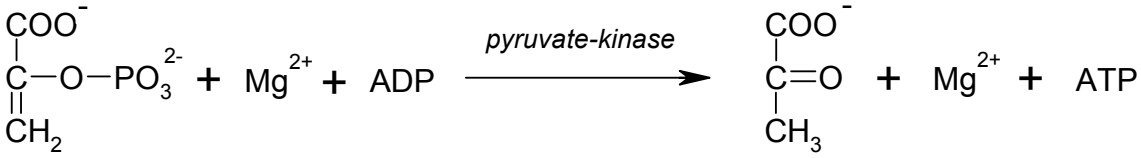
DIFFERENTIAL REGULATORY BEHAVIOUR OF L- AND M-TYPE ISOENZYMES OF PYRUVATE-KINASE: EFFECTS OF ALLOSTERIC LIGANDS ON ACTIVITY

INTRODUCTION

It is known that glycolysis and gluconeogenesis have most of their enzymes in common, and that they catalyze reversible reactions at rates which are controlled by the actual concentrations of substrates and products. There are, however, several control steps, where these two pathways are reciprocally regulated, providing a co-ordination so that one pathway is relatively inactive while the other is highly active.

Glycolysis is known to occur in all tissues of an animal, but the occurrence of gluconeogenesis is limited to the liver and kidney. This fact appears not only in the expression of enzymes catalyzing the reactions found in gluconeogenesis and not in glycolysis, but also in the essential difference in the way several key reactions of glycolysis in the liver (and kidney) compared to those in muscle are regulated. One of the control steps of glycolysis lies at the cross-over of the metabolites phosphoenolpyruvate/pyruvate, in the reaction catalyzed by pyruvate-kinase. Tissue-specific isoenzymes of pyruvate-kinase play an important role in this regulation. Isoenzymes of pyruvate-kinase are tetrameric proteins with a molecular weight of 250 kD. The reaction catalysed by the enzymes (ATP:piruvate phosphotransferase, EC 2.7.1.40.) produces ATP and pyruvate:



phosphoenol-pyruvate (PEP)

pyruvate

Isoenzymes of pyruvate-kinase isolated from liver (type L), kidney, adipose tissue, lung, red cells (type A) and muscle (type M), can be distinguished by their kinetic and immunochemical properties. While type M isoenzyme shows no allosteric behaviour, types A, and L bind phosphoenolpyruvate cooperatively, are activated by fructose 1,6-bisphosphate, as well as inhibited by ATP, alanine and several other amino acids. (These effects result from the shift of Km value of pyruvate-kinase for phosphoenolpyruvate, as shown in Fig.1.) Moreover, glucagon / cAMP initiates protein-kinase- and ATP-dependent phosphorylation of type L and A (but not the type M) isoenzymes, resulting in decreased affinity for the activating ligands, and increased affinity for the inhibitors. In the presence of the activating ligands, however, the phosphorylation of the protein is hindered. Further regulation is obtained by 'de novo' protein synthesis: feeding an animal on a high carbohydrate diet increases mRNA-level of type L isoenzyme up to fivefold, while starvation decreases its level to one third of the normal value. This complex regulation serves to co-ordinate the metabolic conversion among carbohydrates, lipids and amino acids by the needs of energy, blood-glucose, and synthetic pathways.

The allosteric behaviour of Type L isoenzyme is being studied using a crude preparation of pyruvate-kinase

from liver, and compared to the characteristics of type M isoenzyme. The rates are measured using a coupled enzyme assay: pyruvate produced in pyruvate-kinase reaction is converted to lactate, consuming an equimolar amount of NADH and the absorbency change at 340 nm is recorded .

PREPARATIONS AND SOLUTIONS

Stock solutions

Medium: 0.1 M KCl, 50 mM TRIS, 10 mM $MgCl_2$, pH 7.6. All reagents are prepared in Medium and adjusted to pH 7.6.

10 mM $NADH^+$

50 mM PEP^+

400 U/ml lactic dehydrogenase (LDH): use pyruvate-kinase-free LDH (check contaminating PK in the preparation in a blank experiment).

100 mM ADP^+

100 mM ATP^+

100 mM D,L-alanine

2,5 mM fructose 1,6-bisphosphate $(FDP)^+$

Piruvate-kinase, type M (muscle-type isoenzyme): generally it is present in crude lactic-dehydrogenase preparations from muscle.

Piruvate-kinase, type L (liver-type isoenzyme).

Preparation: The first step is to prepare postmitochondrial supernatant from rat liver in the following way: Livers of two rats are quickly excised, rinsed in 0.15 M NaCl and chopped into approx. 3-mm cubes with scissors, then washed free of contaminating blood with 0.15 M NaCl solution. Tissue is then homogenized with 4-fold volumes of ice-cold Medium and centrifuged at 10,000xg for 20 min. Supernatant is filtered through 4 layers of cheese cloth, then 2-mercaptoethanol and fructose 1,6-bisphosphate, 10 mM and 0.2 mM, respectively, are added. This postmitochondrial supernatant is then fractionated by adding ammonium sulfate between the percentage saturations of 33 and 45, in the following way: to 100 ml of supernatant add 50 ml ammonium sulfate solution saturated at 0 ^oC , stir in ice for 30 min, then centrifuge at 10,000xg for 20 min. Pour supernatant into a measuring cylinder to get the volume. Add 7.5 g powdered ammonium sulfate slowly to every 100 ml of supernatant, while stirring in ice. After 30 min stirring in ice, centrifuge slurry as above, and resuspend pellet in a few ml of a solution of 1.8 M ammonium sulfate, also containing 50 mM TRIS, 0.2 mM fructose 1,6-bisphosphate, and 10 mM 2-mercaptoethanol, pH 7.6. Store preparation at 0 ^oC; it is stable for 2-3 weeks at these conditions.

Equipments and Tools

Recording spectrophotometer, able to measure at 340 nm,

semi-microcuvettes with light path of 1 cm,

L-shaped stirring rod made of glass, or plastic (it should fit into the cuvettes, and be able to carry at least 30 μl of reagent solution),

micropipettes with tips.

PROCEDURE

Note: Keep stock solutions (except Medium), and all enzymes on ice, but carry out measurements at room temperature.

1. Regulation of pyruvate-kinase activity by ATP and FDP

Pipette reagents into a cuvette in the following sequence:

Medium	0.82 ml
PEP	20 μl
NADH	20 μl
LDH	20 μl
PK-L	20 μl

Mix the reaction with a stirring rod. Adjust the potentiometric recorder to 100 %, and chart the speed to 60 cm/h (=1cm/min); start the pen and paper moving. When the addition of a reagent is needed, simply open the lid of photometer. Make notes on data of measuring conditions.

Record baseline for about 2 min, then add the next reagent in the given sequence by pipetting reagent onto the stem of the rod, close to its end. When the time comes to add the reagent to the reaction, lift the lid of the photometer, immerse the glass rod into the cuvette, move it twice up and down to mix the content, then take out the rod, and carefully, but quickly, close the lid of the photometer. Usually there is no need to make any adjustment on the recorder later on.

To start the reaction

(1): Add ADP: 20 μl. Mix quickly, and record the optical change

(2): Add ATP: 20 μl. Follow the same procedure.

(3):Add FDP: 20 μl. Mix, and record changes, while NADH has been consumed. Write down the data of the equipment you used (absorbency range, chart speed of recorder).

2. Effect of alanine and fructose 1,6-bisphosphate

Prepare reaction mixture as above (Medium, PEP, NADH, LDH, PK-L), and add ADP (start, step (1)).

(2): Add alanine: 20 μl. Record changes for 3 minutes, then add another 80 μl alanine. Record trace for an additional 3 minutes. The reaction can finally be accelerated by adding:

(3): FDP: 20 μl. Observe the change in rate.

Repeat experiments with M-type pyruvate-kinase. Attach recordings to your notebook, and evaluate results.

EVALUATION OF RESULTS

The rate is calculated from the slope of the linear portion of the trace after each addition. Specific activities in units of μmol ATP formed/min/ml enzyme solution are computed from μmol NADH oxidized/min/ml enzyme) using the rate of absorbency change:

$$v = \frac{V * \Delta Ext/perc}{6.22 * W * l}$$

Where V is the total volume reaction mixture, millimolar extinction coefficient of NADH is 6.22 $cm^2/\mu mol$, W the volume of the added enzyme and l is the path of light (1cm).

To compare the velocities obtained in the presence of effectors, relative values can also be calculated. Take the velocity after the addition of ADP to 1.0, and calculate relative velocities obtained after the following additions. (See "f" in Table I.)

Table I. EVALUATION OF RESULTS

			VELOCITY			
		PK-L			**PK-M**	
Reagents	**ΔExt\min**	**μmol\p\ml**	**f**	**ΔExt\min**	**μmol\p\ml**	**f**
ADP			1.0			1.0
ATP						
FDP						
Ala						

CONCLUSION AND PROBLEMS

1. Compare your results to the plot shown in Fig 1. (Find the concentration of PEP you used in the experiment and check the extent of possible activation or inhibition caused by the ligands.) Do your results support the theory of the allosteric behaviour of PK isoenzymes? Have you observed any difference in the regulation between the types L and M isoenzyme in the presence of ATP, alanine, and FDP?

2. Give an explanation on the actions of possible physiological regulators of pyruvate-kinase activity in liver following a carbohydrate-rich diet. Describe the possible physiological significance which can be attributed to the inhibitory effect of alanine on pyruvate-kinase activity in liver cells?

3. The allosteric inhibitory effect of ATP on the type L isoenzyme has been demonstrated. The dissociation constant of ATP from the allosteric *site* on enzyme is about 0.15 mM Should you expect any significant inhibition on kinase activity caused by ATP generated by the reaction, itself? (The concentration of ATP formed e.g. in the first few minutes can be calculated from the decrease of absorbency from the start.) Even if your expectation is "yes", you probably did not observe any inhibition, because, by the time the ATP formed in the reaction could have been accumulated you have just added FDP (which releases any kind of inhibition). It is worth mentioning that type

M isoenzyme is also inhibited by ATP. However, that inhibition is not of allosteric type, but an inhibition of the product release step at the active centre, with a significantly higher K_i (3.5 mM).

4. Type L isoenzyme is stabilized by FDP. How could you explain this?

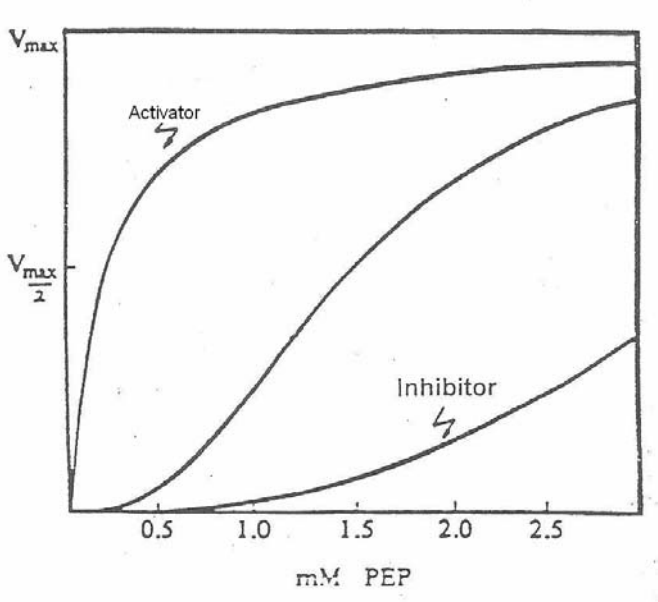


Fig. 1. Dependence of the rate of L-type isoenzyme of pyruvate-kinase on phosphoenolpyruvate (PEP) concentration measured at saturating concentration of ADP, in the presence of allosteric effectors