Kinetics of a self-amplifying substrate cycle: ADP–ATP cycling assay

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A kinetic study of an ATP–ADP amplification cyclic system involving the enzymes adenylate kinase, pyruvate kinase and L-lactate dehydrogenase has been made. The stoichiometry of the cycle is 2:1, because two molecules of ADP are synthesized from one each of ATP and AMP, and one molecule of ADP is converted back into one of ATP at each turn of the cycle. This results in a continuous exponential increase in the concentrations of ATP and ADP in the reaction medium, according to the equations obtained. This is therefore a substrate cycle that

amplifies itself, the cycling rate increasing continuously with time. The background signal of the reagent was reduced by using apyrase to degrade ATP and ADP in the reagent, permitting detection limits as low as 16 pmol of ATP and/or ADP in a continuous spectrophotometric assay.

Key words: adenylate kinase, amplification, enzyme kinetics, pyruvate kinase.

INTRODUCTION

Substrate cycling is an extensively used tool in biochemistry for the amplified quantitative determination of low levels of a metabolite or an enzyme activity. In this reaction scheme, a metabolite is sequentially interconverted into another by means of two two-substrate enzyme-catalysed reactions in opposite directions. In this way the concentration of the recycling substrates remains constant during the reaction, while other products of the enzymic reactions are accumulated at each turn of the cycle. This technique, which significantly increases the sensitivity of enzymic assays, is really a further application of the great amplification capacity shown by futile cycles and cascades in metabolism [1,2]. The kinetics of these systems has been well studied, in both the steady state [3] and the transient phase [4,5], and equations have been reported for calculating enzyme quantities that minimize the cost of assays [6]. In general, with a 1:1 stoichiometry a steady state is reached with a constant product accumulation rate that is directly proportional to the initial concentration of recycling metabolite in the sample, the proportionality constant being the general rate constant for the cycle.

It is common practice to measure low levels of ATP by means of a cycling system involving the enzymes pyruvate kinase (EC 2.7.1.40) and hexokinase (EC 2.7.1.1) [7]. The stoichiometry of the cycle in this case is 1:1; the indicator reaction is the coupling of glucose-6-phosphate dehydrogenase, which measures the appearance of NADPH, or the use of firefly luciferase, which measures luminescence [8]. This latter is a more sensitive technique, although it has the disadvantage of high product inhibition and consequent signal decay [9,10].

Another possibility is the replacement of hexokinase by adenylate kinase (EC 2.7.4.3) in the presence of AMP [11,12]. The kinetics of the system in this case is complex because two molecules of ADP are generated at each turn of the cycle from one of ATP and one of AMP. Adenylate kinase equilibrium is driven towards ADP formation in the presence of an excess of

AMP. In the subsequent step of the reaction, one molecule of ADP is recycled towards ATP at the same time as phosphoenolpyruvate (PEP) is converted to pyruvate by means of the catalytic action of pyruvate kinase. The rate of this step, and therefore the cycling rate, cannot be constant because the concentration of substrate (ADP) is increasing continuously as a consequence of the stoichiometry of the process, i.e. the cycle is indeed amplifying itself. This situation also arises in the assay with glucokinase and adenylate kinase [13]. Two theoretical models yielding an approximate solution have recently been reported [14], using firefly luciferase as reaction indicator. However, both models show deviations from the experimentally obtained progress curves owing to the nature of the approximations performed.

The aim of the present paper is to make a thorough kinetic analysis of this cycling system, using L-lactate dehydrogenase (EC 1.1.1.27) as the coupled enzyme. The model and the equations derived show good agreement with experimental results, and a single procedure for determining the kinetic parameters involved in the system is described. The background signal of reagent was decreased with the use of apyrase (EC 3.6.1.5), which degraded endogenous ATP and ADP in the reagent.

KINETIC ANALYSIS

The mechanism under study is described by the following chemical equations:

Abbreviation used: PEP, phosphoenolpyruvate.

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where Pyr is pyruvate, Lac is L-lactate, and AK, PK and LDH are adenylate kinase, pyruvate kinase and L-lactate dehydrogenase respectively. To derive analytical expressions for the time dependence of the species involved in eqns (1)–(3), the following assumptions were made.

1. AMP, PEP and NADH concentrations are high enough to be saturating or remain constant during the reaction time.

2. During cycling, the concentration of ATP, ADP and pyruvate must clearly be lower than their respective Michaelis–Menten constants ($K_{m,1}$, $K_{m,2}$ and $K_{m,3}$ towards adenylate kinase, pyruvate kinase and L-lactate dehydrogenase respectively), so that the reaction rates of the three steps remain first-order with regard to their respective concentrations.

3. The rate of the backwards reaction catalysed by adenylate kinase is considered negligible because the equilibrium is driven towards ADP formation in two ways: by the presence of an excess of AMP in the reaction medium and by the catalytic action of pyruvate kinase, which consumes part of the present ADP.

Taking into account these assumptions, the differential equation system that describes the mechanism shown in eqns (1)–(3) is:

$$\frac{d[\text{ADP}]}{dt} = -k_2[\text{ADP}] + 2k_1[\text{ATP}] \quad (4)$$

$$\frac{d[\text{ATP}]}{dt} = k_2[\text{ADP}] - k_1[\text{ATP}] \quad (5)$$

$$\frac{d[\text{Pyr}]}{dt} = k_2[\text{ADP}] - k_3[\text{Pyr}] \quad (6)$$

$$\frac{d[\text{Lac}]}{dt} = -\frac{d[\text{NADH}]}{dt} = k_3[\text{Pyr}] \quad (7)$$

where k_i ($i = 1, 2, 3$) are apparent first-order rate constants, and $k_i = V_{\max,i}/K_{m,i}$ ($V_{\max,i}$ is the reaction rate of step i at saturating concentrations of both substrates; $K_{m,i}$ is a function of the Michaelis–Menten constants of the enzyme E_i towards its substrates and of the initial concentrations of AMP, PEP and NADH respectively, with $K_{m,i} \approx K_m^{\text{ATP}}$, K_m^{ADP} or K_m^{Pyr} if AMP, PEP or NADH respectively are saturating).

The solution to the differential equations (4) and (5), whose initial conditions are, at $t = 0$, $[\text{ATP}] = [\text{ATP}]_0$ and $[\text{ADP}] = [\text{ADP}]_0$, is:

$$[\text{ADP}] = \frac{(\lambda_2 + k_2)[\text{ADP}]_0 - 2k_1[\text{ATP}]_0}{\lambda_1 + \lambda_2} e^{-\lambda_1 t} + \frac{(\lambda_1 - k_2)[\text{ADP}]_0 + 2k_1[\text{ATP}]_0}{\lambda_1 + \lambda_2} e^{\lambda_2 t} \quad (8)$$

$$[\text{ATP}] = \frac{-k_2[\text{ADP}]_0 + (\lambda_2 + k_1)[\text{ATP}]_0}{\lambda_1 + \lambda_2} e^{-\lambda_1 t} + \frac{k_2[\text{ADP}]_0 + (\lambda_1 - k_1)[\text{ATP}]_0}{\lambda_1 + \lambda_2} e^{\lambda_2 t} \quad (9)$$

where:

$$\lambda_1 = \frac{1}{2}[k_1 + k_2 + \sqrt{(k_1 + k_2)^2 + 4k_1 k_2}] \quad (10)$$

$$\lambda_2 = \frac{1}{2}[-k_1 - k_2 + \sqrt{(k_1 + k_2)^2 + 4k_1 k_2}] \quad (11)$$

Note that, whereas the first term in eqns (8) and (9) is a time-decreasing exponential function, the second is a time-increasing exponential function. These equations are very different from those obtained for a normal cycling system with a 1:1 stoichiometry, in which the concentration of recycling substrates reaches a constant value after a transition phase that is governed by a negative exponential [6]. Here, ADP and ATP concentrations do not reach a constant value but increase continuously with time while the conditions assumed are fulfilled. At sufficiently high values of t , the first exponential term becomes negligible and recycling substrates would accumulate in accordance with a single-exponential curve.

Now, by inserting eqn (8) into eqn (6), a non-homogeneous linear first-order-type differential equation is obtained whose solution, taking into account that, at $t = 0$, $[\text{pyruvate}] = 0$, is:

$$[\text{Pyr}] = k_2 \left[\frac{(\lambda_2 + k_2)[\text{ADP}]_0 - 2k_1[\text{ATP}]_0}{(\lambda_1 + \lambda_2)(k_3 - \lambda_1)} e^{-\lambda_1 t} + \frac{(\lambda_1 - k_2)[\text{ADP}]_0 + 2k_1[\text{ATP}]_0}{(\lambda_1 + \lambda_2)(k_3 + \lambda_2)} e^{\lambda_2 t} - \frac{(k_3 - k_1)[\text{ADP}]_0 - 2k_1[\text{ATP}]_0}{(k_3 - \lambda_1)(k_3 + \lambda_2)} e^{-k_3 t} \right] \quad (12)$$

Substituting eqn (12) into eqn (7) and integrating again with the boundary condition of $[\text{Lac}] = 0$ at $t = 0$, one obtains:

$$[\text{Lac}] = k_2 k_3 \left[\frac{-(\lambda_2 + k_2)[\text{ADP}]_0 - 2k_1[\text{ATP}]_0}{(\lambda_1 + \lambda_2)(k_3 - \lambda_1)\lambda_1} e^{-\lambda_1 t} + \frac{(\lambda_1 - k_2)[\text{ADP}]_0 + 2k_1[\text{ATP}]_0}{(\lambda_1 + \lambda_2)(k_3 + \lambda_2)\lambda_2} e^{\lambda_2 t} + \frac{(k_3 - k_1)[\text{ADP}]_0 - 2k_1[\text{ATP}]_0}{(k_3 - \lambda_1)(k_3 + \lambda_2)k_3} e^{-k_3 t} \right] - [\text{ADP}]_0 - 2[\text{ATP}]_0 \quad (13)$$

This equation clearly indicates that product accumulation is not constant with time, and so in this cycling system there is no general rate constant for the cycle. For sufficiently high values of t , the two negative exponential terms in eqn (13) can be neglected. Thus in the ‘steady state’, L-lactate continues to increase exponentially with time according to:

$$[\text{Lac}]_{t \gg 0} = k_2 k_3 \left[\frac{(\lambda_1 - k_2)[\text{ADP}]_0 + 2k_1[\text{ATP}]_0}{(\lambda_1 + \lambda_2)(k_3 + \lambda_2)\lambda_2} e^{\lambda_2 t} \right] - [\text{ADP}]_0 - 2[\text{ATP}]_0 \quad (14)$$

However, it must be taken into account that this is not a real steady state because the cycling rate is increasing continuously in exponential form. This is the first time that an equation of this type, with a positive exponential term, has been described for a cyclic system. A similar expression was obtained for autocatalytic zymogen activation processes, which are unidirectional cascades in metabolism where the activating protease and the activated enzyme coincide, two molecules of active enzyme thus being obtained by the catalytic cycle [15,16]. We are therefore dealing with an amplification system that is amplifying itself, its sensitivity therefore being very much greater. Thus, in principle, even only one molecule of ATP or ADP would be sufficient to initiate the cycle and produce a measurable signal.

When the reaction is followed by measuring the disappearance of NADH, eqns (13) and (14) become respectively:

$$[\text{NADH}] = k_2 k_3 \left[\frac{(\lambda_2 + k_2)[\text{ADP}]_0 - 2k_1[\text{ATP}]_0}{(\lambda_1 + \lambda_2)(k_3 - \lambda_1)\lambda_1} e^{-\lambda_1 t} - \frac{(\lambda_1 - k_2)[\text{ADP}]_0 + 2k_1[\text{ATP}]_0}{(\lambda_1 + \lambda_2)(k_3 + \lambda_2)\lambda_2} e^{-\lambda_2 t} - \frac{(k_3 - k_1)[\text{ADP}]_0 - 2k_1[\text{ATP}]_0}{(k_3 - \lambda_1)(k_3 + \lambda_2)k_3} e^{-k_3 t} \right] + [\text{NADH}]_0 + [\text{ADP}]_0 + 2[\text{ATP}]_0 \quad (15)$$

$$[\text{NADH}]_{t \gg 0} = -k_2 k_3 \frac{(\lambda_1 - k_2)[\text{ADP}]_0 + 2k_1[\text{ATP}]_0}{(\lambda_1 + \lambda_2)(k_3 + \lambda_2)\lambda_2} e^{-\lambda_2 t} + [\text{NADH}]_0 + [\text{ADP}]_0 + 2[\text{ATP}]_0 \quad (16)$$

EXPERIMENTAL

Materials

β -NADH, AMP, ADP, ATP, PEP (sodium salt), imidazole, pyruvate kinase (500 units/mg) from chicken muscle, adenylate kinase (2000 units/mg) from rabbit muscle, L-lactate dehydrogenase (830 units/mg) from rabbit muscle, apyrase (216 units/mg) from potato (*Solanum tuberosum*) grade VII with a low ATPase-to-ADPase ratio, and BSA were purchased from Sigma. Stock solutions of enzymes (19.6, 19.2, 535.35 and 108 units/ml respectively) were prepared in 30 mM imidazole/acetic acid buffer (pH 7.5) containing 2 mg/ml BSA. All other buffers and reagents were of analytical grade and were used without further purification. All solutions were prepared in ultrapure deionized non-pyrogenic water (Milli Q, Millipore).

Spectrophotometric readings were obtained on a Uvikon 940 spectrophotometer from Kontron Instruments.

Decreasing the background signal

To decrease the background signal obtained in the absence of externally added ATP and ADP, apyrase was added to a mixture of NADH, AMP, potassium acetate and MgCl_2 , in 30 mM imidazole/acetic acid buffer (pH 7.5), to reach final concentrations of 1.066 mM NADH, 4.66 mM AMP, 250 mM potassium acetate, 6.66 mM MgCl_2 and 12.58 units/ml apyrase. This cocktail was freshly prepared daily and incubated for 30 min at ambient temperature before being heated for 5 min in a near-boiling water bath at 95 °C to denature the apyrase.

Cycling assay

Unless otherwise stated, the cycling reagent contained the following final concentrations: 0.23 mM NADH, 75 mM potassium acetate, 2 mM MgCl_2 , 1.4 mM AMP (all previously treated with apyrase), 1.6 mM PEP, 0.784 unit of pyruvate kinase, 0.768 unit of adenylate kinase, 48.2 units of L-lactate dehydrogenase and different volumes of a stock solution of 2.5 μM ATP or ADP. When ATP was included in the reaction medium, the reaction was started by the addition of adenylate kinase; when ADP was included in the assay cuvette, the reaction was started by the addition of pyruvate kinase, the final volume being 0.5 ml. The time course of the reaction was followed by measuring the disappearance of NADH at 340 nm (ϵ_{340} 6270 $\text{M}^{-1} \cdot \text{cm}^{-1}$) at 37 °C.

The data from experimental progress curves corresponding to the disappearance of NADH were fitted, by non-linear regression with the SigmaPlot Scientific Graphing System, to eqn (16), written in the following form:

$$y = -\gamma e^{At} + c \quad (17)$$

Measurement of adenylate kinase and pyruvate kinase activity

The activity of the two enzymes involved in the cycle was also measured individually to evaluate the apparent first-order rate constants k_1 and k_2 in a classical way, thus checking the correspondence to the values obtained by the cyclic method.

Adenylate kinase activity was measured in a reaction medium including 256 μM NADH, 75 mM potassium acetate, 2 mM MgCl_2 , 1.4 mM AMP (not treated with apyrase), 1.6 mM PEP, 0.1–1.25 mM ATP and 48.2 units of L-lactate dehydrogenase, in 30 mM imidazole/acetic acid buffer (pH 7.5). The reaction was started by adding 15.68 units of pyruvate kinase and 0.096 unit of adenylate kinase (premixed), the final volume being 0.5 ml. Data thus obtained were fitted by non-linear regression to the Michaelis–Menten equation, obtaining the corresponding values of K_m^{ATP} (0.18 mM) and V_{max} .

The linear relation between the amount of adenylate kinase and any activity expressed was checked by performing a set of experiments in which the adenylate kinase concentration was varied from 0.096 to 0.864 unit, in a reaction medium similar to the above, including ATP at a final concentration of 350 μM .

Pyruvate kinase activity was measured in a reaction medium including 256 μM NADH, 75 mM potassium acetate, 2 mM MgCl_2 (not treated with apyrase), 1.6 mM PEP, 0.1–1.25 mM ADP and 48.2 units of L-lactate dehydrogenase, in 30 mM imidazole/acetic acid buffer (pH 7.5). The reaction was started by the addition of 0.098 unit of pyruvate kinase, the final volume being 0.5 ml. Data thus obtained were fitted by non-linear regression to the Michaelis–Menten equation, obtaining the corresponding values of K_m^{ADP} (0.24 mM) and V_{max} .

The linear relation between the amount of pyruvate kinase and any activity expressed was checked by performing a set of experiments in which the pyruvate kinase concentration was varied from 0.098 to 0.882 unit, in a reaction medium similar to the above, including ADP at a final concentration of 1.25 mM.

Numerical integration

Simulated progress curves were obtained by numerical solution of the non-linear set of differential equations (A1)–(A6) listed in the Appendix, with the use of the Runge–Kutta–Fehlberg algorithm [17].

RESULTS AND DISCUSSION

Decreasing the background signal

One of the most common causes of erratic results when performing amplified assays is the presence of a background signal. The higher the signal, the greater the error it is likely to introduce; it might even alter the kinetics of the reaction. It is therefore of use to know where it comes from and, if possible, to decrease it. In the present study, the background signal was decreased by using apyrase to degrade ATP and ADP in a reagent containing NADH, AMP, K^+ and Mg^{2+} under the conditions expressed in the Experimental section. Apyrase is an adenosine 5'-triphosphatase and an adenosine 5'-diphosphatase, the general reaction being: $\text{ATP} \rightarrow \text{ADP} + \text{P}_i \rightarrow \text{AMP} + 2\text{P}_i$. It can therefore remove intrinsic ATP and ADP simultaneously. The product of the

Figure 1 Progress curves corresponding to NADH disappearance obtained both experimentally (A) and theoretically (B)

(A) Experimental progress curves corresponding to NADH disappearance at different initial ATP and ADP concentrations. The concentrations used were 60 nM ATP (curve a), 200 nM ADP (curve b), 200 nM ATP (curve c) and 600 nM ATP (curve d). The lines are experimental progress curves; the points correspond to data obtained by non-linear regression analysis with eqn (17) (for greater clarity, only a selection of points obtained by regression has been plotted, because both curves practically overlap). (B) Theoretical progress curves corresponding to NADH consumption obtained by numerical integration of the non-linear set of differential equations shown in the Appendix (lines) and from eqn (16) (points) at different initial ATP concentrations. The values of the rate constants used were: $k_1 = 3.7 \text{ min}^{-1}$, $k_2 = 2.25 \text{ min}^{-1}$ and $k_3 = 50 \text{ min}^{-1}$, at the following initial concentrations: $[\text{NADH}]_0 = 0.23 \text{ mM}$ and $[\text{ATP}]_0 = 60 \text{ nM}$ (curve a), 100 nM (curve b), 200 nM (curve c) and 600 nM (curve d). For the numerical integration the following initial conditions were also introduced: $[\text{AMP}]_0 = 1.4 \text{ mM}$ and $[\text{PEP}]_0 = 1.6 \text{ mM}$, where $k'_1 = 2.64 \text{ mM}^{-1} \cdot \text{min}^{-1}$, $k'_2 = 1.40 \text{ mM}^{-1} \cdot \text{min}^{-1}$ and $k'_3 = 217.39 \text{ mM}^{-1} \cdot \text{min}^{-1}$.

reaction, AMP, is present in excess in the reaction medium, as has been explained above in the Kinetic analysis section; it therefore does not interfere with the assay. The excess apyrase activity was suppressed by denaturing the enzyme by heat so as not to degrade ATP and/or ADP in the sample. However, PEP could not be included in the cocktail because it was partly dephosphorylated and might have introduced a new initial background curve.

Another common source of contamination is the enzymes used in the amplified assay. The three enzymes involved in the cycling assay were therefore applied to a Sephadex G-25 superfine HiTrap desalting column (Supelco) to remove low-molecular-mass contaminants. However, the background curve did not improve to any great extent, probably because nucleotides were

strongly bound to the proteins; the enzymes were therefore used as supplied.

An analysis of background curves obtained before and after treatment with apyrase showed that they corresponded to the presence of 228.3 and 18.4 pmol of ATP plus ADP respectively, although other nucleoside diphosphates or triphosphates might have been present because they might also react, albeit at a slower rate [8,18].

Time course of the reaction

The time course of the cyclic reaction was followed by monitoring the decrease in A_{340} caused by the consumption of NADH. Figure 1(A) shows a selection of progress curves obtained experimentally for different initial concentrations of ATP or ADP under the conditions described in the Experimental section, including the results obtained by non-linear regression analysis to eqn (17). Taking into account assumption (1) in the kinetic analysis, curves were registered in all cases until an A_{340} of 0.9 (143.5 μM NADH), a concentration much higher than the apparent K_m^{NADH} towards L-lactate dehydrogenase (approx. 1 μM with 3 μM pyruvate [8]). It can be seen that the NADH consumption rate increased continuously and exponentially, in accordance with the equations obtained. The good concordance obtained between experimental and fitted data indicated that the negative exponential terms in eqn (15) can be considered negligible and so eqn (16) is valid from near the beginning of the reaction.

The reaction time over which equations obtained are valid depends on the initial concentrations, on the rate constants and also on the respective Michaelis–Menten constants. Figure 1(B) shows a selection of theoretical progress curves obtained by numerical integration of the non-linear set of differential equations shown in the Appendix (which takes into account the depletion of NADH, AMP and PEP), compared with data obtained from eqn (16), at different initial concentrations of ATP. In the same way, and taking into account assumptions (2) and (3) in the kinetic analysis, as well as the exponential increase of recycling substrates concentration, Table 1 shows the levels of ATP, ADP and pyruvate attained at the times corresponding to a determined depletion of NADH (35%, approximately the same that has been found experimentally), as obtained by numerical integration. Relative errors of NADH values predicted from eqn (16) with regard to those obtained from the numerical solution are also shown at these times. As can be seen, a good concordance between analytical and numerical solutions was obtained, because relative errors calculated at the times corresponding to a consumption of NADH as high as 35% were approx. 3.3% in all cases under these conditions. Furthermore, it can be seen that the levels of ATP, ADP and pyruvate attained at these times were under $0.2 K_m$ in all cases (the apparent K_m^{Pyr} towards L-lactate dehydrogenase is 30 μM with 100 μM NADH [8]), indicating that the assumptions made can be considered valid during the reaction time used in each case. The fact that relative errors and substrate concentrations were approximately constant at a determined consumption of NADH also reveals that, from an experimental point of view, it is more advisable to take into account a critical consumption of NADH than a critical time for the validity of the assumptions made.

Sensitivity and linearity of the assay

An examination of eqns (11) and (16) reveals that the parameter λ is independent of the initial ATP concentration, whereas γ is

Table 1 Data corresponding to theoretical progress curves shown in Figure 1(B)

The subscript t in $[ATP]_t$, $[ADP]_t$ and $[Pyr]_t$ indicates the concentrations of ATP, ADP and pyruvate attained at the times indicated in the second column. These values have been obtained by numerical integration of the set of differential equations shown in the Appendix (conditions as in Figure 1B). The relative errors shown in the last column are referred to NADH concentrations predicted from an analytical solution [eqn (16)] with respect to the values predicted by the numerical integration [eqns (A1)–(A6)].

$[ATP]_0$ (nM)	Time corresponding to a 35% depletion of NADH (min)	$[ATP]_t$ (μ M)	$[ADP]_t$ (μ M)	$[Pyr]_t$ (μ M)	Relative error (%)
60	5.77	19.95	43.42	2.716	3.36
100	5.33	19.97	43.46	2.718	3.35
200	4.74	20.03	43.57	2.725	3.33
600	3.81	20.23	44.00	2.753	3.27

Figure 2 Dependence of kinetic parameters γ (○) and λ (●) on initial concentrations of ATP (A) and ADP (B)

The points represent experimental data and the lines correspond to regression analysis.

linearly dependent on initial ATP concentration in the absence of initial ADP. This condition can be achieved by preincubating the reaction mixture with pyruvate kinase and starting the cycle with adenylate kinase. The data thus obtained are shown in Figure 2(A), in which the linearity and sensitivity of the amplified assay can be seen. With the value λ used here (1.17 min^{-1}), good agreement with the equations obtained was found over a range of 16–500 pmol of added ATP. The presence of a remaining

Figure 3 Variation of λ with the concentrations of the enzymes pyruvate kinase (A) and adenylate kinase (B) in the presence of an initial concentration of 100 nM ATP

The points represent experimental data and the lines correspond to regression analysis.

background signal caused by contaminants can be observed, their concentration being evaluated by extrapolating to $\gamma = 0$, in accordance with eqn (16). The continuity of the assay and the possibility of directly obtaining the kinetic parameter γ from progress curve data permitted the sensitivity of the assay to be increased with respect to discontinuous spectrophotometric methods, which is usually in the range of nmol/ml [8]. Sensitivity might be increased further in several ways, such as by increasing

the enzyme concentrations to increase λ , by immobilizing enzymes or by decreasing the cycling volume. The limit is governed by the validity of the assumptions made in the kinetic analysis and the presence of endogenous substrates. An improvement in the detection limit would entail further purification of the reagent to decrease the background signal.

Figure 2(B) shows the results obtained when ADP was included in the reaction medium in the absence of initial ATP, pre-incubating the reaction mixture with adenylate kinase and starting the cycle with pyruvate kinase. It can be seen that λ remained constant when the initial ADP concentration was varied, whereas γ showed a linear dependence over a range of 20–500 pmol of ADP, in accordance with the equations obtained.

Determination of k_1 and k_2

As mentioned above, there is no general rate constant in this cycling system because the reaction velocity is not constant. It might therefore be convenient, for example in optimizing the cost of the assay, to know the apparent first-order rate constants k_1 and k_2 for the two enzymes involved in the cycle. The best way of determining these is from the cycle itself because experimental conditions are the same, although they can also be determined in a classical way from K_m and V_{max} . In the present study, k_1 and k_2 were determined in the two ways. In the cyclic method, several sets of experiments were performed in which the concentration of adenylate kinase was varied in the range 0.096–1.92 units, while the pyruvate kinase concentration was kept constant, and vice versa (pyruvate kinase being varied in the range 0.098–1.96 units while adenylate kinase was kept constant), in the presence of ATP or ADP (Figure 3). The values of λ thus obtained for each set of experiments were fitted by non-linear regression to eqn (11), after a preliminary check of linearity between the activity expressed and the amount of enzyme for the two kinases involved in the cycle (as explained in the Experimental section), taking for

k_1 and k_2 the mean of the results obtained; their respective values were 3.69 ± 0.56 and $2.24 \pm 0.38 \text{ min}^{-1}$. The values obtained by the classical method were 3.14 and 1.95 min^{-1} , indicating a good agreement between the two methods.

Conclusion

The analytical equations obtained for this system show that the concentration of recycling substrates increases continuously with time in a single-exponential form as a consequence of the stoichiometry of the process. The cycling rate is therefore not constant with time and progress curves fit an exponential equation, which demonstrates the importance of making continuous measurements in these systems. The coefficient γ in eqn (17) is directly related to the initial metabolite concentration, whereas λ is directly related to the concentration of the recycling enzymes. The specific first-order rate constants involved in the cycle (k_1 and k_2) were evaluated from the cycle, i.e. in the same reaction medium in which they will be used afterwards, although they can also be evaluated with the enzymes acting individually.

The amplification capacity of this cycle is therefore much greater and, at least in principle, one molecule of ATP (or ADP) is sufficient to 'catalyse' the formation of many molecules of cycling product (pyruvate) to produce a measurable signal. In practice, however, sensitivity is limited by the presence of endogenous substrates. The use of the enzyme apyrase to degrade endogenous ATP and/or ADP, as well as a knowledge of the kinetic behaviour of the cyclic reaction, has allowed us to achieve sensitivity levels near those attained by bioluminescent methods in spectrophotometric assays. An improvement in the detection limit would entail further purification of the cycling reagent to decrease the background signal.

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APPENDIX

The system of differential equations corresponding to chemical eqns (1)–(3), taking into account the depletion of AMP, PEP and NADH, is the following:

$$\frac{d[\text{AMP}]}{dt} = -k'_1[\text{AMP}][\text{ATP}] \quad (\text{A } 1)$$

$$\frac{d[\text{ATP}]}{dt} = -k'_1[\text{AMP}][\text{ATP}] + k'_2[\text{PEP}][\text{ADP}] \quad (\text{A } 2)$$

$$\frac{d[\text{ADP}]}{dt} = 2k'_1[\text{AMP}][\text{ATP}] - k'_2[\text{PEP}][\text{ADP}] \quad (\text{A } 3)$$

$$\frac{d[\text{PEP}]}{dt} = -k'_2[\text{PEP}][\text{ADP}] \quad (\text{A } 4)$$

$$\frac{d[\text{Pyr}]}{dt} = k'_2[\text{PEP}][\text{ADP}] - k'_3[\text{NADH}][\text{Pyr}] \quad (\text{A } 5)$$

$$\frac{d[\text{NADH}]}{dt} = -k'_3[\text{NADH}][\text{Pyr}] \quad (\text{A } 6)$$

where $k'_1[\text{AMP}]$, $k'_2[\text{PEP}]$ and $k'_3[\text{NADH}]$ correspond to k_1 , k_2 and k_3 in the text respectively.

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