown. The single band of arginine kinase activity present in this stages corresponds to the one found in adult flies. A developmental profile is presented in Fig. 2. Similar profiles, with low activity in the egg, an increase during larval development, low activity again during metamorphosis, followed by a new increase after hatching, have been reported from other *Drosophila* enzymes [20]. The observed activity fluctuation correlates with the presence of functional muscle. At 144 h, according to Bodenstein [21], the last of the larval abdominal muscles have becomes histolyzed, while none of the imaginal muscles have yet become striated. The minimum in the developmental profile would thus appear to correspond to a point at which the larval musculature has been dissolved and at which its replacement by functional imaginal (adult) muscle has not yet begun.

Purification and Characterization

Arginine kinase was extracted and purified from 45 g frozen flies of the strain Berlin$^+$ of *D. melanogaster*. The nature of the starting material probably accounts for the comparatively low specific activity of arginine kinase in the crude extract of whole flies. This activity is up to 10 times lower than in other reported purification procedures of arthropod arginine kinase where muscle tissue alone instead of whole animals could be used [6, 22, 23]. A typical purification protocol is summarized in Table 2. Unless otherwise indicated all operations were carried out at 4 °C. The homogenate was centrifuged at $20000 \times g$ for 20 min and the supernatant was clarified by pouring through glass wool, adjusted to pH 7.5

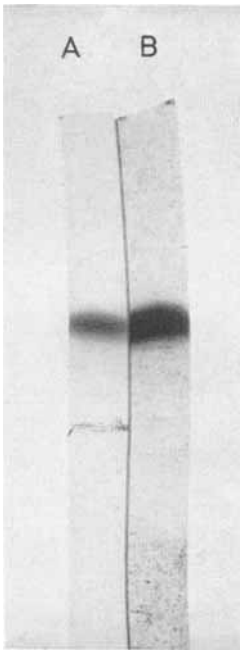

Fig. 3. *7.5% polyacrylamide-gel electrophoresis of purified arginine kinase.* Conditions as described in Methods. (A) 50 μg enzyme protein, (B) 100 μg enzyme protein

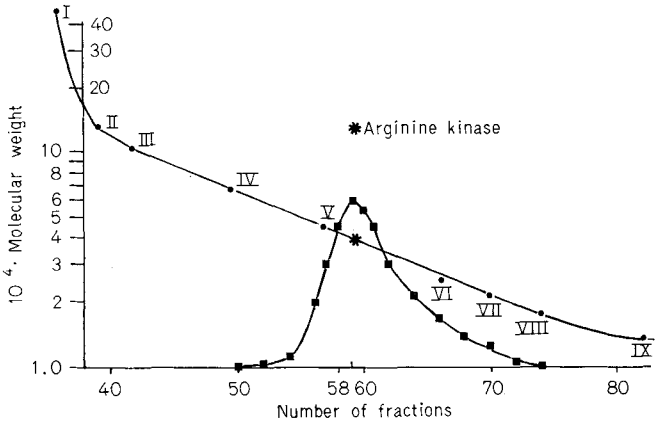

Fig. 4. *Molecular weight determination of* Drosophila melanogaster *arginine kinase by gel filtration through Sephadex G-100.* Conditions as described in Methods. (I) ferritin, (II) bovine serum albumin dimer, (III) lactate dehydrogenase, (IV) bovine serum albumin monomer, (V) egg albumin, (VI) chymotrypsinogen A, (VII) trypsin inhibitor, (VIII) myoglobin and (IX) lysozyme

Table 3. *Effect of pH and sulfhydryl reagent dithiothreitol on the stability of arginine kinase*
Specific activity of crude extract: 1.8 IU/mg protein (= 100%). The buffers used were: Tris-citrate pH 5.5/6.0, Tris-HCl pH 7.0/8.0 and glycine-NaOH pH 9.0/9.5

pH	Activity of enzyme dialyzed for 12 h	
	Without sulfhydryl reagent	With 0.5 mM dithiothreitol
	%	%
5.5	0.6	11
6.0	1.1	22.2
7.0	15	83.3
8.0	40.6	94.4
9.0	59.4	98.3
9.5	74.4	99.4

with ammonia and fractionated by addition of solid ammonium sulfate. The 50—90% cut was desalted on a Sephadex G-25 column and adsorbed to the top 2 cm of a DEAE-cellulose column (2.5×30 cm) previously equilibrated with a starting buffer of 5 mM glycine-NaOH pH 9.4 (4 °C), containing 0.5% phenylthiourea, 0.5 mM dithiothreitol and 1 mM EDTA. Elution was effected by a linear NaCl gradient. The fractions containing arginine kinase activity were pooled and concentrated in an Amicon pressure cell (PM-30 membrane). The concentrate was further chromatographed on a Sephadex G-100 column (1.5×120 cm) as described in Methods. The arginine kinase activity eluate as a single peak coinciding with the only protein peak. Acrylamide-gel electrophoresis of the concentrated peak fractions revealed one strong zone of protein, showing arginine kinase activity. There is also a minor fraction of a contaminating protein visible on the gel (Fig. 3). The purification achieved was about 60-fold with a rather low yield of 12%. This may have been caused by the high sensitivity of the enzyme towards oxidation. Since this behaviour is quite different from what is known of most of the other arthropod arginine kinases [6, 22, 23] we tested the stability of the enzyme by dialyzing it for 12 h in the presence and absence of sulfhydryl reagents at different pH values (Table 3). In the presence of the sulfhydryl reagent the enzyme was stable at pH values above 8.0. Blethen [7] reports on a similar behaviour of the so-called neutral arginine kinase isoenzyme from *Limulus*. The pH optimum of the arginine-kinase-catalyzed reaction was determined using Tris-citrate, Tris-HCl and glycine-NaOH buffer (0.1 M) ranging from pH 5 to 9.5. An optimum had been found between pH 8.6 and 8.9. Using 1:100 diluted samples of purified enzyme, the K_m value for L-arginine was measured being 0.15 mM. The heat stability was tested using extracts which were incubated for 15 min at temperatures ranging from 20 to 70 °C. The enzyme remained stable up to 45 °C but lost all its activity at 55 °C. Mg^{2+} ions (5—10 mM) were essential for the catalytic reaction. Mn^{2+} ions could replace Mg^{2+} but the enzyme activity was lower as compared to the activity with Mg^{2+}. These effects are comparable with those reported for *Limulus* [6] and housefly [8]. Finally we determined the molecular weight of the purified enzyme. Fig. 4 shows the gel-filtration profile from

which a value of 40000 ± 1500 was extrapolated. Nine reference proteins, mentioned in Methods, with known molecular weights had been used. There was no evidence of subunits. Therefore, arginine kinase seems to be a single-chain polypeptide as has been shown for various other arthropod arginine kinases.

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