Adenosine 5'-Triphosphate-Arginine Phosphotransferase from Lobster Muscle: Purification and Properties

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1. A simple chromatographic method is described for the purification of arginine kinase from lobster (*Homarus vulgaris*) muscle. 2. Some physical properties and the effects on enzyme activity of ionic strength, pH, buffer salts, metal ions and substrates are reported. 3. The kinetic parameters, evaluated by variation of the concentration of one of the substrates, are dependent on the concentration of the other substrate. 4. The properties of the enzyme are discussed in relation to previous findings about phosphagen phosphotransferases.

Arginine kinase (EC 2.7.3.3), which catalyses the reaction:

was first discovered in crab-muscle extracts by Lohmann (1935). The enzyme from the marsh crab, *Potomobius astacus*, was purified by salt fractionation and crystallized by Szorenyi, Dvornikova & Degtyar (1949). Morrison, Griffiths & Ennor (1957) tried to use this purification procedure on muscle extracts of the Australian sea crayfish, *Jasus verreauxi*, and freshwater crayfish, *Cherix albidus*, but found that it was unsuitable for these species.

The salt fractionation procedure gave a 5–7-fold purification with the British freshwater crayfish, *Astacus fluviatilis* (Watts, 1961), but the preparation showed a high degree of heterogeneity and could not be crystallized. However, a further purification was achieved by column chromatography on DEAE-cellulose.

In the present work DEAE-cellulose columns have been used to obtain highly purified preparations of arginine kinase from muscle extracts of the common lobster, *Homarus vulgaris*. Some properties of this enzyme are described and the results discussed in the light of findings by other workers.

MATERIALS AND METHODS

Materials

Chemicals. These were purified as described by Virden & Watts (1964) or were of AnalaR grade where obtainable.

Acetone was purified by the method of Vogel (1948).

Buffers. Quartz-distilled water was used throughout. All pH measurements were made at room temperature with

an EIL model 23a pH-meter fitted with a temperature compensator.

Glycylglycine-NaOH buffer, pH 7.8 and *I* 0.001, for muscle extraction was prepared by dissolving 1.337 g. of the dipeptide in water, adjusting the pH with saturated NaOH, added with a micrometer syringe, and making the final volume up to 2 l. The pH was checked at the final dilution.

Glycylglycine-NaOH buffer, pH 8.5 and *I* 0.06, for use in the enzyme assay was prepared as above except that 1.275 g. of the dipeptide was used and the final volume made up to 100 ml.

Sodium chloroacetate-perchloric acid buffer, pH 2.5 and *I* 0.2, for stopping the enzyme reaction was prepared by dissolving 2.33 g. of sodium chloroacetate in water, adjusting the pH with 60% (w/w) perchloric acid and making the final volume up to 100 ml.

The Na₂HPO₄-H₃PO₄ buffers, pH 6.2, for starch-gel electrophoresis, were prepared as described by Gammack, Huehns, Shooter & Gerald (1960).

Acetic acid-NaOH buffers for starch-gel electrophoresis, pH 3.8, 5.0 and 5.4, and *I* 0.02, for preparing gels, and *I* 0.06, for electrode vessels, were prepared by adjusting solutions of NaOH to the required pH with acetic acid, and diluting to give final Na⁺ concentrations of 20 and 60 mM respectively.

Methods

Protein estimation. Unless otherwise stated this was carried out by the method of Gornall, Bardawill & David (1949), with ovalbumin as standard.

Enzyme assays. For determining the specific activities of enzyme fractions and for measuring initial velocities a modification of the method used by Kuby, Noda & Lardy (1954) for creatine kinase was used. The enzyme solution was suitably diluted with glycylglycine buffer, pH 8.5 and *I* 0.06, and 0.1 ml. was added to a mixture containing: arginine (pH 8.5), 25 mM; MgSO₄, 6 mM; glycylglycine buffer, pH 8.5 and *I* 0.06; total volume, 9.0 ml. After equilibration for 5 min. at 30° the reaction was started by the addition of 1 ml. of 10 mM-ATP. Samples (2 ml.) of the

assay mixture were transferred after $\frac{1}{2}$, 5, 10 and 15 min. to 2 ml. of ice-cold sodium chloroacetate-perchloric acid buffer and stored in an ice bath. Phosphoarginine was hydrolysed by placing the tubes in a boiling-water bath for 90 sec. and then returning them to the ice bath. After 5 min. the phosphate liberated was determined by the method of King & Wootton (1956) in a final volume made to 10 ml. with water. The extinction (red filter) was read after 15 min. at room temperature and the reading obtained converted into $\mu\text{g. equiv.}$ by using a calibration curve. From the progress curves apparent second-order rate constants were calculated as described by Kuby *et al.* (1954) (Table 1).

Where large numbers of enzyme samples, such as fractions from a column, were being assayed for activity, single-tube determinations were made. The conditions used were as described above except that a reaction volume of 2 ml. was used and the final concentration of ATP was 1.25 mM. The tubes were incubated for 10 min. at 30° and the stopping agent added from a 2 ml. syringe. The tubes were then assayed as described above. The results obtained were corrected for the non-linearity of phosphoarginine production as a function of enzyme concentration by using a calibration curve. Results are expressed as apparent second-order velocity constants in the manner described by Kuby *et al.* (1954) (Table 1).

Purification of arginine kinase

(1) *Treatment of animals.* A lobster was killed by placing it in the cold room at -30° and leaving it for several hours. The tail muscle was removed and passed through a chilled mincer. Since column chromatography utilizes only a small fraction of the muscle available, the possibility was investigated of preparing an acetone-dried powder by the method of Green, Needham & Dewan (1937) as an intermediate step so that the bulk of the material could be stored. However, this resulted in a considerable loss of enzyme activity and the method was abandoned. It was subsequently found that the unminced muscle would keep for several months in the deep-freeze without measurable loss of arginine-kinase activity, provided that it was freed from any digestive gland.

(2) *Choice of salts and buffers.* For preliminary experiments, aqueous muscle extracts were prepared. Because some ions have been shown to inhibit phosphagen phosphotransferases (Noda, Nihei & Morales, 1960), experiments were carried out to determine which salts were suitable for use in the purification procedure and assay. The effects on the reaction rate of nitrate, chloride and acetate as the sodium and potassium salts are shown in Fig. 1. With all salts, activity decreased with increasing concentration. Nitrate had a specific inhibitory effect, with only 2% of the activity remaining in the presence of 0.5 M-nitrate. No significant difference was found between chloride and acetate. In subsequent experiments the ionic strength was therefore kept as low as possible and adjusted with NaCl.

Of the buffers examined, boric acid-NaOH buffers were found to be inhibitory, whereas either potassium phenol-p-sulphonate-NaOH or glycylglycine buffers caused no significant inhibition over the range used (up to 0.02 M). Glycylglycine buffers have been used throughout in the purification procedure and assay.

Fig. 1. Effect of ionic strength on the reaction velocity of phosphoarginine synthesis. The ionic strength was adjusted with one of the following salts: Δ , NaCl; $\blacktriangle$, KCl; $\circ$, NaNO₃; $\bullet$, KNO₃; $\square$, sodium acetate. The final volume was 2 ml., containing: glycylglycine buffer, pH 8.6 and *I* 0.006; arginine hydrochloride, 10 mM; MgSO₄, 1.25 mM; ATP, 1.25 mM; muscle extract, 5.3 $\mu\text{g./ml.}$

(3) *Extraction of enzyme from muscle.* Minced muscle (30–40 g.) was ground in a mortar with a little acid-washed sand and 40 ml. of 0.1 mM-EDTA in glycylglycine buffer, pH 7.8 and *I* 0.001. The insoluble matter was removed by centrifugation for 25 min. at 26000 *g* and 2° and the extraction procedure repeated on the pellet with a further 40 ml. of EDTA buffer. The combined supernatants were then concentrated by dialysis against 2 l. of glycylglycine buffer, pH 7.8 and *I* 0.001, at 4° under reduced pressure (550 mm.Hg) until the volume was about 15 ml. Dialysis against 2 l. of fresh buffer was continued at atmospheric pressure for a further 24 hr.

(4) *Chromatography on a DEAE-cellulose column.* DEAE-cellulose was washed free from fines, packed in a column (1 cm. $\times$ 60 cm.), washed with 2 l. of 1 mM-EDTA and 2 l. of water, and equilibrated at 4° with glycylglycine buffer, pH 7.8 and *I* 0.001. The protein solution was applied to the column and washed through with at least 100 ml. to remove the protein that does not adsorb to the column. Gradient elution was then commenced with 500 ml. of the equilibrating buffer in a magnetically-stirred straight-sided mixing chamber connected by a siphon to 250 ml. of 75 mM-NaCl solution in the equilibrating buffer in a conical reservoir. Fractions (5 ml.) were collected until elution of the enzyme was complete. Fig. 2 shows an

Fig. 2. Purification of lobster arginine kinase on a DEAE-cellulose column. The experiment was carried out as described in the text. —, $E_{280m\mu}$; ----, concn. of NaCl in the effluent; ●, total activity units/fraction; ○, specific activity.

Table 1. *Purification of arginine kinase*

Summary of the effects of the purification stages in the preparation of arginine kinase from 35 g. of lobster tail muscle, as described in the text. One unit of enzyme activity is defined as that amount of enzyme/ml. of reaction mixture that will catalyse phosphoryl transfer from 1 mM-ATP to 25 mM-arginine in the presence of 6 mM- $MgSO_4$ in glycylglycine buffer, pH 8.5, at 30° and yield an apparent second-order velocity constant equal to 1 ml. μM^{-1} min.⁻¹ (Kuby *et al.* 1954).

Stage	Protein (mg.)	Total activity (units)	Specific activity (units/mg.)	Recovery (%)
(3) Concentrated dialysed EDTA extract	695	56990	82	100
(4) Total enzyme recovered	215	42580	198	75
(5) Combined fractions 45-57	139	29200	210	51
(5) Combined fractions 58-75	34	6660	199	12
Overall recovery	—	—	—	63

elution diagram, and the specific activities of combined fractions are shown in Table 1.

(5) *Concentration of the enzyme preparation.* The fractions possessing the highest specific activities of arginine kinase were pooled and concentrated by dialysis under reduced pressure against the same buffer as described

above. Dialysis was stopped before the protein concentration of the enzyme solution had exceeded 10 mg./ml.

The purification and recovery obtained by this procedure are shown in Table 1.

Homogeneity and further purification of arginine kinase

Starch-gel electrophoresis. The final preparation was analysed by horizontal starch-gel electrophoresis at pH 8.5, as described by Virden & Watts (1964). The electrophoretogram showed a single strong band, and, in some preparations, a more rapidly migrating faint band. Enzyme activity was shown to coincide with the position of the main band by cutting the gel into transverse strips and assaying them by the single-tube method. The strips were placed in the assay tubes before incubation and were removed before hydrolysis of the phosphoarginine. The position of enzyme activity agreed with that reported for crude muscle extracts by Virden & Watts (1964). With very heavy loading, traces of more slowly migrating components could be detected.

The effect of pH on the mobility of the enzyme in the gel was then investigated. This yielded no evidence suggesting further heterogeneity, although at low pH values considerable trailing and some denaturation occurred. Table 2 summarizes the electrophoretic mobilities found, which suggest that the isoelectric point of arginine kinase is about pH 5.4.

Gel filtration on Sephadex. The purified enzyme was passed through columns of Sephadex G-100 [Pharmacia (Great Britain) Ltd., London] as a further criterion of purity and in an attempt to remove the trace impurities found by starch-gel electrophoresis. Columns (3.5 cm. × 44 cm.) were prepared and equilibrated with glycylglycine

Table 2. *Electrophoretic mobilities of arginine kinase at different pH values*

All experiments were carried out in the cold room at 4° at a constant current of 3.85 mA/cm. width of gel.

pH	Buffer	Distance moved from the origin in 3.5 hr. (cm.)
3.84	Acetate	-2.5
4.98	Acetate	-1.7
5.40	Acetate	+0.1
6.00	Phosphate	+1.9

Fig. 3. Gel filtration of purified arginine kinase on a Sephadex G-100 column. The enzyme solution was dialysed against glycylglycine buffer, pH 7.8 and I 0.001, adjusted with NaCl to give total I 0.05. Protein (21.5 mg.) was applied in a volume of 2.5 ml. The temperature was 2°. —, $E_{280m\mu}$; ●, total enzyme activity (units) per fraction.

Fig. 4. Fractionation of lobster muscle extract, prepared as described in the text, on a Sephadex G-100 column (3 cm. $\times$ 18 cm.). The column was equilibrated and eluted with glycylglycine buffer, pH 7.8 and I 0.001. The temperature was 20°. Protein (23 mg.) was applied in a volume of 1 ml. —, $E_{280m\mu}$; ●, total enzyme activity (units) per fraction.

Fig. 5. Ultracentrifuge analysis of arginine kinase. Schlieren patterns were obtained by sedimentation of the enzyme in a synthetic-boundary cell (a) 3 min., (b) 19 min. and (c) 35 min. after reaching full speed. The rotor speed was 59780 rev./min., the temperature 4.1° and the phase-plate angle 65°. The initial enzyme concentration was 6.7 mg./ml. in glycylglycine buffer, pH 7.8 and I 0.001, adjusted to I 0.1 with NaCl.

buffer, pH 7.8 and I 0.001, adjusted with NaCl to total I 0.05. Samples were applied and eluted with the same buffer. The elution diagram (Fig. 3) reveals the presence of about 5% of high-molecular-weight contaminants with the enzyme.

Although Sephadex is most usefully employed as a second step after chromatography on DEAE-cellulose, it may be used as a preliminary purification procedure so that more of the partially purified enzyme may be loaded on to the DEAE-cellulose column. Fig. 4 shows the elution diagram of a crude muscle extract prepared as in section (3) of the purification procedure. This purifies 90% of the arginine kinase 2½-fold, giving a preparation about 30% pure, depending on the activity of the initial extract. It has the advantage that the complete fractionation takes only 3 hr. and can be carried out at room temperature.

Sedimentation analysis. A single moving boundary was apparent in schlieren patterns obtained during sedimentation of the purified enzyme in a Spinco model E analytical ultracentrifuge (Fig. 5).

RESULTS

Properties of arginine kinase

Absorption spectrum and extinction coefficient. The absorption spectrum was measured in a Cary model 14 recording spectrophotometer at room temperature. Fig. 6 shows that there is a negligible difference between the spectra obtained in glycylglycine buffer, pH 7.8, and in borate buffer, pH 9.0. The extinction at 280 $m\mu$ was a linear function of protein concentration, and $E_{1\%}^{1\text{cm}}$ was found to be 6.71.

Fig. 6 also shows that in 0.1 N -sodium hydroxide a marked change in the absorption spectrum occurs. From this, the tyrosine/tryptophan ratio was calculated (Beaven & Holiday, 1952; Goodwin & Morton, 1946). The mean of four determinations at different protein concentrations was 5.23 (S.D. $\pm$ 0.01), equal to 0.23 m-mole of tyrosine/g. and 0.045 m-mole of tryptophan/g. of protein.

Specificity for substrates and metal ions. As reported by Virden & Watts (1964), arginine kinase

Fig. 6. Absorption spectra of arginine kinase and creatine kinase, obtained as described in the text. Arginine kinase (0.507 mg./ml.) in: —, glycylglycine buffer, pH 7.8 and I 0.001; ----, boric acid-NaOH buffer, pH 9.0 and I 0.023; - · - · -, 0.1 N -NaOH solution. · · · · ·, Absorption spectrum of rabbit-muscle creatine kinase (3.5 mg./ml.) in boric acid-NaOH buffer, pH 9.0 and I 0.023, purified by the method of Kuby *et al.* (1954).

was found to be active only with L-arginine, being inactive towards D-arginine, a range of L-arginine analogues, and creatine, glycocyamine and taurocymamine.

Among the metal ions tested, the enzyme was more active with Mn^{2+} than with Mg^{2+} . Results with active metal ions are shown in Table 3. No activity was found with Ni^{2+} , Cu^{2+} , Al^{3+} , Ba^{2+} , Cd^{2+} or Zn^{2+} . Omission of an activating metal ion resulted in a negligible activity.

Stability and storage. In glycylglycine buffer, pH 8.5 and I 0.06, the enzyme showed no loss of activity after 3 hr. at 30° . At 25° the enzyme was stable over 15 min. between pH 9.1 and pH 6.0; but at pH 5.4 and pH 5.1 the enzyme lost 20 and 70% respectively of its initial activity.

The enzyme, at a concentration of about 1%, dialysed against glycylglycine buffer, pH 7.8 and I 0.001, was unaffected in its activity by storage for 3 months in a refrigerator, either at 4° or at -10° . Repeated freezing and thawing also had no effect. The same preparation could also be stored as a freeze-dried powder without loss of activity, but, if it was first dialysed against water adjusted to pH 8.5 with ammonia, the freeze-dried product was insoluble.

Enzyme activity was unaffected by the addition of 1 mM-cysteine.

Effect of pH on activity. The effect of pH was determined for the forward reaction (phospho-arginine synthesis). Between pH 8.2 and 9.0 the buffer power of arginine alone was used (Fig. 7).

Table 3. Activation of arginine kinase by bivalent metal ions

The final volume was 0.4 ml., containing: glycylglycine buffer, pH 8.5 and I 0.024; arginine hydrochloride, 25 mM; ATP, 4 mM; enzyme, 0.151 μ g. The metal ion was added as either the chloride or as the acetate to give a final concentration of 4 mM. The calcium salt was Specpure reagent, from Johnson Matthey and Co. Ltd. Enzyme activity is given as μ moles of phosphoarginine/min./mg. of protein.

Activating metal ion	Enzyme activity	Percentage activity
Mn^{2+}	315	120
Mg^{2+}	262	(100)
Co^{2+}	69	26
Ca^{2+}	50	19
Sn^{2+}	33	13
Fe^{2+}	31	12
None	7	3

Fig. 7. Effect of pH on the reaction velocity of phospho-arginine synthesis by arginine kinase at 30° . The reaction volume was 5 ml., containing: arginine, 25 mM; $MgSO_4$, 6 mM; ATP, 1 mM; enzyme, 0.18 μ g./ml. Samples (2 ml.) were withdrawn at 0.5 and 10 min. and assayed as described in the text. O, The arginine used as buffer; Δ , glycylglycine-NaOH buffer, I 0.05, adjusted with NaCl: total glycylglycine concentration, 50 mM; $\blacktriangle$, glycylglycine buffer as above, with EDTA, 60 μ M; ∇ , glycylglycine-NaOH buffer, I 0.05, adjusted with NaCl: total NaOH concentration, 10 mM. The pH of the remaining reaction medium was measured at 20° at the end of the incubation, and corrected for the temperature of the assay.

Over the range pH 7.1–9.0 glycylglycine buffer was used. Because of the high glycylglycine concentrations required to achieve the necessary buffering power, the effect of this buffer was reinvestigated. The results revealed significant inhibition at high glycylglycine concentrations (Fig. 8). The pH-activity curve was therefore determined under two

Fig. 8. Effect of glycylglycine concentration on the rate of phosphoarginine synthesis by arginine kinase at 30°. The reaction volume was 5 ml., containing: arginine, 25 mM; MgSO_4 , 6 mM; ATP, 1 mM; enzyme, 0.24 $\mu\text{g./ml.}$ Samples (2 ml.) were withdrawn at 0.5 and 10 min. and assayed as described in the text. Glycylglycine-NaOH buffer, pH 8.28, at 20° was adjusted with NaCl to give total I 0.1

Fig. 10. Effect on the initial velocity of phosphoarginine synthesis of varying the ATP- Mg^{2+} concentration in the presence of several fixed concentrations of arginine, as described in the text. The reaction volume was 0.4 ml., containing: glycylglycine buffer, pH 8.5 and I 0.025; NaCl to give total I 0.1; arginine kinase, 0.36 $\mu\text{g./ml.}$; arginine: ○, 0.4 mM; ●, 0.5 mM; △, 0.67 mM; ▲, 1 mM; ▼, 2 mM; ▼, 5 mM.

Fig. 9. Effect on the initial velocity of phosphoarginine synthesis of varying the arginine concentration in the presence of several fixed concentrations of ATP, as described in the text. The reaction volume was 0.4 ml., containing: glycylglycine buffer, pH 8.5 and I 0.025; NaCl to give total I 0.1; arginine kinase, 0.36 $\mu\text{g./ml.}$; ATP and MgSO_4 : ○, 0.67 mM; ●, 1 mM; ▽, 2.5 mM; ▼, 5 mM.

Fig. 11. Effect on the initial velocity of phosphoarginine synthesis of varying the arginine concentration in the presence of a fixed concentration of ATP (0.67 mM) with MgSO_4 either 0.67 mM (○), or 10 mM (●), as described in the text. The reaction volume was 0.4 ml. containing: glycylglycine buffer, pH 8.5 and I 0.025; NaCl to give total I 0.1; arginine kinase, 0.36 $\mu\text{g./ml.}$

sets of conditions: those in which the total glycylglycine was held constant, and those in which the total sodium hydroxide was held constant. Essentially the same results were obtained with both series of buffers (Fig. 7).

Effects of substrate concentration. Figs. 9 and 10 show the effects on the initial velocity of the enzyme reaction of varying in turn the concentration of each substrate in the presence of several fixed concentrations of the other substrate. In these experiments the total Mg^{2+} concentration was kept equal to the total ATP concentration. The ionic strength of the assay mixture was computed by using the stability constant for ATP of O'Sullivan & Perrin (1961) and was adjusted to 0.1 with sodium chloride so that the ionic contribution of ATP plus Mg^{2+} at their maximum concentrations did not exceed 25% of the total ionic strength. In this way, possible errors introduced in the ionic strength by

assumptions in the calculations were kept to a minimum. The initial velocities under each set of conditions were determined from the tangents to progress curves obtained from four measurements of the phosphoarginine produced after various time-intervals.

Fig. 11 shows the inhibitory effect observed when a high Mg^{2+} concentration was used in the presence of a fixed ATP concentration at various arginine concentrations.

DISCUSSION

Lobster arginine kinase appears to constitute about 13% of the water-soluble muscle proteins. However, the degree of purification varied from animal to animal between 2½- and 10-fold. This was due to variable behaviour of the muscle in the initial extraction and concentration step, where the specific activity of the resulting protein solution varied from 20 to over 80 units. However, the specific activity of the purified enzyme was constant within experimental error.

The simple procedure of column chromatography (Fig. 2) gave an enzyme with a high degree of purity (Figs. 2, 3 and 5, and Table 2). Rechromatography on Sephadex G-100 (Fig. 3) removed a small amount of heavier material; this would not significantly increase the specific activity, which cannot be determined with an accuracy greater than this.

It is difficult usefully to compare the specific activity of the purified enzyme with other preparations because of the very large ionic-strength effect and the specific effects of nitrate (Fig. 1). Informative comparisons can only be made under identical assay conditions. It was with this end in view that the procedure for the measurement of the specific activity of creatine kinase (Kuby *et al.* 1954) was adapted to arginine kinase. On this basis the specific activity of arginine kinase is about 2.5 times as great as that of pure creatine kinase. Since under these conditions neither enzyme is working at maximum rate, part of the greater activity found with arginine kinase may relate to the lower K_m for arginine (Fig. 9).

Comparison of the absorption spectrum of lobster arginine kinase with that of rabbit creatine kinase (Fig. 6) reveals interesting features. The inflexions between 250 and 270 $m\mu$ associated with the presence of phenylalanine in creatine kinase are evident in arginine kinase, but the shoulder at 290 $m\mu$ is barely discernible in the latter, suggesting a relatively lower tryptophan content. This is supported by the tyrosine/tryptophan ratio, 5.2, which compares with the ratio, 2.7, for creatine kinase, computed from the amino acid-analysis data of Noltmann, Mahowald & Kuby (1962). Since the two proteins contain similar amounts of tyrosine/g. of protein, this difference is largely accounted for by a lower tryptophan content. This explains the lower value of $E_{1cm}^{1\%}$ at 280 $m\mu$, namely 6.7, for arginine kinase, as against 8.9 for creatine kinase (Noda, Kuby & Lardy, 1954).

Lobster arginine kinase possesses several interesting points of comparison with purified crustacean kinases reported from *Jasus verreauxi* (Morrison *et al.* 1957) and from *Potomobius astacus* (Szorenyi, Elodi & Devenyi, 1956; Elodi & Szorenyi, 1956). Only *Potomobius* used D-arginine as a substrate.

Among 18 different species, only the kinase from an annelid, *Sabella pavonina*, showed significant activity with D-arginine (Virden & Watts, 1964).

Unlike the lobster enzyme (Table 3), that from *Jasus* is not activated by Ca^{2+} , Co^{2+} or Fe^{2+} . Cohn (1963), on the basis of nuclear-magnetic-resonance studies, has divided metal-ion-activated enzymes into two groups: type 1, which are Ca^{2+} -activated, and for which the metal ion does not function as a bridge between nucleotide and enzyme; type 2, which are inactive with Ca^{2+} , and where the metal ion forms a bridge between the nucleotide and enzyme. It is possible that there is a real difference between the structure of the ATP- Mg^{2+} -binding sites of the kinase from lobster and *Jasus*. This finding underlines the importance of establishing species identity when comparing kinetic data for metal ion-activated enzymes.

The pH data for the three kinases are difficult to compare, since the precise conditions under which pH measurements were made have not been reported. The broad pH optimum found for the *Jasus* enzyme is very similar to that found for the lobster enzyme (Fig. 7), although at higher pH values the activity of the latter falls off more rapidly. The *Potomobius* enzyme is reported to have a sharp optimum near pH 9.

The 20% inhibition caused by high concentrations of glycylglycine at constant ionic strength (Fig. 8) is possibly due to competitive inhibition, although the pH curves in the two sets of glycylglycine buffers (Fig. 7) do not indicate a specific inhibition by a particular ionic species. Glycylglycocyamine has been found to act as a rather poor substrate with some arginine kinases (Virden & Watts, 1964).

Above pH 8.3 the scatter found in the data for the lobster enzyme was more than could be accounted for by normal experimental error. This appeared to be independent of the buffer system used, suggesting that a pH-sensitive phenomenon such as conformational changes in the enzyme, or reversible aggregation, might be associated with the decrease in activity.

The Lineweaver-Burk plots (Figs. 9 and 10) show that for either substrate the Michaelis constant, K_m , is a function of the concentration of the other substrate. These experiments were carried out with the ATP/ Mg^{2+} ratio maintained at 1:1. When the concentration of Mg^{2+} (as magnesium sulphate) was increased to make the ATP/ Mg^{2+} ratio 1:10, the Lineweaver-Burk plot (Fig. 11) resembled that for classical competitive inhibition, without a significant effect on the K_m for arginine.

Florini & Vestling (1957) have presented a theoretical treatment for kinetics of this type, assuming that equilibrium conditions apply. The

equation for an enzyme converting two substrates into two products is described by:

$$v = \frac{V_{\max.}}{1 + \frac{K_{a'}}{a} + \frac{K_{b'}}{b} + \frac{K_a K_{b'}}{ab}}$$

where for arginine kinase K_a and K_b are the dissociation constants respectively for arginine and ATP-Mg²⁺ from the enzyme, and $K_{a'}$ and $K_{b'}$ are

the dissociation constants for arginine and ATP-Mg²⁺ respectively from the enzyme-arginine and the enzyme-ATP-Mg²⁺ complexes. $V_{\max.}$ is the maximum velocity. The four constants are related by the equation:

$$K_a K_{b'} = K_{a'} K_b.$$

K_a and K_b (Table 3) were calculated from the abscissa of the point of intersection of the two series of Lineweaver-Burk plots. A plot of $1/V_{\max.}$ against $1/[\text{arginine}]$ and $1/[\text{ATP-Mg}^{2+}]$ should give straight lines both crossing the ordinate at $1/V_{\max.}$ where both substrates are saturating, and crossing the abscissa at $-1/K_{a'}$ and $-1/K_{b'}$. Fig. 12 shows this plot, and the constants are listed in Table 4. The calculation of $K_a K_{b'}$ and $K_{a'} K_b$ gives 1.20 and 1.33 respectively. This is reasonable agreement in view of the difficulties in making accurate measurements at low substrate concentrations.

The above calculations have been made on the basis of the total ATP-Mg²⁺ present in the assay mixtures. The hypothesis has been presented for creatine kinase (Noda *et al.* 1960) that the (ATP-Mg)²⁻ complex is the true substrate for that enzyme. If the constants for arginine kinase are calculated on this basis, by using the stability constant of O'Sullivan & Perrin (1961), essentially the same results are obtained (Table 4), and $V_{\max.}$ with both substrates saturating is unaltered. However, the agreement of $K_a K_{b'}$ (0.72) with $K_{a'} K_b$ (0.89) becomes less satisfactory.

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Fig. 12. Effect on the observed maximum velocities of phosphoarginine synthesis of varying the arginine concentration in the presence of various fixed concentrations of ATP (●), and of varying the concentration of ATP in the presence of various fixed concentrations of arginine (▲). As described in the text, the data are taken from Figs. 10 and 11.

Table 4. Apparent kinetic constants for arginine kinase measured in the forward reaction

The experiments were carried out at 30° in glycylglycine buffer, pH 8.5 and at total I 0.1, as described in the text. Details of the calculation are given in the Discussion section. In the equations E, A and B are the enzyme, arginine and ATP-Mg²⁺ respectively.

Type of constant	Equation	Value for constant (mm)		$V_{\max.}$ (μmoles/mg./min.)
		Based on total ATP-Mg ²⁺	Based on the calculated concn. of (ATP-Mg) ²⁻ complex	
K_a	$E + A \rightleftharpoons EA$	5.2	5.2	—
K_b	$E + B \rightleftharpoons EB$	3.8	2.38	—
$K_{a'}$	$EB + A \rightleftharpoons EAB$	0.35	0.375	—
$K_{b'}$	$EA + B \rightleftharpoons EAB$	0.23	0.138	—
$V_{\max.}$ with A and B saturating	—	—	—	255

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