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COMPARATIVE STUDY OF TWO ATP : L-ARGININE PHOSPHOTRANSFERASES OF MOLECULAR WEIGHT 84 000

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Summary

Solen ensisensis muscle arginine kinase (ATP : L-arginine phosphotransferase, EC 2.7.3.3) was isolated in an homogeneous state. Its molecular weight was found to be about 80 000.

The properties of this enzyme were compared with those of arginine kinase from *Sipunculus nudus*, an enzyme which also has a molecular weight of about 80 000.

Both enzymes have several reactive thiol groups (8 thiol groups in the *Solen* kinase and 12 in the *Sipunculus* enzyme were titrateable with 5,5'-dithio—*bis*-(2-nitrobenzoic) acid and histidine residues (both enzymes have 6 reactive histidine residues). These kinases were, therefore, highly susceptible to oxidation. Both enzymes show the same pH optimum and absolute specificity towards the guanidine substrate, L-arginine. The reaction kinetics of both enzymes are of the sequential type. In the presence of α-aminoacids or Mg^{2+}—ADP, similar spectral effects were obtained.

The enzymes differ in their enzymic activities and in their rate of recovery following urea denaturation. The most important difference that appeared to be a special feature of the *Sipunculus* enzyme is that the spectrum of the Mg^{2+}—ADP—enzyme complex is strongly intensified by L-arginine.

Introduction

In previous publications, we showed that *Sipunculus nudus* muscle arginine kinase (ATP : L-arginine phosphotransferase, EC 2.7.3.3) has a molecular weight of about 80 000, twice that of the crustacean enzyme [1,2]. Here we report the isolation in an homogeneous state of a second arginine kinase of this type from *Solen ensisensis* muscle and the comparative study of both enzymes.

Materials

ADP, ATP, NADH, phosphoenolpyruvate, muscle lactate dehydrogenase (EC 1.1.1.27), muscle pyruvate kinase (EC 2.7.1.40), diethylpyrocarbonate, 5,5′-dithio—*bis*-(2-nitrobenzoic) acid (DTNB) were obtained as previously described [3]. *Sipunculus*, *Homarus* arginine kinases and creatine kinase (EC 2.7.3.2) were prepared as described [2,4,5].

Methods

The creatine and arginine kinase activities were carried out in the forward reaction, estimating creatine and arginine phosphate as previously described [2]. For kinetic studies in which very low substrate concentrations were required, the arginine kinase assay was performed by estimating the rate of ADP release with the coupled enzyme method (pyruvate kinase and lactate dehydrogenase [3]. A unit of enzyme activity is defined, for the first estimation method, as the amount of enzyme which transfers 1 μmole of phosphate per min and for the second method, as the amount of enzyme which releases 1 μmole of ADP per min. Specific activities are expressed as units per mg of protein.

The extinction coefficients of *Solen* and *Sipunculus* arginine kinases were determined from gravimetry and optical absorbance (280 nm, Cary 14 spectrophotometer).

Ultraviolet difference spectra, titration of histidyl groups with diethylpyrocarbonate and titration of cysteinyl groups with DTNB were performed as previously described [3].

Determination of disulfide bonds was carried out by the technique of Cavallini [6].

The reversibility of 8 M urea denaturation of *Sipunculus* and *Solen* arginine kinases was performed as follows: after incubation for 4.5 h at 20 °C with 8 M urea, 50 mM Tris—HCl, 50 mM NaCl, 20 mM L-arginine, 1 mM EDTA and 200 mM β-mercaptoethanol (pH 7.5), the *Sipunculus* and *Solen* arginine kinases at various concentrations (2000, 200, 100, 50 μg per ml) were dialyzed against 50 mM Tris—HCl, 20 mM L-arginine, 1 mM EDTA, 100 mM β-mercaptoethanol and 1 M NaCl (pH 7.5) for 1 h at 20 °C, and then overnight at 5 °C and against the same medium without NaCl for 3 h at 5 °C. Controls of the native enzymes without urea were run in the same conditions. The specific activities of the resulting preparations were then compared.

Results

Purification of Solen arginine kinase

The following solution and buffers were used. Solution A: 20 mM arginine, 1 mM EDTA, 10 mM mercaptoethanol (pH 7.5). Buffer B: 50 mM Tris—HCl (pH 7.5), 100 mM KCl, 20 mM arginine, 1 mM EDTA, 10 mM mercaptoethanol. Buffer C: 5 mM Tris—HCl (pH 7.5), 20 mM arginine, 1 mM EDTA, 10 mM mercaptoethanol. Buffer D: 50 mM Tris—HCl (pH 7.5), 20 mM arginine, 1 mM EDTA, 10 mM mercaptoethanol.

The enzyme was prepared from *Solen* foot and body muscle which had

been preserved at −20 °C. All operations were accomplished at 5 °C in the presence of the protecting agents L-arginine, EDTA and 2-mercaptoethanol. 200 g of muscle were minced and homogenized in a "Waring blendor" for 1 min with 2 vols of solution A. After stirring for 30 min and maintaining the pH at 7.5 by additions of 3 M NH_4OH, the homogenate was centrifuged for 30 min at 48 000 × *g*. The residue was extracted as above. The pooled extracts, adjusted to pH 7.5 (3 M NH_4OH), were allowed to settle overnight and the precipitate of impurities was discarded by centrifuging at 48 000 × *g* for 1 h. The supernantant, which contained about 25 mg protein per ml, was fractionated with solid ammonium sulphate, the pH being maintained at 7.5 by the cautious addition of 3 M NH_4OH. Accordingly, 28 g ammonium sulphate per 100 ml were added. After stirring for 1 h, the precipitate was removed by centrifugation at 14 000 × *g* for 30 min. To the supernatant was added 21 g of ammonium sulphate per 100 ml and the precipitation was performed as above. The precipitate thus obtained was dissolved in a minimal volume (20—30 ml) of buffer B. The enzyme solution (20—30 ml containing 70—80 mg protein per ml) was filtered on a Sephadex G 100 column (5 × 100 cm) previously equilibrated with the buffer B. The fractions having a specific activity above 100 (assayed by the arginine phosphate estimation method) were pooled and precipitated with 56 g ammonium sulphate per 100 ml. The precipitate was dissolved in about 15 ml of buffer C and dialyzed against the same buffer overnight. After centrifuging, the supernatant was adjusted with 1 M Tris—HCl (pH 7.5) to provide the buffer D. The enzyme solution (about 800 mg protein in 15 ml) was filtered on a DEAE—Sephadex column (2 × 40 cm) which had been equilibrated with the buffer D. The fractions of optimum activity, about 270, were pooled. The yield and degree of purification at different stages are given in Table I. At this step, the preparation showed traces of impurities on polyacrylamide-gel electrophoresis. For experiments where the use of a pure enzyme was found necessary, the preparation was treated again with DEAE—Sephadex. The pooled fractions having a specific activity, about 270, were precipitated with 56 g ammonium sulphate per 100 ml. The precipitate was dissolved in about 20 ml of buffer C and dialyzed against the same buffer overnight. After centrifuging, the solution (about 400 mg protein in 20 ml) was applied to a DEAE—Sephadex column (2 × 40 cm) previously equilibrated with buffer C. The elution was performed with linear gradient of 5 mM (400

TABLE I

PURIFICATION OF *SOLEN* L-ARGININE PHOSPHOKINASE FROM 200 g OF MUSCLE

Purification step	Total protein (mg)	Total (units)	Spec. act.	Purification	Yield (%)
Extraction	20400	238000	11.7	1.0	100
Centrifugation	14800	236000	16.0	1.3	99
Ammonium sulphate fractionation	2140	220000	103.0	8.8	92
Sephadex G 100 filtration	1000	190000	190.0	16.2	80
Dialysis	805	165000	204.0	17.4	69
DEAE—Sephadex filtration	400	111000	274.0	23.4	46

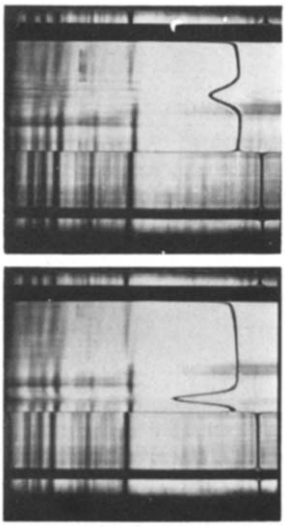

Fig. 1. Sedimentation patterns of *Solen* argenine kinase (7.45 mg/ml) in 50 mM Tris—HCl, 20 mM L-arginine, 1 mM EDTA and 20 mM β-mercaptoethanol (pH 7.5) at 20°C. 60 000 rev./min; exposures taken 9 and 57 min after speed attained.

ml)—200 mM (400 ml) Tris—HCl (pH 7.5), both buffers contained 20 mM arginine, 1 mM EDTA, 10 mM mercaptoethanol. The fractions having a specific activity of 280 were pooled and precipitated with 56 g ammonium sulphate per 100 ml. The precipitate were dissolved in a minimal volume of buffer D and dialyzed against the same buffer. The enzyme was stored in dialysis tubing and the dialysis fluid was changed every 3 days. The preparation thus obtained was homogeneous according to polyacrylamide-gel electrophoresis. A single sedimenting component was observed in analytical ultracentrifugation (Fig. 1).

Molecular properties

The molecular weight estimation of *Solen* arginine kinase by the Yphantis ultracentrifugation technique was not successful because the enzyme had a certain tendency to form aggregates. On a Sephadex G 100 column (3 × 43 cm) equilibrated with 50 mM Tris—HCl buffer (pH 7.5) containing 100 mM KCl, 20 mM L-arginine, 1 mM EDTA, 0.1 mM dithiothreitol, the elution volumes of *Solen* enzyme and of rabbit muscle creatine kinase were the same (120 ml), while the elution volume of *Homarus* arginine kinase was higher (170 ml). This fact suggested that the molecular weight of *Solen* arginine kinase is close to that of creatine kinase, namely 82 000 [7].

The molecular weight of *Sipunculus* arginine kinase as estimated by the Yphantis technique was 84 000. The partial specific volume calculated according to the amino acid composition [8] was 0.730. The experimental conditions were described in an earlier paper [9].

The extinction coefficients $E^{1\%}_{1\,cm}$ at 280 nm of *Solen* and *Sipunculus* enzymes were 12 and 10 respectively.

At pH 7.0, the DTNB titrated 8 thiol groups in the *Solen* enzyme. In the presence either of 6 M urea or 8 M urea and borohydride, the same number of

TABLE II

REVERSIBILITY OF 8 M UREA DENATURATION OF *SIPUNCULUS* AND *SOLEN* ARGININE KINASES

The experimental conditions for the denaturation and the recovery of activity are indicated in Methods.

	Sipunculus arginine kinase				*Solen* arginine kinase			
µg/ml arginine kinase	2000	200	100	50	2000	200	100	50
% recovery	53	59	72	72	0	9	18	20

thiol groups was found. The autooxidability of *Solen* enzyme is explained by the great reactivity of its 8 SH groups, like the 12 SH groups of *Sipunculus* enzyme. Neither enzyme possesses disulphide bonds. The presence of mercaptoethanol is necessary to maintain the active conformation.

Both enzymes were completely inactivated when 6 histidyl groups were titrated by the diethylpyrocarbonate at pH 7.0.

The recovery of enzyme activity following denaturation in 8 M urea differentiates the two enzymes. It varied dramatically from the *Sipunculus* enzyme to the *Solen* one, even when they were denatured under identical conditions as indicated in the Methods, that were found to be the most favourable. The percentages of recovery of activity following urea denaturation obtained at protein concentration of 100 µg per ml were 18 and 72 for *Solen* and *Sipunculus* enzymes respectively (Table II).

Catalytic properties

The optimum pH in the forward reaction of *Solen* arginine kinase, determined in 200 mM glycine—NaOH buffer in the pH range 8.0—9.5, is 8.5, a value identical with that found with the *Sipunculus* enzyme.

The specific activities of *Solen* and *Sipunculus* arginine kinases were 280 and 120 respectively at pH 8.5 and 30°C, as assayed by the method of phosphate estimation [2], but they were 350 and 210 respectively at pH 7.6, and 30°C, when the ADP estimation method was used [3]. The fact that the activities estimated by the first method were low is explained by the rapid autooxidation of these enzymes in the course of the reaction. We have studied the effect of mercaptoethanol on their activities with the second method. The mercaptoethanol concentration that gives the optimum activities is as high as 10 mM. The first method does not permit the use of such mercaptoethanol concentration. With the second method the assay was performed at pH 7.6, because the coupled enzyme system (pyruvate kinase and lactate dehydrogenase) cannot be used with satisfaction at high pH values.

The specificity of *Solen* enzyme with respect to guanidine substrates is absolute, as for *Sipunculus* enzyme [2]. Thus, L-arginine is the only substrate and the enzyme is not active towards the following guanidine derivatives : D-arginine, *N*-acetylarginine, agmatine, L-homoarginine, L-canavanine, glycocyamine, γ-guanidinobutyric acid, δ-guanidinovaleric acid, arginic acid.

Lineweaver and Burk plots gave a family of concurring straight lines for both enzymes. Fig. 2 shows such plots of *Sipunculus* enzyme where the variable substrate was L-arginine. A similar pattern was obtained with *Solen* kinase.

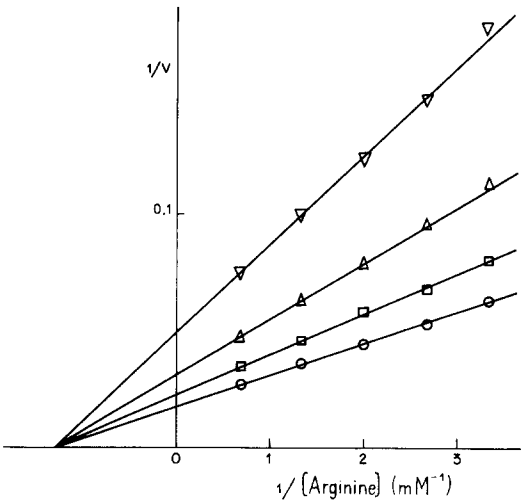

Fig. 2. Kinetics of the forward reaction of *Sipunculus* arginine kinase at low substrates concentrations. Temp. 30°C. 1 mM magnesium acetate, 10 mM KCl, 100 mM Tris—acetate (pH 7.6). Enzyme assay was carried out by the coupled enzyme method (3). 0.3, 0.375, 0.5, 0.75, 1.5 mM L-arginine as variable substrate. Mg^{2+}—ATP as fixed substrate at concentrations: ○, 0.5 mM; □, 0.375 mM; △, 0.25 mM; ▽, 0.125 mM.

Previously, with the *Sipunculus* enzyme, the straight lines obtained being parallel, we interpreted the results as an indication of a "ping-pong" mechanism [2]. These different results can be explained by the experimental conditions. The parallelism should be caused by the fact that the concentrations of substrates Mg^{2+}—ATP and arginine were high relative to their K_m values. Thus the changes in the slopes of the straight lines of a double reciprocal plot were too small to be detected. These views must be abandoned: the mechanism of the reaction catalyzed by *Solen* and *Sipunculus* arginine kinases is of sequential type.

The spectrophotometric study of L-arginine and Mg^{2+}—ADP binding was carried out with both enzymes. Binding of L-arginine on both enzymes at pH 7.5 gave rise to a difference spectrum (Fig. 3A), the features of which are identical to those obtained with *Homarus* arginine kinase, namely two minima at 287 and 279 nm, and another minimum at 239 nm [10]. The substrate analogues, L-isoleucine and L-citrulline produced similar difference spectra (Fig. 3B and C). The interaction of Mg^{2+}—ADP with both enzymes was revealed by a difference spectrum showing two maxima at 295 and 278 nm, and a minimum at 254 nm. This spectrum is analogous to that obtained with *Homarus* enzyme [3].

However, for the *Sipunculus* enzyme only, the spectrum of the Mg^{2+}—ADP—enzyme complex at non-saturating Mg^{2+}—ADP concentration was strongly intensified by the saturation of the enzyme with L-arginine (Fig. 4). The presence of arginine did not modify the spectrum features, only the amplitude of the three peaks were increased. This enhancement by arginine of the spectral effect of the Mg^{2+}—ADP—enzyme complex corresponded to a strong increase of the affinity for Mg^{2+}—ADP. Although giving rise to the same binding spectrum as L-arginine, L-isoleucine did not increase the enzyme affinity for Mg^{2+}—ADP.

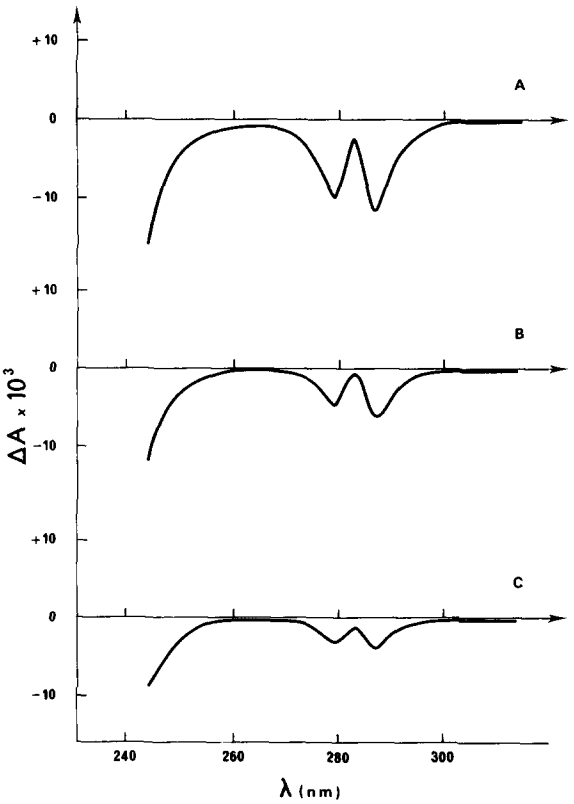

Fig. 3. Various α-amino acids—*Sipunculus* arginine kinase complexes difference spectra. Temp. 23°C. 50 mM Tris-acetate, 1 μM EDTA (pH 7.5). Enzyme 1.6 mg/ml. A, L-arginine 3.4 mM; B, L-isoleucine 8.5 mM; C, L-citrulline 10.5 mM.

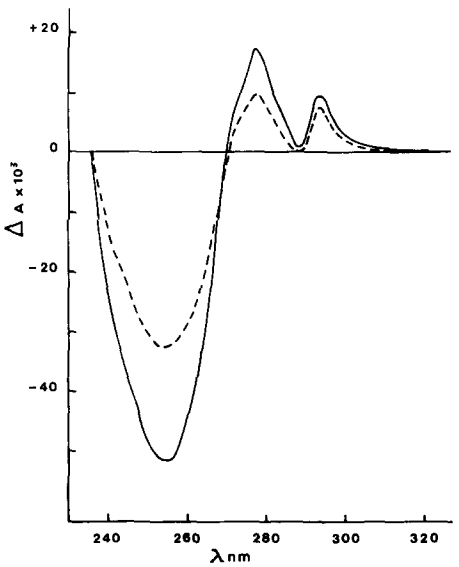

Fig. 4. Interaction of L-arginine with Mg^{2+}—ADP—*Sipunculus* arginine kinase complex. Temp. 23°C. 50 mM Tris-acetate, 1 μM EDTA (pH 7.5); - - - - -, difference spectrum of enzyme (1.41 mg/ml) with 0.085 mM Mg^{2+}—ADP; ———, difference spectrum of enzyme (1.34 mg/ml) with 0.081 mM Mg^{2+}—ADP and 5.18 mM arginine.

Discussion

The interest of this work is to emphasize the differences and similarities between the two forms of arginine kinases of molecular weight of 80 000. They have in common an excessive autooxidability and a great reactivity of their SH and histidine groups so that it was not possible to characterize the essential cysteinyl and histidyl residues as in the case of *Homarus* arginine kinase [11,12,13]. In contrast, their behaviour during renaturation after 8 M urea treatment clearly denotes structural differences. The latter have been also observed in a previous study of the effect of salts on both enzymes [3] and they are shown in particular in the catalysis reaction rates that are different. However the catalytic and binding sites of the kinase from *Sipunculus* are probably similar to the corresponding sites in the enzyme from *Solen* and also those from lobster. This hypothesis relies on the strict specificity towards L-arginine and on the identity of the spectral effect occurring upon binding of L-arginine and Mg^{2+}—ADP. However, emphasis must be laid on the interaction of arginine with the Mg^{2+}—ADP—enzyme complex, a property that characterizes only the *Sipunculus* enzyme.

Acknowledgements

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References

1 Thoai, N. v., Thiem, N. v., Lacombe, G. and Roche, J. (1966) Biochim. Biophys. Acta 122, 547—550
2 Lacombe, G., Thiem, N. v. and Thoai, N. v. (1969) Eur. J. Biochem. 9, 237—244
3 Thiem, N. v., Lacombe, G. and Thoai, N. v. (1972) Biochim. Biophys. Acta 258, 422—435
4 Der Terrossian, E., Kassab, R., Pradel, L.A. and Thoai, N. v. (1966) Biochim. Biophys. Acta 122, 462—469
5 Kuby, S.A., Noda, L. and Lardy, H.A. (1954) J. Biol. Chem. 209, 203—210
6 Cavallini, D., Graziani, M.T. and Dupre, S. (1966) Nature 212, 294—295
7 Yue, R.H., Palmieri, R.H., Olson, O.E. and Kuby, S.A. (1967) Biochemistry 6, 3204—3227
8 Thoai, N. v., Der Terrossian, E., Pradel, L.A., Kassab, R., Robin, Y., Landon, M.F., Lacombe, G. and Thiem, N. v. (1967) Bull. Soc. Chim. Biol. 50, 63—67
9 Oriol, C., Landon, M.F. and Thoai, N. v. (1970) Biochim. Biophys. Acta 207, 514—521
10 Roustan, C., Kassab, R., Pradel, L.A. and Thoai, N.v. (1968) Biochim. Biophys. Acta 167, 326—338
11 Virden, R. amd Watts, D.C. (1966) Biochem. J. 99, 162—172
12 Kassab, R., Pradel, L.A., Der Terossian, E. and Thoai, N. v. (1967) Biochim. Biophys. Acta 132, 347—360
13 Pradel, L.A. and Kassab, R. (1968) Biochim. Biophys. Acta 167, 317—325