TRANSIENT TIME OF THE PYRUVATE KINASE-LACTATE DEHYDROGENASE SYSTEM OF RABBIT MUSCLE *IN VITRO*

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1. Introduction

Biochemical networks are based on the specific organisation of enzymes, which are ordered in series catalyzing linear or cyclic transformations of matter according to the feedback principle. The fundamental, organisational problem of a metabolic pathway is the question of the nature of the coupling of an enzyme sequence in terms of a possible structural organisation of the enzymes as well as of its time structure. A structural organisation, such as aggregation of the enzymes or the intermolecular induction of conformational changes, should be reflected in the concentration dependancy of a metabolic flux. The time structure of the system is given in the transient time of an enzyme sequence, which is defined as the time required for the system to approach to within $1/e$ of the new steady state.

Following a preliminary report [1, 2], we here describe our observations of kinetic properties of a simple two-enzyme-system of glycolysis, namely pyruvate kinase (EC 2.7.1.40) and lactate dehydrogenase (EC 1.1.1.27) of rabbit muscle over a wide concentration range up to physiological concentrations under saturating conditions of the first enzyme. The paper is based on a separate report [3], in which it was shown that the steady state kinetics of lactate dehydrogenase of the rabbit muscle follow a proportionality between the enzyme concentration and its activity in the concentration range of 10^{-9}–10^{-5} M under specified conditions indicating a homogeneous reaction mechanism.

Abbreviations:
PK : pyruvate kinase
LDH : lactate dehydrogenase
NADH: reduced nicotinamide adenine dinucleotide
Pyr : pyruvate
ADP : adenosine diphosphate
PEP : phosphoenol pyruvate.

2. Material and methods

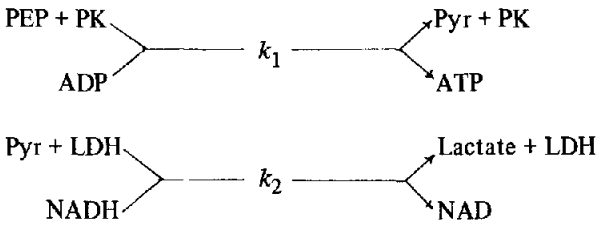
Lactate dehydrogenase and pyruvate kinase of rabbit muscle, nicotinamide adenine dinucleotide reduced, pyruvate (sodium salt), adenosine diphosphate (free acid) and phosphoenol pyruvate tricyclohexyl ammonium salt were purchased from C.F.Boehringer and Söhne GmbH, Mannheim. Lab-trol was obtained from DADE Division, Miami, Florida. The enzymes were passed through Sephadex G-25 and, free of ammonium sulphate, kept in the buffer given below. In a concentration range between 1–10 mg/ml the enzymes were stable over many weeks at 4°. Protein concentrations were determined by the Biuret method [4] using lab-trol as a standard. The activity of lactate dehydrogenase was assayed in 3 mM pyruvate and 0.25 mM NADH and the activity of pyruvate kinase was determined in the coupled assay modified according to [5]. Pyruvate was determined as described in [6].

All experiments were carried out in 50 mM imidazol-HCl-buffer (pH 6.4), 50 mM KCl and 8 mM magnesium acetate at 25°. The kinetic experiments at low enzyme concentrations as well as the determination of the substrate concentrations were carried out in an Eppendorf photometer at 366 nm. For stop-flow experiments with high enzyme concentrations the equipment described elsewhere [7] was used with the mono-

chrometer set at 366 nm. The flow chamber of the apparatus allows a mixing ratio of 1:1 in a volume approximately 22 μl, a total dead time of 2 msec and flow velocity of 5 m/sec, d = 1 cm. Reactions were started by mixing equal volumes of pyruvate kinase + lactate dehydrogenase + phosphoenol pyruvate + NADH and pyruvate kinase + lactate dehydrogenase + ADP + NADH in both drive syringes, respectively. Final conditions: PEP 1 mM, ADP 2 mM, NADH 0.25 mM. A molecular weight of 140,000 for lactate dehydrogenase [8] with a specific activity of 500 I.U./mg and a molecular weight of 237,000 for pyruvate kinase [9] with a specific activity of 190 I.U./mg were used in the calculations of the molar enzyme concentrations.

3. Theory

If the substrates, phosphoenolpyruvate, adenosine diphosphate and NADH are supplied in concentrations saturating their respective enzymes, the general treatment of the two-enzyme-system



can be simplified and yields a zero order reaction of pyruvate kinase and a first order reaction of lactate dehydrogenase as long as the stationary concentration of pyruvate $[Pyr]_{ss}$ is small compared to the Michaelis constant of lactate dehydrogenase for pyruvate K_{Pyr} [10, 11].

Neglecting the inhibition of pyruvate kinase by ATP [for references see 12 and 13] and the inhibition of lactate dehydrogenase by the LDH-NAD-Pyruvate-complex [14, 15], the pyruvate concentration of the system is at any time

$$[Pyr] = \frac{k_1[PK]}{k_2[LDH]}(1-e^{-k_2[LDH]t}) \quad (1)$$

For the steady-state ($t \to \infty$) it follows

$$[Pyr]_{ss} = \frac{k_1[PK]}{k_2[LDH]} \quad (2)$$

The NAD-concentration (the lactate concentration, respectively) is at any time

$$[NAD] = k_1[PK]\left(t + \frac{1}{k_2[LDH]} \times e^{-k_2[LDH]t} - \frac{1}{k_2[LDH]}\right) \quad (3)$$

The intersection of the asymptote with the time axis yields the transient time

$$\tau = \frac{1}{k_2[LDH]} \quad (4)$$

Inserting conventional kinetic constants gives

$$k_1[PK] = V_{max(PK)} \quad (5) \text{ and } k_2[LDH] = \frac{V_{max(LDH)}}{K_{Pyr}} \quad (6)$$

Inserting (5) and (6) into (2) resp. (4) the following relationships hold:

1) The steady-state concentration of pyruvate depends on the K_m of LDH for pyruvate as well as on the activity ratio of PK/LDH.
2) The transient time τ is dependent only on the LDH activity at constant K_{Pyr}; being independent from the activity ratio PK/LDH. τ is a measure of the LDH activity.
3) Under steady-state conditions the NAD formation per unit time is constant and proportional to the concentration of pyruvate kinase.

4. Experimental results

The various parameters were measured with an activity ratio of pyruvate kinase/lactate dehydrogenase = 1/8 respectively a molarity ratio of 1:5 corresponding to an approximately physiological enzyme ratio [16]. Under this condition, a steady state is reached after a turnover of 0.1 mM substrate. Since under this condition $[Pyr]_{ss} \ll K_{Pyr}$, indeed, the lactate dehydrogenase reaction follows a first order course. In separate experiments, a Michaelis constant of LDH for pyruvate of 0.17 mM was determined under the experimental conditions used in the experiments.

Fig. 1 shows the time course of a typical experiment with 0.026 I.U./ml pyruvate kinase and 0.210 I.U./ml lactate dehydrogenase as recorded with an Eppendorf photometer. The extrapolation of the steady state region of the progress curve to the time axis is drawn into the record. The steady state concentration of pyruvate $[Pyr]_{ss}$ was determined at the time indicated by the arrow, when the reaction in the steady state was stopped with $HClO_4$. The pyruvate level of 22 μM (see table 1) is sufficiently small to fulfil the condition of $[Pyr]_{ss} \ll K_{Pyr}$ for a first order treatment of the LDH reaction.

Using equations (1) and (3) and the data summarized in table 1 (see below), values for NAD (cross) and pyruvate (open circle) were computed and drawn into the record giving a good agreement of the transient state parameter between experimental and calculated data.

Table 1 summarizes the results of experiments obtained with small enzyme concentrations. From the steady state turnover ($V_{max(PK)}$) and the steady-state pyruvate concentration ($[Pyr]_{ss}$) the transient times were computed according to equation (2) and (4). Computed and graphically determined transient times agree well within the experimental error.

Fig. 2 demonstrates the time course of a typical stop-flow experiment with 1.6×10^{-6} M pyruvate kinase and 8×10^{-6} M lactate dehydrogenase. From this experiment a steady state turnover of 73 I.U./ml and a transient time of 20 msec was estimated.

Fig. 3 summarizes the activity of pyruvate kinase vs. molarity of pyruvate kinase in a double log net. A clear proportionality in the concentration range between 10^{-9}–10^{-6} M is found. From the same series of experiments a linear dependency of the reciprocal transient time from the LDH molarity over the corresponding concentration range is found as shown in fig. 4 in agreement with the theory.

The experiments demonstrate that V_{max}-values of lactate dehydrogenase as well as pyruvate kinase of rabbit muscle do not exhibit a significant concentration-dependency over a wide concentration range up to physiological levels. Our results are only a necessary, but not conclusive prerequisit for the assumption that no protein-protein interactions between LDH-LDH, LDH-PK or PK-PK exist. Thus, if such an interaction affects only the affinity of pyruvate kinase for PEP or ADP or the affinity of LDH for NADH, we would not recognize it in our experimental design.

Since, however, both possibilities are excluded by earlier, independent experiments, we are justified to exclude protein-protein interactions in our experiments. Reynard et al. [17] as well as Mildvan and Cohn [18] have shown in binding studies with approximately 10^{-4} M pyruvate kinase and in kinetic experiments with 10^{-8} M pyruvate kinase that the dissociation constants of PK-ADP as well as PK-PEP complexes are in good agreement at both concentrations. We have shown [3] that the Michaelis constant of LDH for NADH and pyruvate at 1.2×10^{-9} M LDH and 7.0×10^{-7} M LDH

Table 1

Enzyme	Experimental conditions: Acitivity (nmoles/min/ml)	Experimental conditions: Molarity (M) $\times 10^9$	Transient time: Exptl.* (sec)	Transient time: Calc. (sec)	$[Pyr]_{ss}$ (μM)	PK-activity (exptl.) (nmoles/min/ml)
PK	26	0.6	50	52	23	26
LDH	210	3				
PK	78	1.8	15	17	22	76
LDH	650	9				
PK	240	5.6	5	5.4	22	243
LDH	2000	28				

* Obtained by graphic extrapolation (see fig. 1).

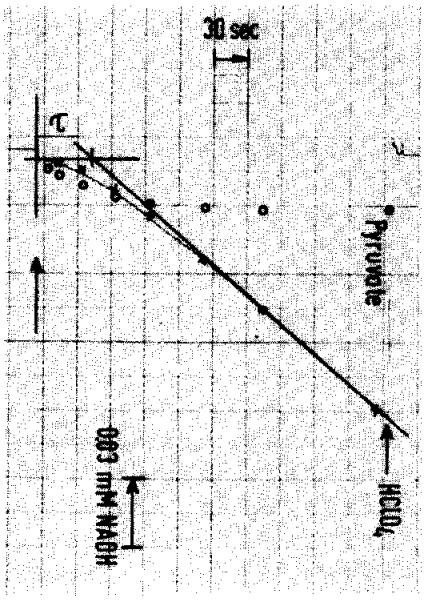


Fig. 1. Record of the time course of the pyruvate kinase-lactate dehydrogenase system after initiation. Time proceeds from left to right. The reaction was started as indicated by the arrow on the left side of the record. The soft line is the trace drawn by the recorder. The drawn line illustrates the extrapolation procedure. The o and x are calculated values for the pyruvate and NADH course, respectively • is the assayed value for pyruvate.

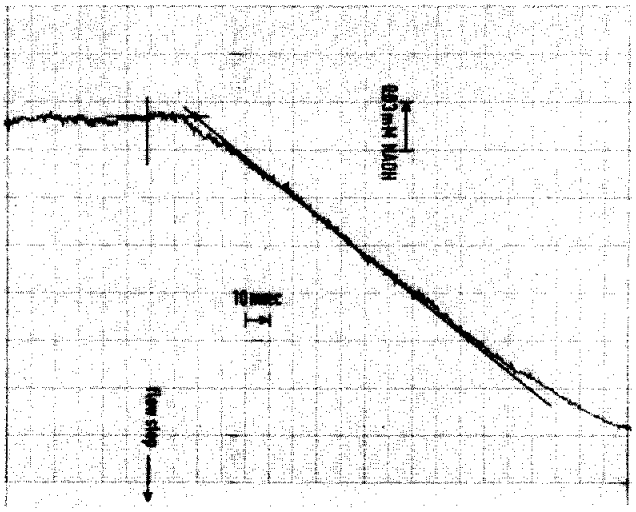


Fig. 2. Record of a stop-flow experiment. Increase in absorbancy is recorded as an upward deflection. Time proceeds from left to right. Instrumental time constant 1 msec. Flow stop is indicated at the bottom of the record by a sharp deviation of the flow indicator trace. The drawn line illustrates the extrapolation procedure.

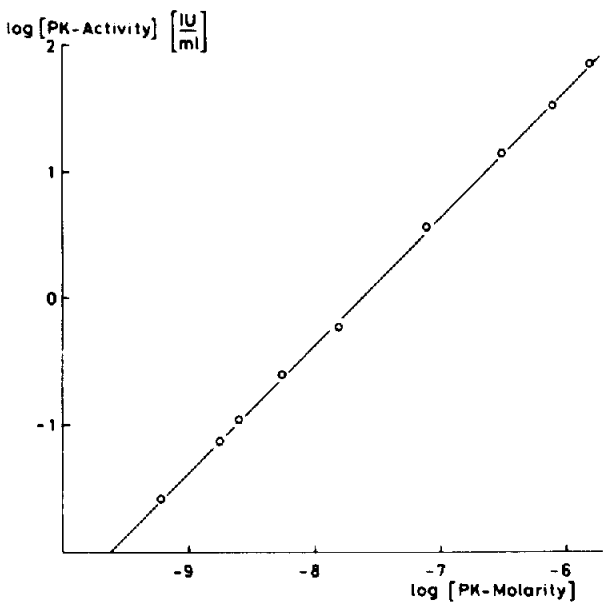


Fig. 3. Relation between activity and molarity of pyruvate kinase given in log initial velocity (I.U./ml) and log[M].

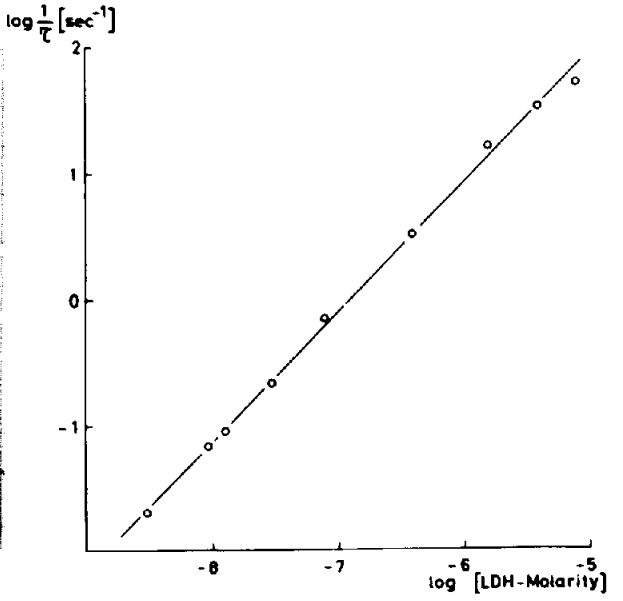


Fig. 4. Relation between the reciprocal transient time and the molarity of lactate dehydrogenase given in log[sec^{-1}] and log[M].

also agree. Whether these results can also be extended to the condition of the intact muscle cell, is, however, questionable. Arnold and Pette [19] have demonstrated that the kinetic properties of aldolase change on reversible binding to F-actin.

The transient time for the two-enzyme-system studied in this investigation at enzyme concentrations corresponding to the condition in the intact rabbit muscle was found in the range of 20 msec. A quantitative application of the results obtained with this simple model to the conditions of the intact muscle system must again be done with caution. *In vivo,* no steady state can be defined over a time difference, covering more than one time domaine. The large number of variables induce steadily consecutive transients moving the system in short time from one steady state to another. However, from our results we can conclude that in the system investigated, transients can occur within a few msec.

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