Characteristics of Arthropod Arginine Kinases*

Sandra L. Blethen† and Nathan O. Kaplan

ABSTRACT: Arginine kinase has been purified from the muscle of two crustaceans, the hermit crab, *Pagurus bernhardus*, and the blue crab, *Callinectus sapidus*. Two forms of arginine kinase were separated and purified from muscle of the horseshoe crab, *Limulus polyphemus*. The two forms are designated "negative" and "neutral," according to their respective charges at pH 8.6. Negative and neutral arginine kinases were also found in extracts from a spider, *Pholucus phalangioides*, and a tarantula, *Dugesiella hentzi*. The negative form of the *Limulus* enzyme is more stable than the neutral, and its cysteine residues are less susceptible to oxidation in the absence of added thiols. Crude extracts from the grasshopper, honey bee, gypsy moth, and dragonfly were also examined electrophoretically, and each showed only one form of arginine kinase. The molecular weights of the purified enzymes, measured by sedimentation equilibrium, were about 38,000, and the molecular weights found for crude preparations using gel filtration ranged from 34,000 to 38,000. The amino acid compositions of the purified enzymes were relatively similar, and they contained 5 moles of cysteine/mole of protein. Rabbit antiserum to "negative" *Limulus* arginine kinase cross-reacted with the "neutral" form of *Limulus* arginine kinase, although semimicrocomplement fixation showed that its reaction with the neutral form was somewhat weaker. Anti-*Limulus* negative did not cross-react with any of the crustacean arginine kinases, nor did antiserum to lobster or blue crab arginine kinase cross-react with either of the *Limulus* enzymes. Antisera to crustacean arginine kinases cross-reacted with all of the insect extracts studied. Anti-*Limulus* negative reacted only slightly with bee extract and did not react with other insect extracts.

Arginine kinase (adenosine 5′-triphosphate–arginine phosphotransferase, EC 2.7.3.3) catalyzes the reversible formation of arginine phosphate and adenosine diphosphate from ATP and L-arginine. The enzyme is found in a wide variety of invertebrate species, and it is especially abundant in muscle where it serves a function analogous to that of creatine kinase in vertebrates. Crustacean muscle is a particularly convenient source of arginine kinase and purified preparations have been obtained from the following species: freshwater crayfish, *Potamobius astacus* and *Potamobius leptodactylus* (Elodi and Szorenyi, 1956), European lobster, *Homarus vulgaris* (Pradel *et al.*, 1964; Virden *et al.*, 1965), sea crayfish, *Jasus verreauxi* (Uhr *et al.*, 1966), and American lobster, *Homarus americanus* (Blethen and Kaplan, 1967). These enzymes have the following properties in common: a requirement for either magnesium or manganese ions, inhibition by sulfhydryl reagents, and a molecular weight between 36,000 and 43,000. Arginine kinase is similar to the other phosphagen kinases with respect to its metal ion requirement and its sensitivity to sulfhydryl reagents, but its molecular weight is about half of that reported for the others (Thoai *et al.*, 1965). However, recent gel filtration experiments with crude extracts from the sea urchin, *Paracentrotus lividus*, the siponcle, *Sipunculus nudus*, and a polycheate worm, *Travisia forbesii*, have indicated the existence of arginine kinases with molecular weights of about 80,000 (Thoai *et al.*, 1966). A detailed survey of echinoderm phosphagen kinases using gel filtration (Moreland *et al.*, 1967) has shown that echinoderm arginine kinases have molecular weights of 80,000.

Recent kinetic studies with sea crayfish arginine kinase (Uhr *et al.*, 1966) indicate that the reaction catalyzed by this enzyme occurs with the formation of a phosphorylated enzyme intermediate. This finding is in contrast to the mechanism proposed for rabbit muscle creatine kinase where kinetic evidence indicates that the reaction does not proceed with the formation of a phosphorylated enzyme as an intermediate but occurs with the formation of a ternary complex between MgATP, enzyme, and creatine (Morrison and James, 1965). This difference in mechanism between arginine kinase and creatine kinase may not hold for all arginine kinases, since kinetic experiments with arginine kinase from *H. vulgaris* (Virden *et al.*, 1965) suggest that this enzymatic reaction also proceeds *via* a ternary complex.

In this paper, we report the purification of arginine kinase from two other crustacean sources, the blue crab, *Callinectus sapidus*, and the hermit crab, *Pagarus bernhardus*. The properties of these two enzymes are compared with those of the other purified crustacean arginine kinases. A positive staining technique was developed and used to study the distribution of arginine kinase in the different organs of the American lobster; no evidence was found for the existence of arginine

* Publication No. 573 from the Graduate Department of Biochemistry, Brandeis University, Waltham, Massachusetts 02154. *Received February 1, 1968.* This study was aided in part by research support from the National Aeronautics and Space Administration (Contract NSG375) and the American Cancer Society, New Jersey Division (Grant P77J).

† Public Health Service postdoctoral fellow. Fellowship No. 5-F2-GM-30,707-02 from the National Institute of General Medical Sciences.

kinase forms with different electrophoretic mobilities in lobster. The arginine kinase activities of species representing different arthropod subphyla were also examined electrophoretically. The grasshopper, *Melanopus bruneri* (Scudder), the honey bee, *Apis millifera*, the gypsy moth, *Porthetria dispar*, and the dragonfly, *Sympetrum rubicundulum*, represented the *Labiata;* each had only one electrophoretically distinct form of arginine kinase. Three representatives of the *Celicerata* were studied. The horseshoe crab, *Limulus polyphemus*, represented the *Merostomata.*[1] A tarantula, *Dugesiella hentzi*,[2] and a spider, *Pholucus phalangioides*, represented the Arachnomorphs. Two arginine kinase forms with different electrophoretic mobilities were found in muscle extracts from *Limulus* and in spider and tarantula extracts. The two *Limulus* forms were separated and purified and some of their chemical and physical properties were studied. As far as we know, the *Limulus* isoenzymes are the first isoenzymes of arginine kinase to be purified although Virden and Watts (1964) have reported the existence of multiple forms of arginine kinase in extracts of some crustaceans. The two isoenzymes from the tarantula were also separated chromatographically and some of their properties have been compared with those of the two *Limulus* isoenzymes.

Experimental Procedure

Enzyme Assays. Arginine kinase activity was measured spectrophotometrically by a modification of the procedure of Wiesmann and Richterich (1964) described previously (Blethen and Kaplan, 1967). When working with crude extracts or other samples with high ATPase activities, the reaction was initiated by addition of L-arginine, and appropriate corrections were made for the reaction observed in the absence of arginine.

Protein. During the course of purification, protein concentrations were determined by the method of Warburg and Christian (1941) on samples which had been dialyzed to remove mercaptoethanol. The protein concentrations of purified enzyme preparations were determined from the absorbance at 215 and 225 mμ of dialyzed samples as described by Waddell (1956). The protein concentrations determined in this fashion were correlated with the absorbance at 280 mμ.

Electrophoresis. Starch gel electrophoresis was performed using buffers described previously (Blethen and Kaplan, 1967). Agar gel electrophoresis was carried out with gels containing 1% agarose (purchased from Fisher Scientific Co.) and 1.5% hydrolyzed starch (Connaught Medical Research Laboratories, distributed by Fisher Scientific Co.) in 0.03 M Veronal (pH 8.6). The gels were stored at 4° for at least 12 hr prior to use and were used within 10 days of preparation. A 1% solution of Amido Black was used to stain for protein. Purified lobster tail muscle arginine kinase was run as a standard.

Localization of Arginine Kinase Activity. Arginine kinase activity was located on the gels by a procedure similar to that of Eppenberger *et al.* (1964) for creatine kinase. A stock solution containing the components necessary for the assay was mixed with a 1.5% solution of agar (Difco Bacto Agar) in 0.23 M glycine (pH 8.6) which had been melted and cooled to about 45°. The final concentrations of reagents in this mixture were agar, 1%; KCl, 100 mM; $MgSO_4$, 10 mM; ATP, 5 mM; phosphoenolpyruvate, 1.5 mM; DPNH, 1.4 mM; L-arginine, 30 mM; lactic dehydrogenase, 70 μg/ml; and pyruvate kinase, 18 μg/ml. As a control for ATPase activity in crude extracts, gels were also stained with the same mixture lacking L-arginine. After the liquid agar mixture was poured over the gel surface, the gels were placed in a refrigerator for 30–45 min in order to allow the substrates to diffuse into the gel. Following this period in the cold, the gels were incubated at 37°. After 10 min at 37°, the gels were examined under an ultraviolet lamp. Those places where DPNH was oxidized to DPN appeared as dark spots on a fluorescent background. The incubation of the gels was continued for longer periods of time to allow weakly staining spots to be seen. In highly active preparations, activity could be detected after 30 min at 4°.

Immunology. Antibodies were prepared by the method of Plescia *et al.* (1964). Ouchterlony double-diffusion studies were carried out by the method of Stollar and Levine (1963), and semimicrocomplement fixation was done by the method of Wasserman and Levine (1961).

Ultracentrifugal Analysis. Sedimentation velocity experiments were done at temperatures between 18 and 21° at a rotor speed of 59,780 rpm. The protein concentrations used ranged from 7 to 10 mg/ml. When the sedimentation behavior of negative *Limulus* arginine kinase was examined at various concentrations from 8.9 to 1.5 mg/ml no concentration dependence of $s_{20,w}$ was found. The sedimentation equilibrium method of Yphantis (1964) was used to determine the molecular weights of purified preparations. Partial specific volumes were calculated from the amino acid composition by the method of Cohn and Edsall (1943). The molecular weights of arginine kinase in crude extracts as well as purified and partially purified preparations were determined by gel filtration on columns of Sephadex G-100 according to the method of Andrews (1964). Bovine serum albumin, ovalbumin, myoglobin, and cytochrome *c* were used as standard; Blue Dextran was used to determine the void volume of the columns.

Amino Acid Composition. Amino acid analyses were done according to the procedure of Spackman *et al.* (1958) on samples which had been dialyzed against 0.05 M ammonium bicarbonate and lyophilized prior to acid hydrolysis. Values for threonine and serine were obtained by extrapolating the values observed after 24- and 48-hr hydrolysis to zero time. Total half-cystine was determined from the cysteic acid content of samples which had been oxidized by performic acid (Schram *et al.*, 1954). Cysteine was determined by titration with hydroxymercuribenzoate in 8 M urea (Boyer, 1954).

[1] Until 1944, the *Limulus* was grouped with the arachnids. At that time, Størmer reclassified *Limulus* into its own class, the *Merostomata*. The *Arachnida* and *Merostomata* together make up the subphylum *Chelicerata* (Savory, 1964).

[2] The term "tarantula" is commonly used to mean any large spider of the family *Theraphosidae* (Savory, 1964).

In some cases, where removal of mercaptoethanol led to a loss of enzymatic activity, cysteine was measured in the presence of 1 mM dithiothreitol using 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB)[3] in a modification of Ellman's (1959) procedure described by Butterworth *et al.* (1967). The tyrosine to tryptophan ratio measured in 0.1 M NaOH (Goodwin and Morton, 1946) was used to calculate the tryptophan content.

Optical rotatory dispersion studies were carried out with a Cary Model 60 spectropolarimeter equipped with a thermostated cell holder. The constants of the Drude and Moffitt equations were calculated as described by Fasman (1963).

Buffers. The pH of Tris buffers used in the cold was determined at 5°. The pH of all other buffers was measured at 20°. All of the buffers used in preparing enzymes contained 1 mM mercaptoethanol and 1 mM EDTA.

Temperature Stability. Studies comparing the temperature stability of different arginine kinase preparations were carried out in 0.1 M potassium phosphate buffer (pH 7.5) containing 10 mM mercaptoethanol and 1 mM EDTA. The protein concentrations used were about 0.1 mg/ml, and samples were diluted into cold buffer prior to assay. Urea reactivation experiments were carried out as described previously (Blethen and Kaplan, 1967). Partially purified preparations which were studied with respect to temperature stability or reactivation on urea treatment were free from contaminating ATPase activity as judged by the oxidation of DPNH in the absence of L-arginine.

Materials

ATP and DPNH were obtained from P-L Laboratories. Phosphoenolpyruvate, phosphoenolpyruvate kinase, and cytochrome *c* were from Boehringer. Chicken heart lactic dehydrogenase was prepared in this laboratory by the method of Pesce *et al.* (1964). Crystalline bovine serum albumin was obtained from Pentex; Sephadex G-25 and G-100, Ficoll, and Blue Dextran were obtained from Pharmacia. DEAE-cellulose was type DE-11 from Reeve Angel. Live lobsters were obtained from a local market. A frozen Alaskan king crab was purchased from Wakefield Food Supply, Mount Vernon, N. Y., and a frozen South African rock lobster was obtained at a market in Vermont. Live blue crabs were from Seaboard Fish Co., Baltimore, Md. Live tarantulas and other spiders were from Carolina Biological Supply, Burlington, N. C. Live *Limulus* and live hermit crabs were obtained from the Marine Biological Laboratory, Woods Hole, Mass. Live honey bees were from Jackson Apiaries, Funston, Ga. Live grasshoppers were obtained from the U. S. Department of Agriculture, Bozeman, Mont., courtesy of Mr. F. Cowan. Dragonflies were caught locally by Mr. H. White. Gypsy moths were obtained from the U. S. Department of Agriculture Pest Control Division, New London, Conn., courtesy of Mr. R. Godin.

Enzyme Purifications. Callinectus sapidus. The muscle from fresh blue crabs was excised and minced with two volumes of distilled water (1 mM EDTA–1 mM mercaptoethanol). In one preparation, the crabs were frozen prior to removing the muscle; this preparation had an initial specific activity which was lower than that of preparations using fresh muscle (15.9 μmoles/min per mg compared with 21.4 μmoles/min per mg), but the electrophoretic properties and final specific activity of the purified preparations were the same. After standing at 4° for 3 hr, the muscle mince was centrifuged for 15 min at 13,200*g*. The precipitate was discarded and solid ammonium sulfate was added to make the supernatant solution 70% saturated in ammonium sulfate.[4] The precipitate which formed on standing was removed by centrifugation at 23,000*g* for 15 min and discarded. Solid ammonium sulfate was added to bring the supernatant solution to 75% saturation. After the supernatant solution from this step had stood at 4° for 2 days, fine crystals appeared. These were removed by centrifugation at 13,200*g* for 30 min. They were dissolved in 0.05 M Tris (pH 8.0); recrystallization was induced by adding solid ammonium sulfate to about 60% saturation and warming the solution gradually to room temperature. A summary of this procedure is given in Table I.

Pagurus bernhardus. The tail and claw muscle of fresh hermit crabs were homogenized separately each in two volumes of 1 mM EDTA–1 mM mercaptoethanol (pH 7.0). After standing at 4° for 1 hr, each mixture was centrifuged for 15 min at 23,000*g*; the crude extracts obtained in this way were examined electrophoretically, but only the tail muscle extract was used in the following purification.

Solid ammonium sulfate was added to make the crude extract 60% saturated. The precipitate obtained after centrifugation at 12,100*g* for 10 min was discarded, and solid ammonium sulfate was added to make the supernatant solution 80% saturated. The precipitate obtained after centrifugation at 12,100*g* was dissolved in 0.05 M Tris (pH 8.0) and dialyzed against this buffer for 3 hr. Inactive protein which precipitated during dialysis was removed by centrifuging and solid ammonium sulfate was added to 70% saturation. The precipitate obtained after centrifugation for 15 min at 12,100*g* was discarded and solid ammonium sulfate was added to make the final saturation of the supernatant solution 80%. The precipitate formed in this step was collected by centrifuging for 15 min at 23,000*g* and dissolved in 0.05 M Tris (pH 8.0). Attempts to crystallize the enzyme from this solution by the addition of ammonium sulfate were unsuccessful as an inactive precipitate formed when the enzyme was allowed to stand in concentrated ammonium sulfate solutions for long periods of time. The results of this purification procedure are shown in Table II.

Paralithodes kamtschatica. Frozen leg muscle from

[3] Abbreviation used that is not given in *Biochemistry 5*, 1445 (1966), is: DTNB, 5,5′-dithiobis(2-nitrobenzoic acid).

[4] Although the purification steps were carried out at temperature between 0 and 4°, the per cent saturation in ammonium sulfate was calculated on the basis of its solubility properties at 25°.

TABLE I: Purification of Blue Crab Arginine Kinase.

Step	Vol (ml)	Act. (units/ml)	Protein (mg/ml)	Sp Act. (μmoles/min per mg)	Total Act.	Yield (%)
Crude extract	1140	255.5	11.1	23.0	2.91×10^5	100
70% $(NH_4)_2SO_4$						
Precipitate	400	33	21.4	1.6	1.32×10^4	4.6
Supernatant solution	800	309	5.2	59.4	2.47×10^5	84.6
75% $(NH_4)_2SO_4$						
Precipitate	73	2180	23.0	95	1.58×10^5	54.3
Supernatant solution	800	35	1.5	24	2.80×10^3	0.96
Crystallization	30	5000	23.9	209	1.5×10^5	51.5

TABLE II: Purification of Hermit Crab Arginine Kinase.

Step	Vol (ml)	Act. (units/ml)	Protein (mg/ml)	Sp Act. (μmoles/min per mg)	Total Act.	Yield (%)
Crude extract	165	373	30.0	12.5	61,500	100
First $(NH_4)_2SO_4$, 60% saturation						
Precipitate	80	63	54.0	1.2	5,040	8.2
Supernatant solution	140	338	8.2	41.2	47,300	77
Second $(NH_4)_2SO_4$, 80% saturation						
Precipitate	11	2,620	15.7	167	28,800	46.9
Supernatant solution	153	2.9	6.0		440	0.7
Third $(NH_4)_2SO_4$, 70% saturation						
Precipitate	5.0	64.3	4.2	15.3	321	0.5
Supernatant solution	18.5	1,280	7.0	183	23,700	38.5
Fourth $(NH_4)_2SO_4$, 80% saturation						
Precipitate	8.2	2,870	14.4	199	23,550	38.3
Supernatant solution	21.5	57.2	0.4	143	1,230	2.0

an Alaskan king crab was homogenized in 0.05 M Tris (pH 8.0). After standing at 0° for 3 hr, the extract was centrifuged for 15 min at 12,100*g*. The supernatant solution which had a specific activity of 17.8 μmoles/min per mg was used for electrophoresis and immunodiffusion experiments.

Jasus lalandii. Frozen tail muscle from a South African rock lobster was homogenized in 0.05 M Tris (pH 8.0). The extract was allowed to stand at 0° for 3 hr and centrifuged for 15 min at 12,100*g*. The supernatant solution was used in electrophoresis and immunodiffusion experiments. Its specific activity was 24.7 μmoles/min per mg.

Homarus americanus. Live lobsters were obtained from a local market. Tail muscle arginine kinase was purified as described previously (Blethen and Kaplan, 1967). Crude extracts of other tissues studied were obtained by homogenizing them in 0.25 M sucrose and centrifuging the homogenate at 30,900*g* for 30 min. The distribution of arginine kinase in the various lobster tissues is shown in Table III.

Limulus polyphemus. The hinge muscle of frozen horseshoe crabs was dissected and homogenized with three volumes of 1 mM EDTA–1 mM mercaptoethanol (pH 7.5). The pellet obtained after centrifuging for 15 min at 4080*g* was reextracted with 1.5 volumes of the same buffer. Solid ammonium sulfate was added to 60% saturation. The precipitate obtained after centrifuging for 15 min at 4080*g* was discarded and solid ammonium sulfate was added to saturate the supernatant solution. The precipitate obtained from this step was collected by centrifugation at 4080*g* for 15 min, dissolved in 5

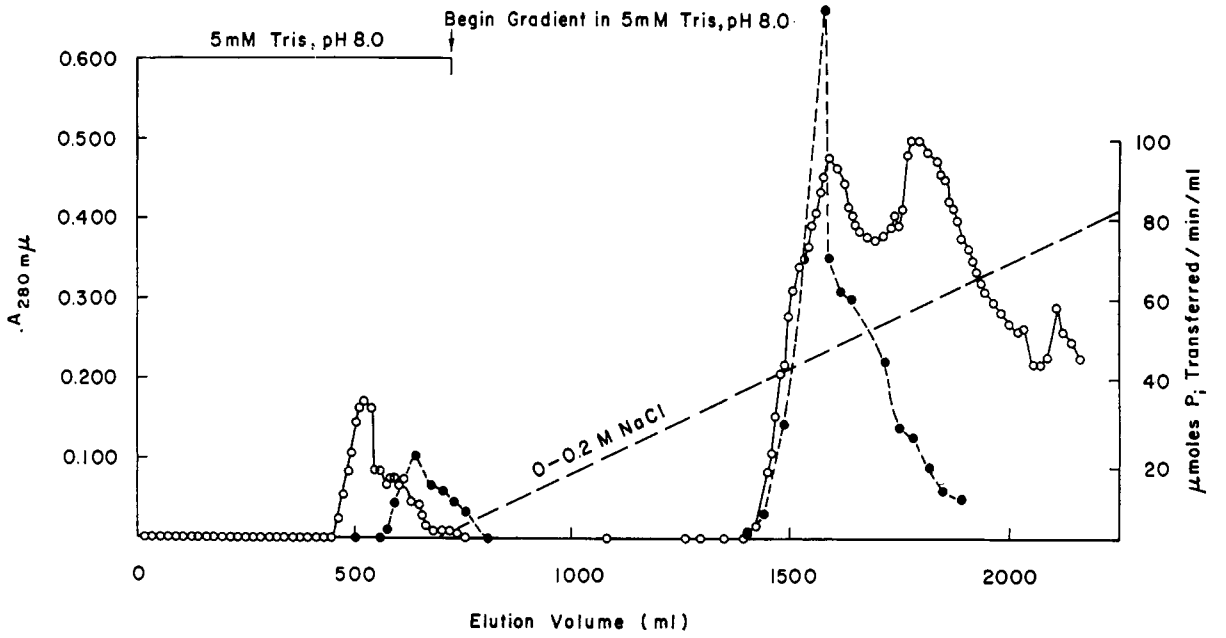

FIGURE 1: Separation of *Limulus* arginine kinase activities on DEAE-cellulose. The buffer used was 5 mM Tris, 1 mM EDTA, and 1 mM mercaptoethanol (pH 8.0); 7.18 g of protein was applied to a 5 × 40 cm column. The column was then washed with 750 ml of this buffer followed by a gradient between 750 ml each of 0 and 0.2 M NaCl. (O—O) A_{280}; (●—●) units of arginine kinase per milliliter.

mM Tris (pH 8.0), and dialyzed against the same buffer for 12 hr. The dialyzed protein solution was applied to a 5 × 40 cm column of DEAE-cellulose which had been equilibrated with the same buffer. The column was washed with 750 ml of this buffer and then with a gradient of NaCl from 0 to 0.2 M. Two peaks having arginine kinase activity were eluted from the column by this procedure (Figure 1). The tubes in each peak were pooled and concentrated with Ficoll. The concentrated solutions were then applied separately to a column of Sephadex G-100 and eluted with 0.05 M Tris (pH 8.0). The fractions with arginine kinase activity from each column were pooled and concentrated with Ficoll. A summary of the results of this procedure is given in Table IV. Both forms of arginine kinase appeared homogeneous on starch and agar gel electrophoresis. Both gave a single peak in sedimentation velocity experiments in the analytical ultracentrifuge and a straight line when the log (fringe displacement) was plotted as a function of the distance from the center of rotation in a sedimentation equilibrium experiment (Yphantis, 1964).

Dugesiella hentzi. The legs and bodies of frozen tarantulas were separated and the legs were split open with a pair of small scissors and homogenized with two volumes of 0.05 M glycine (pH 8.5). The bodies were cut open and the body wall muscle was scraped free with a scalpel and homogenized in two volumes of the same buffer. The initial specific activity was 32.1 μmoles/min per mg; 2 ml of extract was applied to a 1 × 23 cm column of DEAE-cellulose that had been equilibrated with the same buffer. The column was washed with 30 ml of this buffer and then with a stepwise gradient from 0.05 to 0.20 M NaCl. Two peaks having arginine kinase activity were separated (Figure 2). The tubes in each of the two arginine kinase fractions were pooled, concentrated with Ficoll, and chromatographed on Sephadex G-100 as described for the *Limulus* arginine kinases. The specific activity of the neutral fraction was 58.5 μmoles/min per mg (ATPase activity was reduced by this procedure to less than 0.1%), that of the negative fraction was 75 μmoles/min per mg.

TABLE III: Distribution of Arginine Kinase Activity in Lobster Tissues.

Tissue	Sp Act.[a] (μmoles/min per mg)	Content of Arginine Kinase (mg of arginine kinase/g wet wt of tissue)[b]
Tail muscle	25.9	24.2
Claw muscle	15.6	16.7
Heart muscle	11.6	31.0
Green gland	0.6	7.6
Gill	0.2	0.2
Brain	27.4	27.4
Intestine	1.1	0.9
Digestive gland	0.4	0.4

[a] Specific activity of a crude extract corrected for ATPase activity. [b] Calcd: a value of 212 units/mg of protein was used for the specific activity of purified lobster tail muscle arginine kinase.

TABLE IV: Purification of *Limulus* Arginine Kinases.

Step	Vol (ml)	Act. (units/ml)	Protein (mg/ml)	Sp Act. (μmoles/min per mg)	Total Act. (μmoles/min)	Yield (%)
Crude extract	2175	422	49.6	8.5	9.2×10^5	100
First $(NH_4)_2SO_4$, 60% saturation						
Precipitate	200	3.1	65.8		616	0.07
Supernatant solution	2400	283	21.2	13.3	6.8×10^5	73.8
Second $(NH_4)_2SO_4$, saturated						
Precipitate	178	1730	40.2	43.0	3.09×10^5	33.6
Supernatant solution[a]	2700	13.4	17.6	0.8	0.36×10^5	3.9
DEAE-cellulose chromatography						
Neutral	44	956	8.8	109	4.22×10^4	4.6
Negative	29	8030	50.7	158	2.33×10^5	25.3
Sephadex G-100						
Neutral	4.1	3440	14.6	235	1.4×10^4	1.5
Negative	34	5950	27.5	216	2.03×10^5	22.1

[a] The arginine kinase activity remaining in solution at this point is the neutral form.

Pholucus phalangioides. A mixed population of spiders was separated into types. The main species used was identified as *P. phalangioides* with the aid of the keys in Kaston and Kaston (1952). The legs and bodies were separated after the animals had been frozen, and each was homogenized with two volumes of 0.05 M glycine (pH 8.5). Crude leg extracts had a specific activity of 15.3 μmoles/min per mg, whereas the specific activity of the body extracts was 0.7.

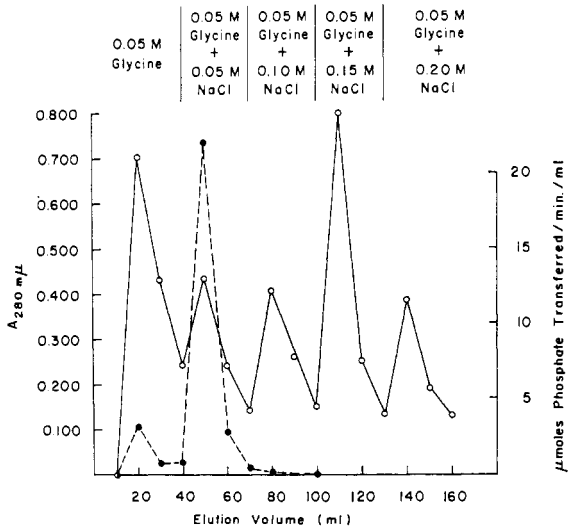

FIGURE 2: Separation of tarantula arginine kinase activities on DEAE-cellulose. The buffer used was 0.05 M glycine, 1 mM EDTA, and 1 mM mercaptoethanol (pH 8.5); 71 mg of protein was applied to a 1 × 23 cm column of DEAE-cellulose. The total volume of each elution step was 30 ml. (○—○) A_{280}; (●—●) units of arginine kinase per milliliter.

Melanopus bruneri. The legs of frozen grasshoppers were separated from their bodies and extracted with two volumes of 0.01 M Tris (pH 8.0). The specific activity of the crude extract was 15.1 μmoles/min per mg.

Apis millifera. Frozen honey bees were homogenized with two volumes of 0.05 M Tris (pH 8.0). The specific activity of a crude extract of whole bees was 0.3 μmole/min per mg.

Porthetria dispar. Whole gypsy moths which had been stored frozen were homogenized with two volumes of 0.05 M Tris (pH 8.0). The crude extract had a specific activity of 0.4 μmole/min per mg.

Sympetrum rubicundulum. Five frozen male dragonflies were homogenized with two volumes of 0.05 M Tris (pH 8.0). The specific activity of the crude extract was 1.6 μmoles/min per mg.

Results

Distribution of Arginine Kinase Activity and Arginine Kinase Isoenzymes. Figure 3 is a composite of the results obtained on agar gel electrophoresis of crude extracts from various arthropod sources. The varying degrees of shading indicate the length of time required for enzymatic activity to be visible. More than one spot with arginine kinase activity was found in the *Limulus*, tarantula, and spider extracts. The two forms are referred to as the negative and neutral indicating their respective charges at pH 8.6. The control stain from the spider and tarantula extracts, developed more slowly than did the section stained with L-arginine. In order to obtain a more quantitative picture of the relative amounts of arginine kinase and ATPase activities represented by the material remaining at the origin, the extracts were

TABLE V: Sedimentation Properties of Purified Arginine Kinases.

Source	$s_{20,w}$ (S)	Mol Wt[a]
Blue crab	3.20	37,000
Hermit crab	3.18	38,000
Limulus		
Negative	3.24	38,000
Neutral	3.24	38,000
American lobster[b]	3.25	40,000
European lobster[c]	2.69	42,000
European lobster[d]	3.18	37,000
Marsh crab[e]	2.5	43,000
Sea crayfish[f]	3.07	

[a] Measured from sedimentation equilibrium data and rounded to the nearest 1000. [b] Blethen and Kaplan (1967). [c] Pradel *et al.* (1964). [d] Virden *et al.* (1966). [e] Elodi and Szorenyi (1956). [f] Uhr *et al.* (1966).

chromatographed on DEAE-cellulose. As shown in Figure 2, two protein peaks with arginine kinase activity were separated from an extract of tarantula bodies. A similar separation of arginine kinase activities was obtained when an extract of spider legs was chromatographed in the same system. The first peak had 9.9 times as much activity in the presence of L-arginine as in its absence; the second had 212 times as much arginine kinase as ATPase activity. Chromatography of the first peak on Sephadex G-100 resulted in the separation of the arginine kinase and ATPase activities.

No evidence for the existence of arginine kinase isoenzymes in the American lobster was found when crude extracts of different tissues were examined electrophoretically. Similarly, no difference was observed in the electrophoretic mobilities of the arginine kinase activities of the claw and tail muscle of the hermit crab. The results obtained with fresh *Limulus*, lobster, and blue crab were identical with those obtained with preparations which had been stored frozen.

TABLE VI: Chromatography of Arthropod Arginine Kinases on Sephadex G-100.

Source	V_e/V_0	Mol Wt[a]
American lobster	1.68	38,000
Hermit crab	1.68	38,000
Blue crab	1.74	35,000
Limulus		
Negative	1.68	38,000
Neutral	1.71	37,000
Tarantula		
Negative	1.71	37,000
Neutral	1.69	38,000
Grasshopper	1.70	38,000

[a] ±2000.

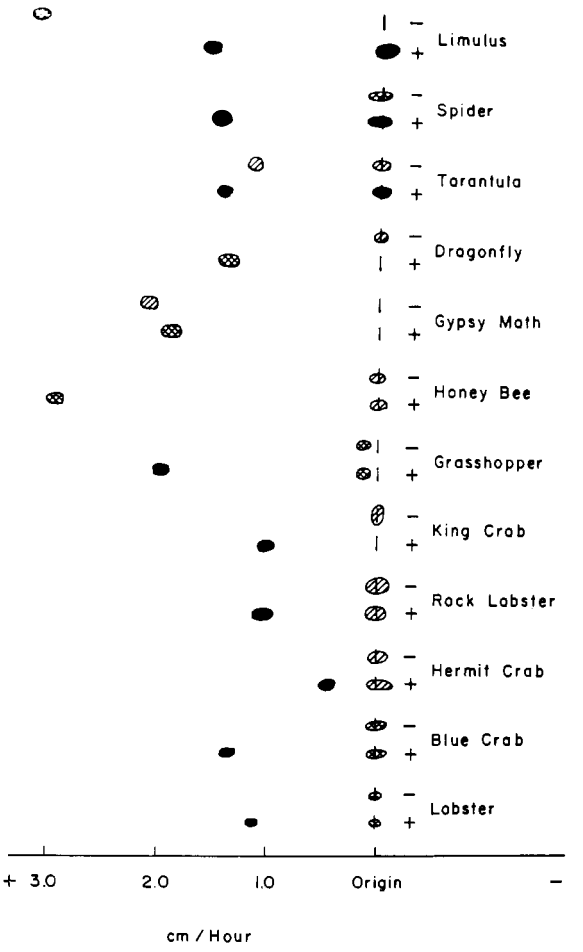

FIGURE 3: Agar–starch gel electrophoresis of arthropod arginine kinases. This is a composite picture of the electrophoresis carried out at pH 8.6 in 0.03 M Veronal buffer. A constant current of 0.32 mA/cm² was used. Purified *H. americanus* arginine kinase was used to standardize the experiments which lasted from 1.5 to 2 hr. Staining patterns obtained in the presence of L-arginine are designated $\pm$; those obtained in its absence, $\mp$.

Molecular Weights and Sedimentation Velocities. The sedimentation constants and molecular weights determined by sedimentation equilibrium of the purified arginine kinases described in this paper are shown in Table V along with the values determined for other purified preparations. The molecular weights of both crude and purified arginine kinases as determined by chromatography on Sephadex G-100 are shown in Table VI, and all gave almost identical results.

Amino Acid Compositions. The amino acid compositions of blue crab, hermit crab, and the two *Limulus* arginine kinases are given in Table VII.

Immunology. Table VIII gives a qualitative indication of the immunological similarities of the various arginine kinases with rabbit antibodies to purified blue crab, lobster, and negative *Limulus* arginine kinase. The reactions of the two *Limulus* arginine kinases with antibody to the negative enzyme as measured by complement fixation are compared in Figure 4a. The reactions of

TABLE VII: Amino Acid Composition of Arginine Kinases.[a]

Amino Acid	Residues/38,000			
	Blue Crab	Hermit Crab	Horseshoe Crab	
			Negative	Neutral
Lysine	29	32	32	29
Histidine	8	6	8	8
Ammonia[b]	16	20	12	22
Arginine	15	15	16	16
Aspartic acid	37	36	44	39
Threonine[b]	20	21	25	22
Serine[b]	16	20	21	19
Glutamic acid	41	33	36	40
Proline	11	13	13	15
Glycine	28	26	25	27
Alanine	21	23	17	21
Half-cystine[c]	5	5	5	5
Valine	23	19	16	17
Methionine	7	7	6	6
Isoleucine	13	13	15	17
Leucine	32	29	34	30
Tyrosine	11	9	9	10
Phenylalanine	15	18	16	18
Tryptophan[d]	2	2	3	3
Partial specific volume[e]	0.73	0.73	0.73	0.73

[a] The data on the blue crab and hermit crab enzymes represent the average of two analyses each after 24 and 48 hr of acid hydrolysis. The data for the *Limulus* isoenzymes represent the average of four determinations on preparations obtained at two different times. [b] Extrapolated to zero time from the values obtained after 24- and 48-hr hydrolysis in 6 N HCl. After hydrolysis for 3 hr in boiling 2 N HCl (Wilcox, 1967), the ammonia content of the negative *Limulus* arginine kinase was determined as 11 moles/mole and that of the neutral was 25 moles/mole. [c] Measured as cysteic acid after performic acid oxidation. [d] Calculated from the tyrosine to tryptophan ratios measured in 0.1 N NaOH. The ratios were 5.2, 3.8, 3.2, and 3.1, respectively. [e] In calculating partial specific volumes, it was assumed that each protein contained equal amounts of asparagine and glutamine.

TABLE VIII: Immunodiffusion Studies of Arginine Kinases.[a]

Antigen	Rabbit Antiserum to		
	Blue crab	Lobster	Negative *Limulus*
Blue crab	+++	++	−
Lobster	++	+++	−
Hermit crab	++	+++	−
Alaskan king crab	+++	+	−
South African rock lobster	+++	+	−
Grasshopper	±	±	±
Dragonfly	±	+	−
Gypsy moth	±	+	−
Honey bee	±	+	+
Spider negative	−	−	+
Spider neutral	−	−	+
Tarantula negative	−	−	+
Tarantula neutral	−	−	+
Limulus negative	−	−	+++
Limulus neutral	−	−	++

[a] Ouchterlony immunodiffusion experiments were performed as described in Stollar and Levine (1963). The following symbols indicate the strength of the reaction: +++, band visible in less than 6 hr; ++, band visible after 6–12 hr; +, band visible in 18–24 hr; ± faint band visible in 24–48 hr; − no reaction visible after 72 hr.

blue crab arginine kinase and the two tarantula isoenzymes with this antibody are included for comparison in Figure 4b.

Effect of Temperature. In Table IX are shown the temperature optima, ΔH values determined from the slopes of Arrhenius plots of log v against $1/T$, and stability at 50° for the arginine kinases purified in this study as well as values for the Hungarian marsh crab. It is interesting to note that the negative isoenzymes of *Limulus* and tarantula are more stable than the neutral forms. The stabilities of the two purified *Limulus* arginine kinases at various temperatures are shown in Figure 5.

Sulfhydryl Groups. The results of titrating purified arginine kinase preparations with hydroxymercuribenzoate in the presence and absence of urea are shown in Table X. All of the enzymes studied lost some activity when dialyzed to remove mercaptoethanol. This loss was particularly marked with the neutral *Limulus* arginine kinase. Since sulfhydryl groups are known to be important in enzymatic activity (Virden and Watts, 1966; Pradel *et al.*, 1965; Blethen and Kaplan, 1967) we investigated to see whether there were really fewer free sulfhydryl groups in the neutral enzyme or if an essential sulfhydryl group in the neutral enzyme was more susceptible to oxidation in the absence of added thiols. When the *Limulus* neutral enzyme was allowed to react with an excess of DTNB in the presence of 1 mM dithiothreitol, it bound 4.9 moles of thionitrophenylate/mole of enzyme. Similar results were obtained with the negative *Limulus* arginine kinase and the hermit crab enzyme. As with lobster arginine kinase (Blethen and Kaplan, 1967), L-arginine protected the enzymes studied here from inactivation by hydroxymercuribenzoate. This protection of the blue crab arginine kinase is illustrated in Figure 6. The K_m values measured for L-arginine in the presence of 5 mM ATP for each of the enzymes are shown in Table XI.

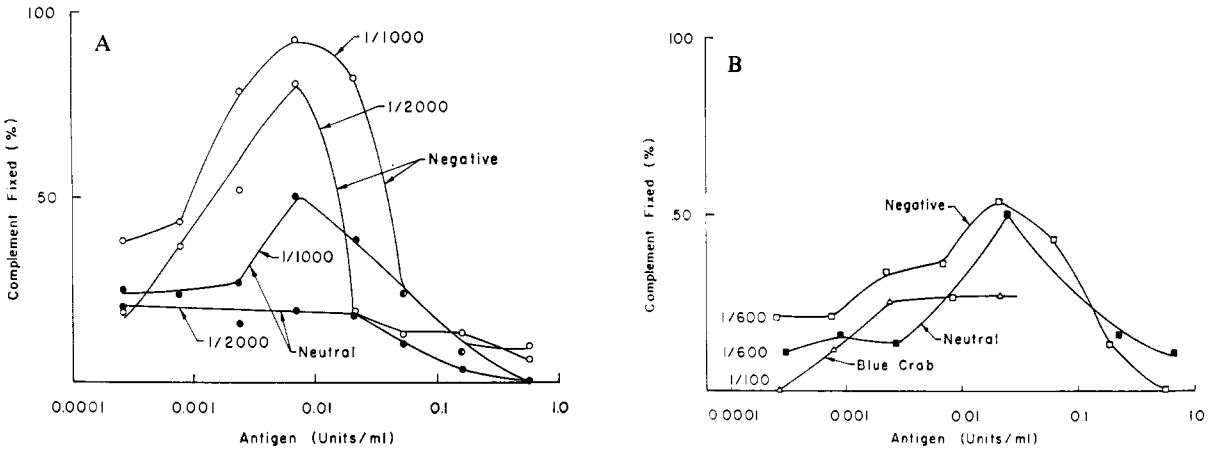

FIGURE 4: Complement fixation with antiserum to negative *Limulus* arginine kinase. The numbers on each curve indicate the antiserum dilution used: (O—O) *Limulus* negative, (●—●) *Limulus* neutral, (□—□) tarantula negative, (■—■) tarantula neutral, and (△—△) blue crab.

Optical Rotatory Dispersion. In Figure 7 are shown the optical rotatory dispersion curves for the two *Limulus* arginine kinases. Both proteins obeyed a one-term Drude equation between 245 and 310 mμ, and both had a λ_c of 242 mμ. Negative arginine kinase had an $[m']_{233}$ equal to −7680° and a b_0 of −293. Neutral *Limulus* arginine kinase had an $[m']_{233}$ of −4590° and a b_0 of −155.

Urea Reactivation. The results of experiments showing the effect of mercaptoethanol and L-arginine on the reactivation of hermit crab and blue crab arginine kinases are shown in Table XII. When diluted from 8 M urea into phosphate buffer containing 0.1 M mercaptoethanol, "negative" *Limulus* arginine kinase recovered 71% of its initial activity, whereas the neutral form recovered only 1.5%. Even the addition of 0.1 M mercaptoethanol to the urea solution did not increase the reactivation that was observed. The most reactivation obtained (4.9%) was found when the enzyme was diluted into 30 mM L-arginine. Negative *Limulus* arginine kinase had the same electrophoretic mobility on agar gel after reactivation as the untreated enzyme both with the activity and protein stains. When the neutral form was subjected to agar gel electrophoresis after treatment with 8 M urea and attempted reactivation, the protein stain gave a smear and no activity was found.

TABLE IX: Effect of Temperature on Arthropod Arginine Kinases.

Source	Optimum Temp (°C)	$-\Delta H$ (kcal/ mole per deg)	Stability at 50° (act. retained after 10 min) (%)
Lobster	40	10.7	2.6
Hermit crab	30	10.3	0.8
Blue crab	43	10.4	13.3
Limulus negative	45	9.6	75.9
Limulus neutral	45	10.0	44.3
Tarantula negative	>45	11.1	94.0
Tarantula neutral	45	10.2	74.0
Hungarian marsh crabs			
P. astacus	30	7.9[a]	
P. leptodactylus	30	9.9[b]	

[a] Calculated from an average Q_{10} of 1.70. [b] Calculated from an average Q_{10} of 1.96 (Elodi and Szorenzi, 1956).

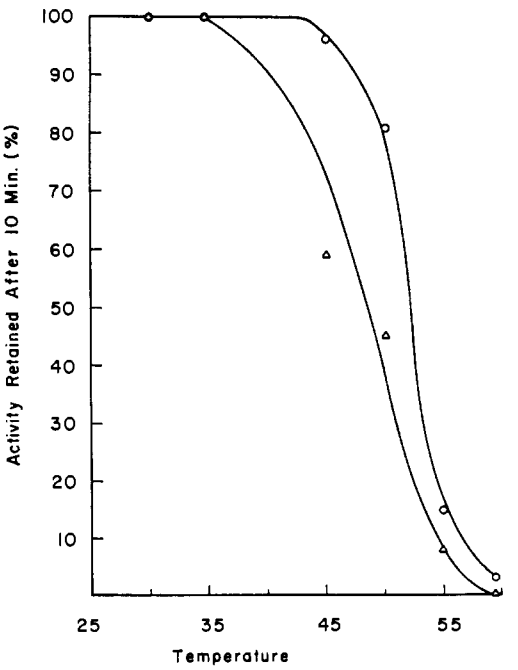

FIGURE 5: Comparative temperature stabilities of the two *Limulus* arginine kinases. (△—△) *Limulus* neutral, 0.048 mg/ml; (O—O) *Limulus* negative, 0.047 mg/ml.

TABLE X: Sulfhydryl Groups of Various Arginine Kinases.

Source	Half-cystine	Act. without Mercaptoethanol (%)	Free SH Groups as Measured by: Hydroxymercuribenzoate in 0.1 M Potassium Phosphate (pH 7.5)	Free SH Groups as Measured by: Hydroxymercuribenzoate in 8 M Urea	Free SH Groups as Measured by: DTNB in 1 mM Dithiothreitol
Blue crab	5.1	85	4.3	4.9	
Hermit crab	4.9	82	2.5[a]	4.0	4.8
Limulus					
Negative	4.8	79	4.5	4.8	5.2
Neutral	4.8	6	3.1	3.6	4.9

[a] When 3.0 equiv of hydroxymercuribenzoate were added to hermit crab arginine kinase in the absence of urea a precipitate was formed.

Discussion

The blue crab, hermit crab, and the two *Limulus* arginine kinase preparations appeared to be homogeneous when examined in the analytical ultracentrifuge and on electrophoresis. The blue crab and negative *Limulus* preparations also appeared to be homogeneous in immunodiffusion studies. All of the arthropod enzymes studied appear to behave similarly when chromatographed on Sephadex G-100. Further evidence for this similarity in size is provided by the close agreement between the sedimentation constants of the purified enzymes. The values reported here (ranging from 3.18 to 3.25 S) are also similar to the value reported by Virden *et al.* (1966) for the European lobster (3.18) and by Uhr *et al.* (1966) for the sea crayfish arginine kinase (3.07). Pradel *et al.* (1964) reported a sedimentation constant of 2.69 S for their arginine kinase preparation from European lobster. This value may be in error due to the low ionic strength of the buffer used (0.01 M glycine). Elodi and Szorenyi (1956) also reported a lower value for the sedimentation constant of their arginine kinase preparation from the Hungarian marsh crab. These sedimentation experiments were performed at pH 8.5 in 0.2 M glycine and 1 mM KCN, but these authors reported some inactivation of their preparation during the experiments.

Since it comprises from 10 to 20% of the readily extractable protein of arthropod muscle, arginine kinase is relatively easy to purify from any arthropod source where the muscle can be obtained in reasonable quantities. Ammonium sulfate fractionation has proved to be an effective means of purification, although the exact concentration of ammonium sulfate required for precipitation of arginine kinase from different species varies. The addition of a thiol, such as β-mercaptoethanol, to the buffers used in enzyme purification increased the yields obtained. The neutral *Limulus* en-

TABLE XI: K_m of L-Arginine for Various Arginine Kinases.[a]

Source	K_m (mM)
Blue crab	0.85
Hermit crab	0.80
Limulus negative	0.35
Limulus neutral	0.40
Tarantula negative	0.51
Tarantula neutral	0.67

[a] The K_m values were measured at 30° in the presence of 5 mM ATP–10 mM $MgSO_4$ by the standard assay system described in the text.

TABLE XII: Effect of Additions on the Reactivation after Urea Treatment of Blue Crab and Hermit Crab Arginine Kinases.[a]

Addition (mM)	Reactivation of Blue Crab (%)	Reactivation of Hermit Crab (%)
None	7.3	4.9
Mercaptoethanol (1)	26.4	24.5
Mercaptoethanol (100)	85.7	83.0
L-Arginine (30)	8.6	46.3
L-Arginine (30) + mercaptoethanol (1)	65.0	68.7
L-Arginine (30) + mercaptoethanol (100)	85.7	83.4

[a] Samples containing approximately 0.3 mg of protein were mixed with 0.95 ml of freshly prepared 8.4 M urea in 0.1 M potassium phosphate (pH 7.5) containing 1 mM EDTA. After 2 min, samples were diluted 1:20 with phosphate buffer containing the additions shown.

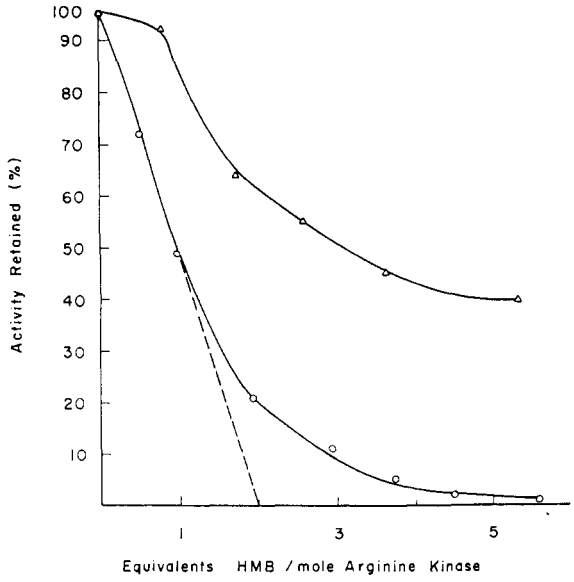

FIGURE 6: Effect of hydroxymercuribenzoate on blue crab arginine kinase. The enzyme (0.28 mg/ml) was allowed to react with hydroxymercuribenzoate in 0.23 M glycine (pH 8.6) for 15 min at 20°. The reaction between the added hydroxymercuribenzoate and the native enzyme was complete within the incubation time used.

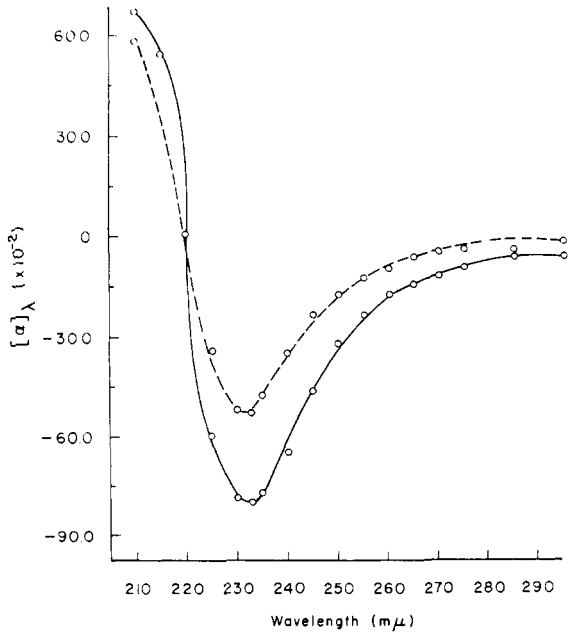

FIGURE 7: Optical rotatory dispersion of *Limulus* arginine kinases. Solid line: negative, enzyme 0.33 mg/ml; dashed line: neutral enzyme, 0.24 mg/ml.

zyme lost almost all activity in the absence of added thiol.

The amino acid compositions of the arginine kinases studied here are similar although the hermit crab enzyme appears to have fewer dicarboxylic amino acids than the others. (The two *Limulus* enzymes have about the same total number of dicarboxylic amino acids, but because of its higher ammonia content, the neutral form may have fewer free carboxyl groups.) All have five half-cystine residues per mole. When the enzymes are allowed to react with DTNB in the presence of an excess of added thiol (*i.e.*, under conditions where the enzymes have their maximal activities), 5 moles of thiophenylate are bound/mole of enzyme. This observation indicates that in the active enzyme all the half-cystines are present as cysteine.

L-Arginine completely protects blue crab arginine kinase from inactivation in the presence of 1 mole of hydroxymercuribenzoate/mole of enzyme. As the amount of hydroxymercuribenzoate present increases, the protection decreases but is still substantial. Similar protective effects have been shown for the enzymes from both the American and European lobsters with a number of sulfhydryl reagents (Pradel *et al.*, 1965; Virden and Watts, 1966; Blethen and Kaplan, 1967). These results indicate that there is a reactive sulfhydryl group at the active site. Arginine may protect by binding to it or by an effect on the protein conformation around it. On the other hand, rabbit muscle creatine kinase required the presence of both creatine and metal–nucleotide for full protection (Lui and Cunningham, 1966; O'Sullivan *et al.*, 1966), and creatine by itself was ineffective in protecting the enzyme from inactivation. These results were interpreted by O'Sullivan *et al.* (1966) as indicating that the binding of the metal–nucleotide complex to the enzyme is necessary before creatine can bind to the reactive sulfhydryl at the active site. This difference in the ability of the guanido substrate to protect the enzyme from inactivation by sulfhydryl reagents may or may not indicate other differences in the mechanisms of the two types of phosphagen kinases. It would be very interesting to know if arginine kinase reactions generally proceed with formation of a phosphorylated enzyme intermediate as the sea crayfish enzyme (Uhr *et al.*, 1966) or whether the general mechanism is similar to that of creatine kinase as was found for the enzyme from European lobster (Virden *et al.*, 1965).[5]

The ability of L-arginine to promote reactivation of *H. americanus* arginine kinase which has been treated with 8 M urea has been shown previously (Blethen and Kaplan, 1967). The importance of free sulfhydryl groups to enzymatic activity and the ability of L-arginine to protect them from hydroxymercuribenzoate and iodoacetamide suggested that one of the ways in which arginine promoted reactivation was by protecting the active site sulfhydryl from oxidation during the refolding of the disordered polypeptide chain. However, there was considerable difference in the ability of the nucleotide substrates (which were equally effective in protecting against sulfhydryl reagents) to promote reactivation; ATP was about as effective as arginine, whereas adenosine diphosphate was ineffective. These results suggest that other factors besides sulfhydryl group might be involved in reactivation. Arginine is about as effective in promoting the reactivation of the

[5] The kinetic behavior observed with arginine kinase purified from the American lobster (W. J. O'Sullivan, R. Virden, and S. L. Blethen, manuscript in preparation) is similar to that of the enzyme from the European lobster.

hermit crab enzyme as it was with lobster, but it did not increase the reactivation of the blue crab enzyme. When coupled with low concentrations of mercaptoethanol, L-arginine promoted the reactivation of both enzymes. This result illustrates the dual nature of the reactivation process, first the importance of reduced sulfhydryl groups to enzymatic activity and second, the role of arginine in promoting recovery of the structure of the active site. This role may be a type of "induced fit" effect (Koshland, 1959) with the substrate promoting the formation of the correct active site conformation from the disordered polypeptide chain.

In contrast to lactic dehydrogenase, which has a different distribution of enzyme types in lobster tail and claw muscle (Wilson and Kaplan, 1964), the arginine kinase activities of the lobster and hermit crab are the same in both tail and claw muscle. In fact, we were unable to demonstrate arginine kinase isoenzymes of the type reported by Virden and Watts (1964) for the claw muscle of *Eupagurus bernhardus* in either the tail or claw muscle of the closely related *Pagurus* species. The staining procedure used by Virden and Watts was based on the disappearance of the positive Sakaguchi reaction given by arginine on the phosphorylation of arginine by ATP. Localization of arginine kinase activity depends on the appearance of a white spot against a red background. The staining procedure we have described allows for a more direct localization of enzymatic activity although problems in its use still arise when an ATPase activity migrates directly with an arginine kinase activity. In this case, it is necessary to isolate the enzymatic activities chromatographically in order to be certain that the spots developed are actually due to arginine kinase.

The separation and purification of the two *Limulus* arginine kinase forms represent the first instance where two forms of arginine kinase from one organism have been separated and purified. The two enzymes have similar amino acid compositions with the ammonia content being the most striking difference. The results of complement fixation experiments indicate that there is some difference in structure and perhaps in amino acid sequence between the two forms.

It is interesting to compare the results obtained with the *Limulus* arginine kinases with those obtained with the isoenzymes of creatine kinase studied in this laboratory (Dawson *et al.*, 1965, 1967; Eppenberger *et al.*, 1967). Only a single form of creatine kinase was found in vertebrate skeletal muscle, whereas the *Limulus* isoenzymes are both found in muscle. Creatine kinase consists of two subunits, and two different types of subunits, the muscle and brain type, exist (Dawson *et al.*, 1965). The combination of these different subunits into enzymatically active dimers leads to the three different isoenzymes observed in different vertebrate tissues. In contrast, the arthropod arginine kinases are half as large, and at least the lobster enzyme is not composed of smaller subunits. In the case of the muscle- and brain-type creatine kinases from chicken, there was no cross-reaction between an antiserum to the muscle-type and brain-type enzyme, and only a very slight cross-reaction at an antiserum dilution of 1:100 was observed in the converse case. Thus, the two arginine kinase forms are much more similar than the two types of creatine kinase subunits.

The negative form has been isolated in higher yield than the neutral. This may reflect a difference in the relative concentrations of the two forms in *Limulus* muscle, but part of the lower yield of the neutral form is due to its greater solubility in saturated ammonium sulfate solutions. In fact, the arginine kinase activity remaining in solution when saturation in ammonium sulfate is attained is the neutral form, which is less stable than the negative, and this may also result in a lower yield. In this respect, there is some analogy between the arginine kinase isoenzymes and those of creatine kinase as the brain-type creatine kinase is less stable than the muscle type (Eppenberger *et al.*, 1967).

Preliminary studies of the catalytic properties of the two *Limulus* arginine kinase forms have not yet shown any differences between them. They have similar ΔH values and similar K_m values for L-arginine. The observed differences in optimum temperature between the two forms probably reflect differences in their temperature stability. The partially purified tarantula arginine kinases are also similar to each other catalytically, and the differences in their temperature stability parallel those of the *Limulus* enzymes. Their behavior on complement fixation with antiserum to the negative *Limulus* enzyme is also analogous. These results indicate that the structural differences between the two tarantula enzymes are similar to those of the *Limulus* arginine kinases. Because their molecular weights are the same these isoenzymes are different from those found in the muscle of some molluscs by Moreland and Watts (1967). Unfortunately, the experiments reported in this paper do not indicate a positive functional difference between the neutral and arginine kinases. However, the presence of negative and neutral (at pH 8.6) forms of arginine kinase may be characteristic of the chelicerates, since no such isoenzymes were found in the insect and crustacean species examined by us, and the crustacean arginine kinase isoenzymes reported by Virden and Watts (1964) were all negatively charged at pH 8.6.

Acknowledgments

We thank Miss Lurley Dillahunt for performing the amino acid analyses, Miss Jean King for doing the complement fixation experiments, and Mr. Fred Chasalow for writing the computer programs used to calculate the amino acid compositions and partial specific volumes of the enzymes studied.

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