MULTIPLE CONTROL OF *HELIX POMATIA* PYRUVATE KINASE BY HIGH-M_r MODULATORS

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Received 21 December 1981; revision received 15 January 1982

1. Introduction

Recent reviews of the biochemistry of anaerobiosis in poikilothermic animals [1–4] have made it clear that under the stress of low pO_2 several metabolic pathways may be switched on in sequence and that the enzyme pair PK/PEPCK assumes a central role in this switching operation. This implies that the PK of an animal subject to ecological anaerobiosis must respond to factors which are of little (sometimes even opposite) significance in homeotherms. Such a factor is the concentration of FBP in the cell. All the major inhibiting effects on the PK which operate at physiological concentrations are removed by FBP. This would appear to be a useful response if high concentrations of FBP always signalled high glycolytic turnover rates. However, during ecological anaerobiosis a relatively high glycolytic flux bypasses the PK reaction and is directed towards succinate and propionate by the PECK reaction. This may lead to high intracellular concentrations of FBP [5] in the face of which, however, the PK of these particular tissues must remain inactive.

The garden snail, *Helix pomatia,* is subjected to anoxic conditions in spring before it leaves its winter quarters. If exposed to a nitrogen atmosphere it accumulates large amounts of D-lactate and succinate in the haemolymph [6]. We shall show that the PK of this species is under the influence of a number of controlling effectors some of them corresponding to well-known principles in other animals, but at least one of them representing an apparently novel mechanism which causes drastic inhibition of PK activity even in the presence of as much as 100 μM FBP.

Abbreviations: PK, pyruvate kinase (EC 2.7.1.40); LDH, D-lactate dehydrogenase (EC 1.1.1.28); FBP, fructose-1,6-diphosphate; PEP, phosphoenolpyruvate; PEPCK, phosphoenolpyruvate carboxykinase (EC 4.1.1.32)

2. Experimental

2.1. *Enzyme purification*

About 10 g frozen tissue were homogenized in 50 ml ice-cold phosphate buffer pH 6.5 or 7.0, 0.067 M, 1 mM EDTA, with an Ultra Turrax homogenizer. The homogenate was centrifuged at 15 000 rev./min for 20 min and the pellet extracted a second time with the same buffer. The combined supernatants were brought to 80% with solid $(NH_4)_2SO_4$ and stirred at 2–4°C for 1 h. The mixture was centrifuged, the pellet suspended in 50 ml triethanolamine buffer 10 mM (pH 7.5) and dialysed against 3 × 2 l of distilled water for 6–12 h. A DEAE-cellulose 2.6 × 30 cm column was equilibrated with triethanolamine buffer 10 mM (pH 7.5) and the dialysed preparation applied to it. Proteins were eluted with a linear gradient of 0–0.5 M KCl in 10 mM triethanolamine buffer, the fractions collected and assayed for enzyme activity or for modulating effects. Protein concentration was monitored at 280 nm. The KCl concentration of the fractions was followed with a refractometer.

2.2. *Preparation of crude homogenates*

Tissues were homogenized in exactly 20 vol. of the above phosphate buffer by means of an Ultra Turrax homogenizer. The homogenate was centrifuged at 15 000 rev./min for 30 min and the supernatant used as an enzyme source. All preparative steps were carried out close to 0°C.

Published by Elsevier Biomedical Press
00145793/82/0000–0000/$02.75

2.3. *Enzyme assay*

Pyruvate kinase activity was measured photometrically in a coupled assay, as indicated in the legend to table 1.

3. Results

3.1. *A tissue-specific inhibitor which forms weak complexes with PK*

The specific activity of PK depends on its concentration in all tissues except the kidney (fig.1). This effect is most pronounced in the mantle, the tissue with the highest 'glycolytic scope' (expressed as the difference between the activities of LDH and PK). In the preparation shown in fig.1, a 10-fold increase in the concentration of the assayed cell fraction lead to a 5-fold decrease in specific enzyme activity. This inhibitory effect is not influenced by the presence of 0.1 mM FBP in the assay medium, and it is just as effective in the protein fraction of a supernatant that had been passed through a G-25 or a G-50 Sephadex column. However, when the homogenate is passed through a DEAE ion-exchange column, the inhibitor is separated from the PK activity (inset in fig.1) and elutes just ahead of the major PK peak (fig.2). These findings suggest the presence of a reversibly bound inhibitor which remains attached to the enzyme during gel filtration.

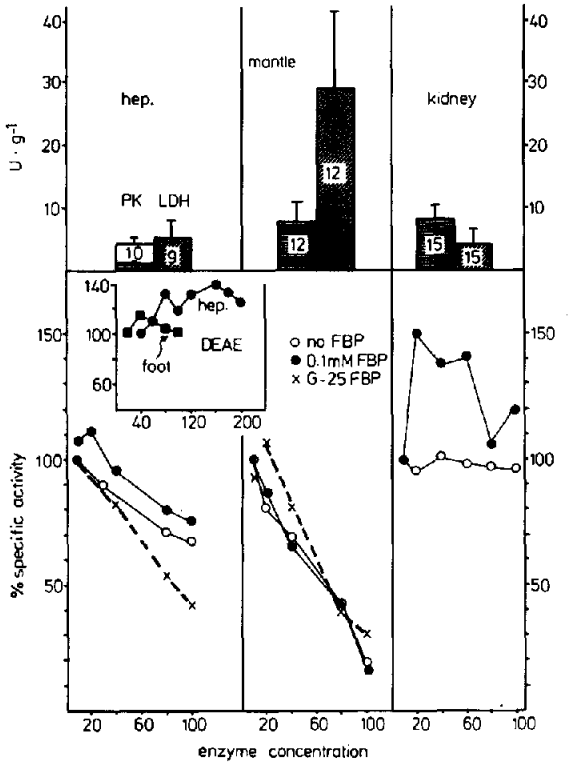

Fig.1. Lower: Dependence of the activity of pyruvate kinase on enzyme concentration (μl of a 20-fold dilution of tissue fresh wt/ml assay volume) in 3 organs of *Helix pomatia*. Enzyme activity is expressed in % of the activity found at the greatest dilution of the sample. Shown are measurements with crude supernatants with and without 0.1 mM FBP, with supernatants that had been passed through a G-25 Sephadex column, and with purified PK fractions after anion exchange chromatography on DEAE (inset). Upper: Activities of pyruvate kinase and lactate dehydrogenase (U/g fresh wt) in the same 3 organs. Means, standard deviations and numbers of determinations are indicated. For the FBP-sensitive PK of the hepatopancreas (hep.) activities without (shaded portion of the column) and with (open portion) 0.1 mM FBP are given.

3.2. *Effect of ADP on inhibited and inhibitor-free PK*

A mechanism that inhibits PK activity in the presence of high concentrations of FBP must itself be under some controlling influence or it would be of little biological interest. One of these factors seems to be the concentration of ADP. Fig.3a shows that concentration-dependent inhibition of PK activity in crude homogenates is strongest at low concentrations (0.5 mM) of ADP but is relaxed at 2.0 mM ADP. This relationship is further strengthened by fig.3b which compares the effect of ADP on a purified PK fraction in the absence and presence of partially purified inhibitor (fig.2). This ADP-dependence of the inhibition links the PK/inhibitor system to the energy content of the cell.

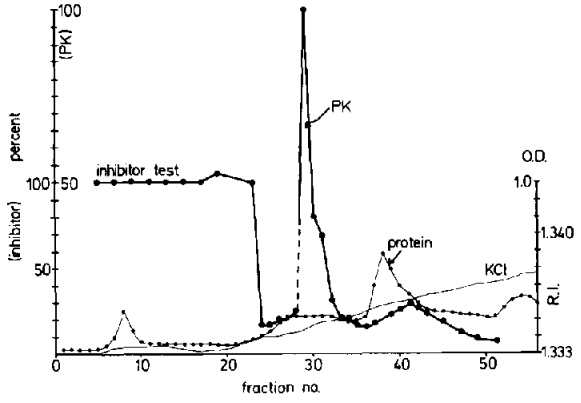

Fig.2. Fractionation of a mantle homogenate on DEAE-cellulose. The inhibitor test consisted of mixing aliquots of the fraction with the highest PK activity with all other fractions and recording the degree of inhibition.

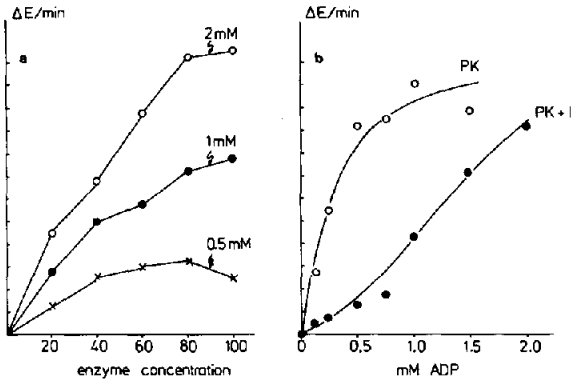

Fig.3.(a) The concentration-dependent inhibition of PK activity in crude homogenates (μl of a 20-fold dilution of tissue fresh wt/ml assay volume) of mantle tissue at 3 concentrations of ADP.

Fig.3.(b) ADP kinetics of a DEAE-purified fraction of PK (○) and of the same fraction mixed with the inhibitor which on DEAE elutes before the major PK peak (●).

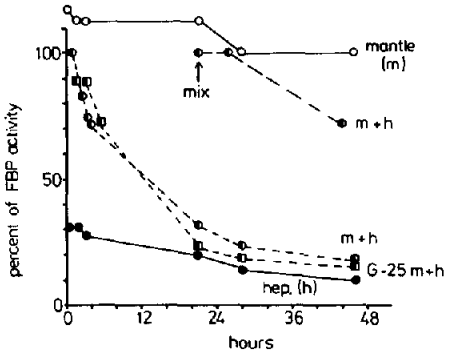

Fig.4. Time course of PK activity in homogenates of mantle (m), hepatopancreas (h), and in 1:1 mixtures of these 2 preparations. In one experiment the protein fraction of a G-25 Sephadex filtered hepatopancreas homogenates was used; in another experiment mixing of the 2 homogenates took place after the preparations had been standing on ice for 24 h. PK activity is expressed in % of the maximum activity elicited by activation with 0.1 mM FBP.

3.3. *Slow inactivation of FBP-insensitive PK*

The PK of a hepatopancreas homogenate always shows allosteric kinetics and is activated by FBP. However, the degree of inactivation (i.e., degree of FBP-sensitivity) varies from preparation to preparation. One finds preparation with a $S_{0.5(PEP)}$ of several mM down to nearly the 'normal' K_m-value of a FBP-activated PK, of 60–90 μM [7,8]. The Hill coefficient may vary from 1.8–1.0. Upon storage of a crude hepatopancreas homogenate PK activity becomes progressively inactivated, i.e., more sensitive to FBP. In the end all hepatopancreas preparations have an activity with 2 mM PEP which is from 10–15% of the activity when 0.1 mM FBP are added. This suggests the presence of an inactivating system in the hepatopancreas which is of different strength in different specimens, and which alters the conformation of the PK from a form with Michaelis-Menten PEP kinetics to one with extreme allosteric behaviour. No other organ displays this type of inactivation. However, if equal volumes of a mantle homogenate and a hepatopancreas homogenate are mixed the PK of the mantle homogenate is also inactivated (fig.4), the rate of inactivation depending on the initial strength of the inactivating principle in the hepatopancreas homogenate as well as on the presence of a third factor to be described below. If the hepatopancreas homogenate is passed through a G-25 Sephadex column, the inactivating strength recovered in the protein fraction is exactly the same as that of the crude homogenate.

3.4. *A principle that delays the inactivation of FBP-insensitive PK*

If a PK partially purified by DEAE-chromatography is mixed with a hepatopancreas homogenate inactivation of the enzyme is much more rapid than that of the unpurified PK in a homogenate. This suggests the presence of a factor in the mantle homogenate which delays or even prevents inactivation of the PK. Preliminary results indicate that the delaying principle elutes on DEAE after the major PK-peak as a broad, perhaps bimodal, shoulder.

The rate at which inactivation is transferred from a hepatopancreas homogenate to any other tissue sys-

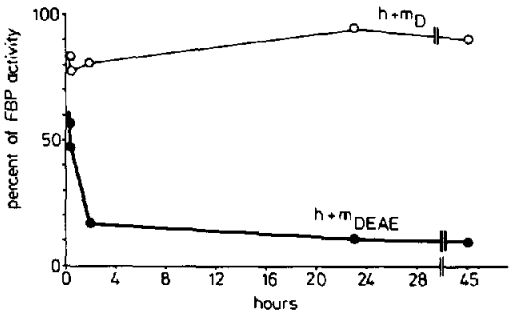

Fig.5. Time course of PK activity in 1:1 mixtures of a hepatopancreas homogenate (h) with 2 preparations of a mantle homogenate. m_D is a preparation which has been precipitated with ammonium sulfate, concentrated and dialysed. m_{DEAE} is a PK preparation purified by DEAE anion exchange chromatography. PK activity expressed as in fig.4.

tem seems to depend on the quantitative relationship between the inactivating and the delaying principle as well as on the PK concentration. After ammonium sulfate precipitation we concentrated a mantle preparation 3-fold compared with the original homogenate and dialysed it. This preparation, when mixed with a hepatopancreas homogenate, did not change in activity for ≥45 h. However, the PK from the same dialysate after DEAE ion-exchange chromatography was inactivated in <2 h (fig.5).

4. Discussion

After ammonium sulfate precipitation, dialysis and DEAE ion-exchange chromatography all tissues tested so far yield one major peak of PK-activity.

In addition to the usual low-M_r modulators (ATP, alanine, FBP) the regulatory and catalytic properties of this enzyme are influenced by 3 components which can be recovered after ammonium sulfate precipitation and dialysis of the homogenate, and thus are considered to be of medium or high M_r. The first of these modulators is an inhibitor which shows concentration-dependent association–dissociation behaviour. Characteristically this inhibitor is most effective in the mantle, the organ with the highest glycolytic scope and absent in the kidney with a low glycolytic scope (fig.1). This supports the contention [9,10] that tissues which are more dependent on anaerobic processes for energy production require a greater degree of metabolic integration than strictly aerobic tissues. Of the greatest importance for this kind of integration is the fact that this effector inhibits PK activity even in the presence of high concentrations of FBP. After prolonged periods of anaerobiosis, up to 60 μM FBP have been reported in tissues of *Mytilus edulis* [5]. Since such a concentration would maximally activate all known PKs (at least at physiological concentration of ATP or alanine) the question arises how the flow of glycolytic intermediates could be directed towards succinate and propionate, the major end products of late stages of anaerobiosis, rather than towards lactate or alanine. The dissociable inhibitor described in this paper would be perfectly poised to fulfill such a task.

Of the other 2 high-M_r modulators, one causes the time-dependent inhibition of PK accompanied by increasing sigmoidicity of the PEP kinetics, whereas the other delays this process. The former principle is maximally expressed in the hepatopancreas, the latter has so far been found only in the mantle, but is likely to be present in other tissues too. Since the rate of inhibition is neither diminished by filtration of the homogenate through G-25 Sephadex nor influenced by ATP, and since FBP-sensitive and FBP-insensitive PK show identical electrophoretic behaviour (R. L., in preparation) we believe that this type of inhibition is not due to a phosphorylation–dephosphorylation mechanism as suggested for the PK from *Mytilus edulis* in [11,12]. Rather, we are thinking along the lines of Hoffmann [13] who stated that the inhibition of the PK of *Tubifex* by phosphoarginine 'requires an additional factor which has either a high M_r or is bound to a corresponding protein that may be separated from the enzyme by an ultracentrifugation step'.

This control of PK activity by several complexing factors of 'high M_r' may be a wide-spread phenomenon in animals in which the succinate pathway operates during anaerobiosis.

Acknowledgements

This investigation was supported by project 3917 of the Fonds zur Förderung der wissenschaftlichen Forschung in Österreich. We thank Ulrike Platzer for technical assistance.

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