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Enzymatic Assays: Optimization of Systems by Using Pyruvate Kinase and Lactate Dehydrogenase as Auxiliary Enzymes

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A method to optimize enzymatic assays by using pyruvate kinase and lactate dehydrogenase enzymes is presented and applied to mitochondrial ATPase as an example. Optimum amounts of auxiliary enzymes, to obtain either a 99 % of the initial rate in a given time (t_{99}) or a given lag period (L), are calculated from their apparent Michaelis constants (K_{app}) in the medium used and their prices per enzymatic international unit.

The planning of enzyme assays with one or two auxiliary enzymes, keeping them at a minimum cost but stil being adequate enough has been the subject of a recent paper written by GARCÍA-CARMONA *et al.* (1). The mathematical treatment of the problem there described, allows such a calculation which will be applied here to a very common case of coupling enzymes system. i.e. pyruvate kinase (PK, EC 2.7.1.40) and lactate dehydrogenase (LDH, EC 1.1.1.27), used together with phosphoenolpyruvate and NADH to regenerate ATP from ADP. This ATP regeneration system has been used since long ago (6) to follow ATPase activities. As a practical example we apply here this methodology, precisely to F_1-ATPase mitochondrial (EC 3.6.1.3).

THEORY

The calculation of the minimum cost for enzymatic assays in which two auxiliary enzymes are used, after their particular kinetics properties, has been recently described by GARCÍA-CARMONA *et al.* (1).

According to these authors it was shown that

$$\frac{t_{99}\sqrt{2}}{4.6} \geqq L \geqq \frac{t_{99}}{4.6} \qquad [1]$$

where, L is the lag period or transient time and t_{99} is the time in which 99 % of initial rate is reached. Then, equations are obtained in which the minimum cost of the assay can be calculated for a given t_{99} or a given L, considering the prices

of each enzyme and their kinetics peculiarities.

In the case of the ATPase assay using pyruvate kinase and lactate dehydrogenase as auxiliary enzymes:

$$V_{ADP} = \frac{4.6\,K_{ADP}}{t_{99}}\left[1+\frac{1}{\alpha^2}\right]^{1/2} \quad [2]$$

$$V_{Pyr} = \frac{4.6\,K_{Pyr}}{t_{99}}\left[1+\frac{1}{\alpha^2}\right]^{1/2} \quad [3]$$

$$V_{ADP} = \frac{K_{ADP}}{L}\left[1+\frac{1}{\alpha}\right] \quad [4]$$

$$V_{Pyr} = \frac{K_{Pyr}}{L}[1+\alpha] \quad [5]$$

where

$$\alpha = \left(\frac{P_{PK}K_{ADP}}{P_{LDH}K_{Pyr}}\right)^{1/3} \quad [6]$$

being V_{APD} and V_{Pyr} the maximum rates for PK with ADP as substrate and LDH using pyruvate as substrate (in I.U./ml) respectively and K_{ADP} and K_{Pyr} the Michaelis constants of PK for ADP and LDH for pyruvate respectively. P_{PK} and P_{LDH} are the cost per unit of PK and LDH respectively.

A practical approach is to plot $L_1 = \frac{K_{APD}}{V_{ADP}}$ (being L_1 the lag period for the activity of PK) versus $L_2 = \frac{K_{Pyr}}{V_{Pyr}}$ (being L_2 the lag period for the LDH activity).

The points corresponding to the optimized cost are given by the intersections of the optimized straight lines and the family of circumferences with centre in the origin and radius (R)

$$R = \frac{t_{99}}{4.6} = \frac{(\alpha^2+1)^{1/2}}{1+\alpha} \quad [7]$$

and where $L = L_1 + L_2$.

The optimized straight lines go through the origin and have a slope α. The circumference of radius R is according to McCLURE (4) and GARCÍA-CARMONA *et al.* (1), the representation of all the possible combinations of L_1 and L_2 allowing to obtain a given t_{99}.

The above theoretical treatment is only true assuming that: 1) the substrate level should not change during the time of the experiment; 2) the auxiliary enzymes, i.e. PK and LDH, catalyze first order, irreversible reactions, i.e. *a*) $ADP_{ss} < 0.1\ K_{ADP}$ where ADP_{ss} is the concentration of ADP in the steady state. *b*) $Pyr_{ss} < 0.1\ K_{Pyr}$ where Pyr_{ss} is the concentration of Pyr in the steady-state; and, 3), any other substrates for the auxiliary enzymes are saturating.

During the economical optimization of an assay, assumption 3 can not be easily accomplished. However it can be substituted by concentrations of the auxiliary substrates which make constant the rate of the whole reaction. This assumption makes necessary to use the apparent K_m for ADP and pyruvate after the actual concentrations of phosphoenolpyruvate and NADH.

A useful way of measuring these apparent Michaelis constants (K_{app}) is by means of using the fact that at a given concentration of an auxiliary enzyme, with a given constant lag period, the changes in L will be a consequence of variations in the other enzyme and so

$$L_1 = L - L_2 = \frac{K_{app\,ADP}}{V_{ADP}} \quad [8]$$

and thus plotting

$$\frac{1}{L_1} = \frac{1}{K_{app\,ADP}}V_{ADP} \quad [9]$$

a straight line with a slope of $\frac{1}{K_{app\,ADP}}$ will result.

On the other hand,

$$\frac{1}{L_2} = \frac{1}{K_{app\,Pyr}}V_{Pyr} \quad [10]$$

and thus $\frac{1}{K_{app\,Pyr}}$ can be calculated.

Assumption 2 made in the chapter of Theory, will limit the maximum initial rate ($V_{o\,max}$) in the assay with two coupling enzymes; therefore the concentrations of ADP and pyruvate in the steady-state must be equal or lower than 0.1 K_{ADP} and 0.1 K_{Pyr} respectively. In consequence:

$$V_{o\,max\,ADP} \leq 0.1\, V_{ADP} \quad \text{and}$$

$$V_{o\,max\,Pyr} \leq 0.1\, V_{Pyr}$$

Materials and Methods

ATP (disodium salt), phosphoenolpyruvate (monopotassium salt), NADH (disodium salt), pyruvate kinase and lactate dehydrogenase were obtained from Sigma. All other chemicals used were analytical reagent grade.

Beef heart mitochondial F_1-ATPase was isolated by the method of KNOWLES and PENEFSKY (2). Enzyme specific activity varied between 90 and 100 μmol of ATP hydrolyzed per min and mg of protein under the described assay conditions.

Initial rate experiments were carried out at 30° C in a total volume of 1 ml at pH 8 containing 50 mM triethanolamine-HCl, 2 mM ATP, 2 mM $MgCl_2$, 0.24 mM NADH, 2 mM phosphoenolpyruvate. The I.U. of pyruvate kinase and lactate dehydrogenase were different in each case.

The reactions were initiated by the addition of purified F_1-ATPase which had been previously centrifuged to remove most of the $(NH_4)_2SO_4$ in which it was stored as a precipitate.

The reactions were followed by observing the disappearance of NADH at 340 nm with an Aminco DW-2a UV/VIS Spectrophotometer.

Protein was estimated by the method of LOWRY *et al.* (3). Crystalline bovine serum albumin was used as standard.

Results and Discussion

The theory here presented was applied to a representative practical case, i.e. mitochondrial F_1-ATPase.

To calculate the K_{app} of PK for ADP in the assay mixture PK concentration was changed between 0.96 and 11.52 I.U./ml whereas LDH concentration was kept at 63.5 I.U./ml. Doubling LDH concentration did not result in any change in the lag period. Therefore, the lag period was independent of LDH concentration, at the concentration stated before.

Reactions were started by adding 0.015 I.U. of F_1-ATPase. Plotting the results according to equation [9] a straight line resulted and from its slope of 0.31 a K_{app} of 3.22 mM of PK for ADP was evaluated (fig. 1).

K_{app} of LDH for pyruvate was similarly calculated. In this case 19.2 I.U./ml of PK were used in all cases and LDH was

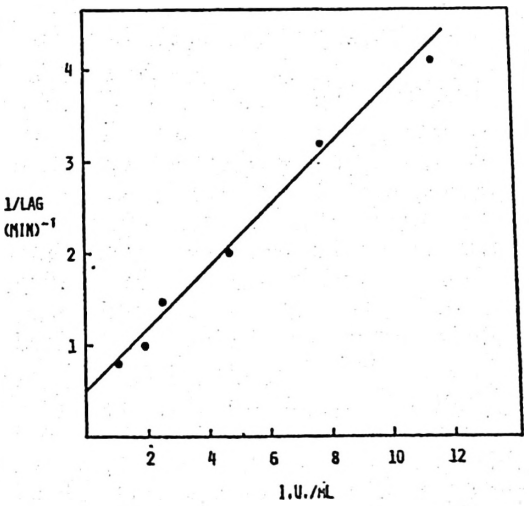

Fig. 1. *Determination of the K_{app} of PK for ADP.*
PK concentration in the assay mixture was changed between 0.96 and 11.52 I.U./ml whereas LDH concentration was kept at 63.5 I.U./ml. Reactions were started by adding 0.015 I.U./ml of F_1-ATPase.

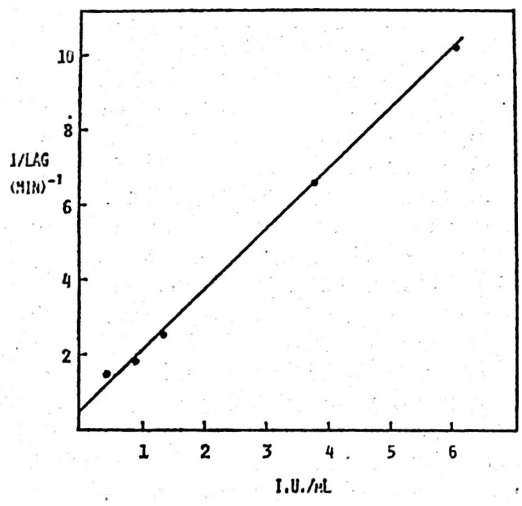

Fig. 2. *Determination of the K_{app} of LDH for pyruvate.*
LDH in the assay mixture was changed between 0.42 and 6.35 I.U./ml whereas PK concentration was kept at 19.2 I.U./ml. Reactions were started by adding 0.055 I.U./ml of F_1-ATPase.

changed between 0.42 and 6.35 I.U./ml (fig. 2). In these conditions a constant lag time of 11 seconds appeared; it was due to PK and so it was subtracted in all assays. This time decreased if PK concentration was increased. However it is not convenient to use this procedure to eliminate it since a lot of enzyme should be necessary.

Reactions were started by adding 0.055 U.I. of F_1. Plotting the results after equations [10] a straight line was obtained and from its slope of 1.48 a K_{app} of 0.67 mM was evaluated.

After equation [6] all optimization conditions for any given L or t_{99}, can be obtained in a plot of L_{PK} versus L_{LDH} by means of a straight line with a slope value of α (straight line of optimization).

After the calculation of α, from K_{app} of PK for ADP and K_{app} of LDH for pyruvate, and the price relation (Sigma) which is 4.24, the corresponding straight line can be plotted as done in figure 3.

In practice, the calculation of optimum conditions can be carried out by fixing either t_{99} or the lag period L. This calculation is easily made using the optimized straight line.

As an example, fixing a t_{99} of 2.15 min, after equation [7], R = 0.46. Plotting a circumference with this radius the point of intersection with the optimized straight line is obtained. From the coordinates of this point a $L_{PK} = 0.439$ min and $L_{LDH} = 0.161$ min are obtained. So, the total lag period of the process will be; $L = L_{PK} + L_{LDH} = 0.439 + 0.161 = 0.6$ min.

After equations [9] and [10], the optimum concentrations of the auxiliary enzymes to obtain a lag of 0.6 min can be calculated. These values are 7.33 I.U./ml of PK and 4.16 I.U./ml of LDH. The enzymatic assay carried out in these condi-

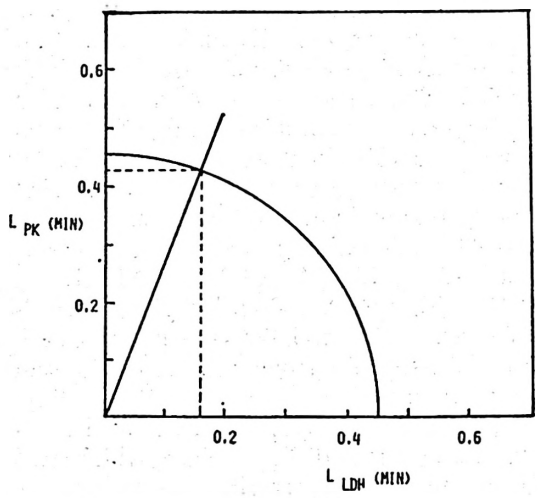

Fig. 3. *Calculation of the optimum conditions for the assay using the straight line of optimization.*
In the example plotted, a t_{99} of 2.15 min was assumed. From this value a R of 0.46 was calculated and used to draw the circumference. From the coordinates of the point of intersection 0.439 min and 0.161 min were the values obtained for L_{PK} and L_{LDH}.

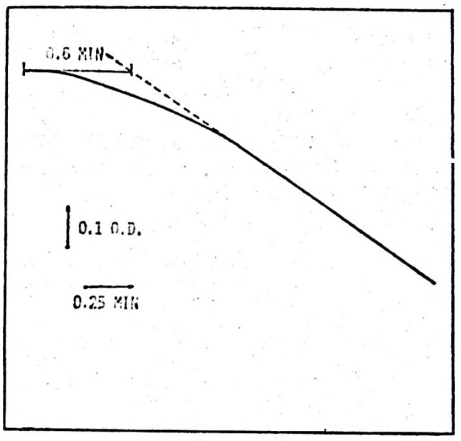

Fig. 4. *Spectrophotometric recorder tracing of the optimized conditions for a lag period of 0.6 min.*
The enzymatic assay was carried out with 7.33 I.U./ml of PK and 4.16 I.U./ml of LDH.

tions really gives 0.6 min as shown in figure 4.

According to V_{ADP} and V_{Pyr}, ATPase maximum activities must be <0.416 I.U. per ml.

The assay mixture was incubated in the spectrophotometric cuvette, for some minutes, before starting the assay, until no variation in O.D. with time was detectable. This is a necessary precaution to avoid the presence of intermediate substrate such as ADP or pyruvate which could interfere with the measurements in a similar form to that described (5).

The optimization procedure here described is useful for any assay in which the kinetics of ADP production is to be measured and pyruvate kinase and lactate dehydrogenase are used for that. Many different enzymes are assayed in this way. We can mention, between them: the different ATPases, i.e. from mitochondria, from sarcoplasmic reticulum, from bacteria, from chloroplast, etc. acetate kinase, fructose-6-phosphate kinase, guanosine-5′-monophosphate kinase, hexokinase, myokinase and others which can be found in the literature.

It is interesting to remark how important is to control the amount of auxiliary enzymes used for a particular assay. Due to their high cost it is a natural tendency to try to reduce their concentration. But to do that has many obvious risks and others not so obvious: for example, in the case of mitochondrial ATPase, lag periods which are related to the activity of the auxiliary enzymes, as discussed here, are used to monitor activation of the primary enzyme when contacting the assay mixture which contains the coupling system as described by VASILYEVA *et al.* (7). It is evident that enough auxiliary enzymes have to be present if the eventual lag period is to be attributed exclusively to the primary enzyme.

In conclusion, when designing enzyme assays in which auxiliary enzymes are used, it is strongly recommended to make use of optimization procedures, as those described here.

Acknowledgement

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Resumen

Se describe una metodología que permite reducir el coste de los ensayos enzimáticos en los que se usen piruvato quinasa y lactato deshidrogenasa. Se utiliza ATPasa mitocondrial como ejemplo. Se calculan las cantidades óptimas de enzimas auxiliares, para obtener un 99 % de la actividad inicial en un tiempo dado (t_{99}) o un período de retardo dado (L), según sus constantes de Michaelis aparentes en el medio usado y sus precios por unidad enzimática internacional.

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