APPARENT INHIBITION OF PYRUVATE KINASE BY ARGININE PHOSPHATE

A problem of kinetic studies on partially purified extracts

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1. Introduction

Inhibition of pyruvate kinase by arginine phosphate is studied [1–4]. Arginine phosphate could act either as a competitive inhibitor [1,2] or by phosphorylating or inducing a protein–protein interaction resulting in a decreased affinity for PEP [4]. In these studies the enzyme source was a crude extract [4], fractionated by ammonium sulphate, or partially-purified by other standard protein isolation procedures [1,2].

This paper reports how arginine phosphate exhibits effects on pyruvate kinase examined in crude extracts of the adductor muscle of the sea mussel *Mytilus edulis* similar to those described [1–4].

Following enzyme activity tests and determination of substrates (PEP, ADP, NADH, PArg) and products (lactate, ATP, Arg) of the LDH-coupled pyruvate kinase and the arginine kinase reaction, we conclude that PArg does not effect pyruvate kinase activity. Due to the arginine kinase activity also present in the preparations used, ADP is converted into ATP which leads to a concomitant reduction of the pyruvate kinase reaction rate. As in [1–4] no evidence is offered to show that arginine kinase is not involved; we doubt its effect on pyruvate kinase.

2. Experimental

Adductor muscles of *Mytilus edulis* were homogenized with 5 vol. ice-cold buffer (glycerol-2-phosphate 50 mM, EDTA 2 mM, mercaptoethanol 5 mM) pH 7.2 in a Sorvall Omnimix. The homogenate was centrifuged at 3000 X *g* for 15 min. An aliquot of the supernatant was taken to use as enzyme source (crude extract), the remainder was brought to 30% ammonium sulphate, stirred for 1.5 h and centrifuged. The supernatant was then brought to 70% ammonium sulphate and the above procedure repeated. The pellet was resuspended in the homogenization buffer and this also served as enzyme source (ammonium sulphate extract). All operations were performed at 2°C or on ice. Pyruvate kinase activity was measured as in [5] at 20°C, pH 7.6. Arginine kinase activity was measured spectrophotometrically in a cuvet containing: triethanolamine buffer 50 mM (pH 7.6); $MgSO_4$ 6.6 mM; NADH 0.12 mM; glycerate-3-phosphate 14.0 mM; ADP 0.1 mM; PArg 5 mM; PGK 4 units; GAPDH 4 units. Arginine was measured spectrophotometrically in a coupled reaction with arginine kinase, pyruvate kinase and LDH. Assay mixture: glycine buffer 100 mM (pH 8.6); mercaptoethanol 10 mM; NADH 0.12 mM; ATP 7 mM; $MgCl_2$ 20 mM; PEP 2 mM; LDH 18 units; arginine kinase 1 unit and pyruvate kinase 10 units. Arginine phosphate was measured spectrophotometrically in a coupled reaction with arginine kinase, PGK and GAPDH; test conditions as for arginine kinase except for PArg which is replaced by arginine kinase (1 unit). ATP and ADP were measured as in [6], lactate as in [7] and PEP as in [8].

3. Results and discussion

Table 1 shows the presence of both pyruvate kinase and arginine kinase in crude extracts of

Table 1
Pyruvate kinase activity (arbitrary units) under various conditions[a]

Condition	ΔE_{340}/min
1. No PArg, 2 mM ADP	0.100
2. 5 mM PArg, 2 mM ADP	0.005
3. No PArg, 12 mM ADP	0.106
4. 5 mM PArg, 12 mM ADP	0.103
5. Standard arginine kinase test	0.178

[a] Activity determined in a test containing: imidazole buffer 100 mM (pH 7.6); KCl 67 mM; $MgSO_4$ 8.3 mM; NADH 0.12 mM; LDH 36 units; PEP 5 mM; PArg and ADP as indicated. When PEP was added to complete the reaction, the mixture without PEP was preincubated for 5 min

M. edulis. Specific activities (μmol/min/mg protein) were 0.207 and 0.356, respectively. In the presence of 5 mM PArg pyruvate kinase activity is almost absent (about 5% control when based on a 5 min reaction time). When the pyruvate kinase test is repeated with 12 mM ADP instead of 2 mM, the specific activity is hardly effected. In contrast, addition of 5 mM PArg in this case exhibits no effect at all. This either means ADP and PArg compete for the same enzyme site or ADP is utilized by a side reaction in the presence of PArg. A likely candidate is the arginine kinase reaction:

$$ADP + PArg \rightleftarrows ATP + Arg$$

By repeating the experiment of table 1 with the ammonium sulphate enzyme extract addition of 5 mM PArg resulted in less inhibition of pyruvate kinase, namely about 11% control. Specific activities for both enzymes in this extract were 0.416 and 0.403, respectively. Due to the ammonium sulphate treatment there is a relative loss of arginine kinase activity which results in less sensitivity of pyruvate kinase towards PArg. This indicates the second suggestion is correct. In order to get stronger evidence the study was extended towards the determination of reactants. The results of this experiment are summarized in table 2. The first 2 conditions are controls, either PEP plus PArg (condition 1) or enzyme extract (condition 2) is omitted. In both cases no conversions occurred. Condition 3 represents an activity test for pyruvate kinase. There is a satisfactory stoichiometric relation between the reactants involved, including NADH and lactate. This proves that the LDH reaction is a reliable

Table 2
Utilization of substrates and formation of products involved in both the pyruvate kinase and the arginine kinase reaction after a 15 min incubation at 20°C

Condition	Utilized				Produced		
	NADH	PEP	ADP	PArg	Lac	ATP	Arg
1. 20 μl extract	0	0	0	0	0	0	0
2. 0 μl extract + PEP	0	0	0	–	0	0	–
3. 20 μl extract + PEP	0.212	0.223	0.230	–	0.206	0.219	–
4. 20 μl extract + PArg	0	0	0.660	0.731	0	0.675	0.625
5. 20 μl extract + PEP + PArg	0.036	0.053	0.690	0.658	0.040	0.675	0.655

When PEP was added to complete the reaction (conditions 2, 3 and 5) the mixture without PEP was preincubated for 5 min. The enzyme source was a 30–70% $(NH_4)_2SO_4$ extract. All cuvettes contained buffer (imidazole 100 mM, KCl 67 mM, $MgSO_4$ 8.3 mM) pH 7.0, NADH 0.56 mM and ADP 0.69 mM, other additions as indicated (PEP 0.38 mM and PArg 3.62 mM). The incubation was stopped by adding 1 vol. 14% (w/v) trichloroacetic acid, followed by neutralization with 5 M K_2CO_3. After centrifugation, the supernatant was used for reactant determinations. Data are expressed as μmol/15 min and are based on a constant amount of enzyme extract

indicator for the pyruvate kinase reaction rate. Condition 4 represents the arginine kinase reaction. Again there is a good stoichiometric relation between substrates and products. In condition 5 the demands are met for the operation of both enzymes. This condition is similar to that of the tests applied in the cited papers in which the effect of PArg on pyruvate kinase is studied and is also comparable to condition 2 of the first experiment. As in condition 3 there was no conversion of PArg and in condition 4 no conversion of NADH or PEP it is easy to interpret the result of condition 4. Due to the 5 min incubation preceding the addition of PEP (start of the pyruvate kinase activity test) followed by the 15 min incubation almost all ADP has been converted into ATP with a concomitant conversion of an almost equimolar amount of PArg into arginine. Therefore the apparent pyruvate kinase inhibition by PArg (compare condition 3 with 5) is not real but a consequence of ADP withdrawn by arginine kinase. This result explains 2 observations:

1. The % inhibition by PArg depended upon the time used for mixing and temperature adjustment preceding the start of the pyruvate kinase reaction by the addition of PEP (table 1, condition 2).
2. The progress curves were not linear but leveled off in an exponential manner.

The same type of progress curves were obtained [4] when the pyruvate kinase test was carried out in the presence of PArg. In fig.3 of [4] progress curves for the pyruvate kinase reaction in the presence and absence of PArg were presented and from the difference inhibition by PArg concluded. By extrapolation towards zero incubation time there would be no obvious reason for this conclusion.

This paper shows that the effect of arginine phosphate on pyruvate kinase is probably due to a side reaction with arginine kinase contaminating the pyruvate kinase preparation. To obtain unequivocal evidence it will be necessary to separate arginine kinase from pyruvate kinase.

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