INHIBITION OF THE PYRUVATE KINASE OF *HELIX POMATIA* L. BY PHOSPHO-L-ARGININE

Phosphorylation or a novel mechanism?

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1. Introduction

It is probably true for all animals that the rate of glycolysis is intimately linked with muscular activity. Activation of the phosphorylase kinases which initiate the breakdown of glycogen constitutes one of these links; another one is the effects of nucleotides and phosphagens on key enzymes of glycolysis. Recently it was shown that the control of carbohydrate metabolism by muscular activity in primitive vertebrates differs considerably from that in mammals [1]. Additional distinguishing features may be expected if control mechanisms are studied in invertebrates.

For example, in a crab, *Oplophorus gracilirostris,* pyruvate kinase (pK) activity is competitively inhibited by ATP but also by the phosphagen of this group of animals, phospho-L-arginine (PArg) [2]. In the snail, *Helix pomatia,* however, it was found that inhibition of the PK by PArg is not competitive but is mediated by a protein that transfers either the phosphagen itself or its phosphate group onto the enzyme. This is the first of a series of reports in which this new phenomenon is described.

2. Experimental

The investigation was carried out in the winter of 1976/77 on hibernating snails which had been obtained from a commercial supplier (Stein, Lauingen, West Germany). The animals were kept in a dark cold room at 4–5°C. One specimen at a time was taken to the laboratory, opened, the foot excised, weighed and quickfrozen at −30°C. After thawing, the foot was cut up with scissors, put into 30 ml of 0.01 M glycine buffer (pH 8.6), with 1 mM EDTA and 14 mM mercaptoethanol, and homogenized in an Ultraturrax mixer. The homogenate was centrifuged at 26 000 × *g* for 25 min, the pellet washed in 30 ml of the same buffer and centrifuged again. The two supernatants were pooled and fractionated by two ammonium sulfate steps at 25% and 80% saturation respectively. After centrifugation at 30 000 × *g* for 15 min the final pellet was resuspended in 4 ml of the homogenization buffer and dialyzed against 1 litre of the same buffer for a maximum of 3 h, with one change of buffer. All preparative steps were carried out at 0–4°C. The dialyzed preparation was separated into about 12 aliquots which were deep-frozen immediately and used, one at a time, for the enzyme tests. Pyruvate kinase (EC 2.7.1.40) activity was assayed spectrophotometrically according to [3]. The following concentrations were used: $MgSO_4$ 3 mM, KCl 30 mM, $NADH_2$ 0.3 mM, ADP 1 mM, phosphoenolpyruvate (PEP) varying concentrations, lactate dehydrogenase (LDH), (H 4 or M 4) 9–10 units, phosphate buffer 0.067 M (pH 6.0 to 7.0). In the inhibition experiments with phospho-L-arginine, PEP was kept at a concentration of 0.4 mM. All the reagents, except H 4 LDH from Boehringer, were from Sigma, St. Louis.

Ion-exchange chromatography on DEAE-cellulose (Whatman DE 52) was carried out with various elution buffers, mainly glycine–NaOH 10 mM (pH 8.6), and Tris–HCl 10 mM (pH 7.5), and with a linear KCl gradient from 0 to 250 mM.

3. Results

3.1. *Pattern of inhibition of PK activity by phospho-L-arginine*

The PK of *Helix pomatia* shows allosteric behaviour and sigmoidal kinetics at pH 6.0–6.2, hyperbolic kinetics at more neutral pH values. Fructose-1,6-diphosphate (FDP) is a strong activator of the enzyme and is capable of altering sigmoidal to hyperbolic kinetics at low pH values (fig.1). These features are the same as those of the PK of the mussel, *Mytilus edulis*, recently studied [4]. ATP is a competitive inhibitor of the reaction, as shown by figs.2a and 3, with a K_i of 1.8×10^{-3} M which agrees well with the value of 1.7×10^{-3} M reported for the crab *Oplophorus gracilirostris* [2]. However, when PArg instead of ATP is added to the reaction system the snail enzyme responds quite differently from the crab enzyme. In the crab, PArg is also a competitive inhibitor of PK with a K_i of 3.5×10^{-3} M. In the snail the initial rate of the reaction (when started with PEP) is similar to that of a reaction with an equimolar amount of ATP but then the rate decreases until after a few minutes a depressed but reasonably linear rate is reached. The degree and the time course of the inhibition depends on the concentration of PArg (fig.4) and on the individual snail preparation. In fig.3, for example, an experiment is illustrated in which PK activity is completely shut down by 5 mM PArg after about 3 min. In the preparation shown in fig.4, on the other hand, it took 12–15 min for the reaction to be slowed down to approximately 5% of the uninhibited rate by 4 mM PArg. In this case no further inhibition was achieved by 5 or 6 mM PArg. A second addition of PEP after 15 min (fig.4) did

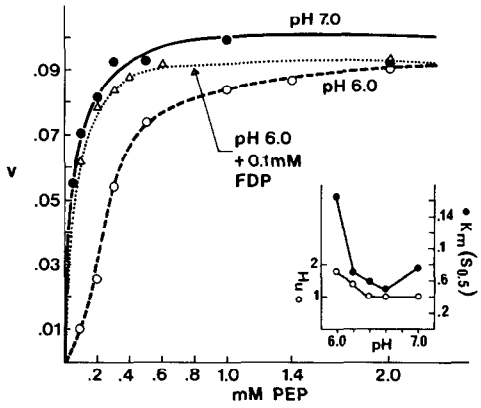

Fig.1. Kinetics of pyruvate kinase from the foot of *Helix pomatia*. Open circles: pH 6.0; closed circles: pH 7.0; open triangles: pH 6.0 + 0.1 mM FDP; phosphate buffers; 20°C. Inset: Hill constant (n_H; open circles) and substrate affinity (K_m or $S_{0.5}$ in mM PEP; closed circles) in relation to pH. Other assay conditions summarized in legend to fig.3. The diagram represents a characteristic experiment with the enzyme from one winter animal.

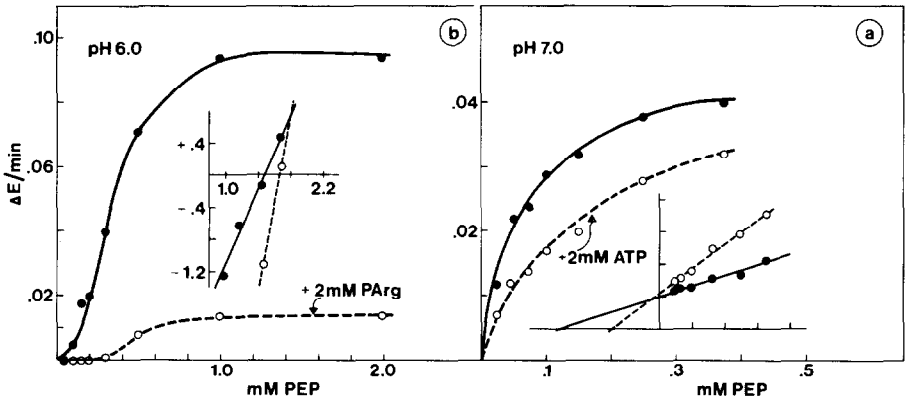

Fig.2. Effects of two inhibitors on the pyruvate kinase from the foot of *Helix pomatia*. (a) Competitive inhibition of PK reaction by ATP at pH 7.0. Inset: Lineweaver–Burk plot of the uninhibited (closed circles) and the inhibited (open circles) reaction. (b) Non-competitive inhibition of PK reaction by PArg at pH 6.0. Inset: Hill plot of the uninhibited and the inhibited reaction. Phosphate buffers; 20°C.

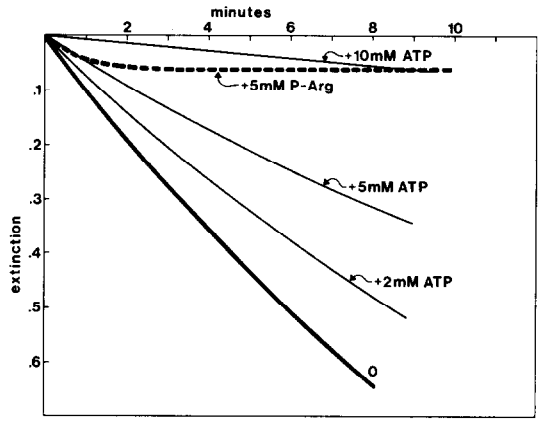

Fig.3. Effect of ATP and PArg on the time course of the pyruvate kinase reaction from the foot of one winter snail. Assay conditions: $MgSO_4$ 3 mM, KCl 30 mM, $NADH_2$ 0.3 mM, ADP 1 mM, PEP 0.4 mM, 10 units LDH, phosphate buffer M/15, pH 7.0; 20°C. All reactions were started with PEP. The end concentrations of the two inhibitors are indicated.

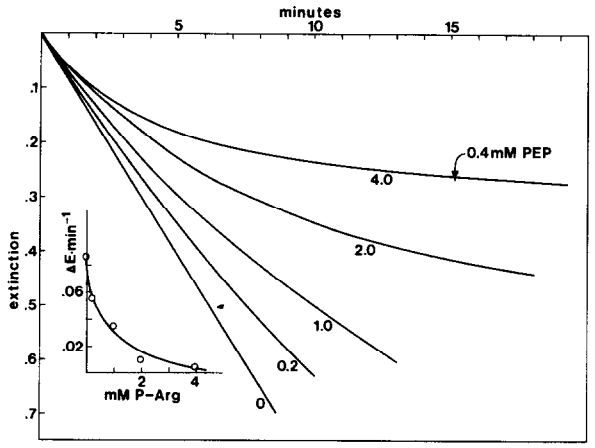

Fig.4. Effect of concentration of phospho-L-arginine on the time course of the pyruvate kinase reaction from the foot of one winter snail (different specimen from the one recorded in fig.3). The concentrations of PArg (in mM) are indicated. At one point 0.4 mM PEP were added to the assay system during the reaction. Inset: PK activity after 12–15 min plotted against concentration of inhibitor. At this time the reaction rate is linear.

not affect the rate of the reaction, proving that the inhibition is non-competitive. Moreover, physiological concentrations of NAD, pyruvate or arginine had no effect on the rate of the uninhibited reaction. At pH 6.0, with sigmoidal kinetics of PK activity, inhibition by PArg is equally effective. If the rates after 12–15 min are taken as the inhibited rates, fig.2b shows that PArg increases the allosteric behaviour of the enzyme, decreases V_{max} but not the substrate affinity of the enzyme. In the preparation illustrated by fig.2b 2 mM of PArg increase the Hill constant from 2.3 to 5.1, but decrease $K_{0.5\ S}$ from 0.47 to 0.34. V_{max} is depressed by approximately 85%. Addition of 0.1 mM of FDP together with PArg did *not* release the inhibition. This is in contrast to the finding [5] that in the PK of the rat FDP counteracted the inhibition caused by phosphorylation of the enzyme.

3.2. *Inhibition by PArg is mediated by a protein*

Ion-exchange chromatography of the dialyzed 30–80% ammonium sulfate precipitate on DEAE-cellulose separates PK activity from a factor which is not retained by the column and is eluted with the front of both 10 mM Tris–HCl pH 7.5 and 10 mM glycine–NaOH pH 8.6. The PK thus purified is not inhibited by 2 mM PArg at all (fig.5b). If to 200 μl of the fraction containing the PK activity 100 μl of the elution front are added, inhibition sets in immediately until after 4 min an approximately linear rate is reached (fig.5c). A second addition of 100 μl of the same fraction did not lead to a further decrease of the inhibited rate.

4. Discussion

The PK from a dialyzed 30–80% ammonium sulfate precipitate of the foot of *Helix pomatia* is competitively inhibited by ATP whereas inhibition by PArg is non-competitive and requires the presence of an additional factor which can be separated from the enzyme by ion-exchange chromatography. The factor is eluted with the elution front of various buffers from pH 7.5 to 8.6 and thus under these conditions seems to be a cationic protein.

If one speculates on the mechanism involved two possibilities come to mind. One possibility is that the

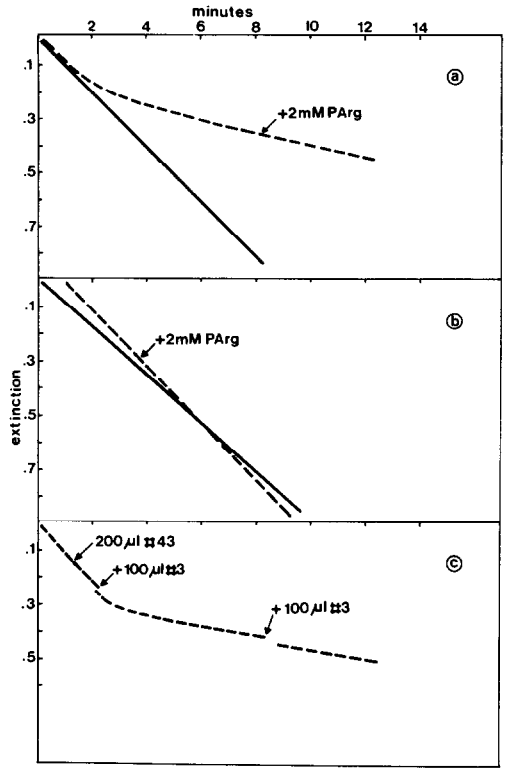

Fig.5. Effects of PArg on time course of pyruvate kinase reaction from the foot of one winter snail. (a) Crude homogenate, fractionated by two ammonium sulfate steps between 25% and 80% of saturation and dialysed against elution buffer. Full curve: uninhibited reaction; dashed curve: inhibition with 2 mM PArg. 35 µl of the preparation were added to the assay system described in the legend to fig.3. (b) Fraction No. 43 after ion-exchange chromatography on DEAE-cellulose. 200 µl of this fraction were added to the assay system. Full curve: without PArg; dashed curve: with 2 mM PArg. (c) Assay containing 2 mM PArg. The reaction was started with 200 µl of fraction No. 43; at the points indicated 100 µl of fraction No.3 (elution front) were added.

protein plays a role similar (although opposite in sign) to α-lactalbumin which increases the affinity of galactosyl-transferase for glucose [6]. The other possibility is that the factor is a protein kinase which assists in phosphorylating the PK of the snail. Recently it has been shown that the PK of rat, chicken and pig liver can be phosphorylated with ATP by a cAMP stimulated protein kinase [7–9], a process showing similarities to the inhibition of the PK of the snail with PArg and the cationic protein. The time constant of inhibition is rather similar for the snail and for the pig enzyme [9], and at pH 6.0 PArg increases the sigmoidicity of the kinetics of the snail enzyme in the same way as ATP in the presence of a protein kinase increases the sigmoidicity of rat liver PK at pH 7.0 [8].

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