Allosteric control of *Zymomonas mobilis* glucose-6-phosphate dehydrogenase by phosphoenolpyruvate

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The second enzyme of the Entner–Doudoroff glycolytic pathway in *Zymomonas mobilis*, glucose-6-phosphate dehydrogenase, has been found to be inhibited by phosphoenolpyruvate (PEP). In the presence of PEP levels in the micromolar range, the response of the enzyme to glucose 6-phosphate concentration becomes sigmoidal, with a Hill coefficient up to 2. At low ionic strength in the absence of PEP, the response to glucose 6-phosphate concentration is Michaelis–Menten, but at physiological ionic strength and pH, a Hill coefficient of 1.3 to 1.4 was found even in the absence of PEP. K_m values for NAD^+ and $NADP^+$ are also ionic-strength-dependent, increasing rapidly as salt concentration increases. Some sigmoidicity was also observed for NAD^+ in the presence of PEP at low glucose 6-phosphate concentrations. The results can be interpreted in a Monod–Wyman–Changeux model, in which glucose 6-phosphate binds principally to the R-state, PEP to the T-state, and NAD^+ to both states. These observations are clearly physiologically significant, and provide an explanation for the control of the balance between glycolytic throughput and ATP consumption in *Z. mobilis*.

INTRODUCTION

Zymomonas mobilis is an anaerobic bacterium which ferments sugars to ethanol and CO_2 through the glycolytic Entner–Doudoroff pathway [1,2]. Pathways such as this normally have some control at the earliest enzymic steps to avoid accumulation of metabolic intermediates when demand for the product is low. The product of glycolysis is ATP, and glycolytic flux must ultimately be controlled by ATP turnover. On the other hand, it has been suggested that glycolysis and the ATP demand are uncoupled in *Z. mobilis* [3], and ATPases that might be responsible for degrading excess ATP production have been reported [3,4]. The maximum rate of glucose consumption, as measured by the rate of ethanol production in optimum fermentation conditions, closely matches the maximum throughput of the first two enzymes, glucokinase and glucose-6-phosphate dehydrogenase [5,6]. Nevertheless, in the natural environment, organisms do not often grow or metabolize at the maximum rates as determined in fermentors, and some control on the glycolytic pathway is to be expected.

Although weak inhibition of glucokinase by glucose 6-phosphate and of glucose-6-phosphate dehydrogenase by ATP has been reported [7,8], these do not appear to be effective enough to provide a strong control on these enzyme activities, and therefore on glucose consumption. The subsequent enzyme, 6-phosphogluconolactonase, is highly exergonic and, as far as is known, has no allosteric control on its activity. In any case, this reaction occurs spontaneously, with the half-life of 6-phosphogluconolactone of the order of only tens of seconds [9,10]. Glucose enters the cell by facilitated diffusion [11], so it can accumulate intracellularly to quite high concentrations. Consequently, without some restraints on glucokinase and glucose-6-phosphate dehydrogenase activity, 6-phosphogluconate and subsequent metabolites would accumulate in the cell and deplete the ATP level.

While developing an enzyme assay, which included both glucose-6-phosphate dehydrogenase and phosphoenolpyruvate (PEP), for another purpose, I discovered that the glucose-6-phosphate dehydrogenase activity was completely inhibited by 0.5 mM PEP. A detailed study of this effect has demonstrated that PEP is an allosteric inhibitor, forcing the enzyme to display a sigmoid response to glucose 6-phosphate concentrations, and it dramatically reduces the activity of the enzyme at likely physiological concentrations of PEP. It is proposed that glucose phosphorylation and further metabolism is controlled in part by the level of PEP in *Z. mobilis* cells.

EXPERIMENTAL

Z. mobilis glucose-6-phosphate dehydrogenase was purified as described elsewhere [12]. The preparation was 98% pure as estimated by capillary electrophoresis, and had a specific activity of 355 μmol/min per mg at 1 mM glucose 6-phosphate and 1 mM NAD^+, pH 8.0. Kinetic measurements were conducted using automated mixing and measuring equipment [13]. with rates determined between 5 and 15 s after mixing of reagents. Substrate consumption was less than 10% at all times, and was corrected for by subtracting two-thirds of the final concentration of NADH from the initial substrate value. All measurements were made at 30 °C. The buffers were A [20 mM Mes (potassium salt) + 2 mM $MgCl_2$, pH 6.6], B (as for buffer A, but including 0.1 M KCl) and C (20 mM Tris/HCl, pH 8.0). Condition B was considered to be a reasonable approximation of ionic conditions *in vivo*. Most experiments involved about 20 separate rate measurements over a substrate concentration range of 100-fold. A BASIC program was written to analyse the data for the Hill coefficient h. This program minimized the root-mean-square (r.m.s.) variation of $\log v/(V_{max}-v)$ against $\log s$ by iterative selection of the best-fitting values for V_{max} and h. When h was found to be 1.00 ± 0.05, the data were further analysed to determine K_m values, using a similar program to minimize r.m.s. variation on a v-against-s plot. Curves to fit data were generated using the determined value of h, and K in the expression $v = V_{max}\, s^h/(K^h + s^h)$ where $K = K_0(1 + i/K_i)$, K_0 being the best-fitting value at inhibitor concentration $i = 0$, and K_i the best-fitting value for the inhibition constant for PEP in the complete set of data.

The non-Michaelis–Menten data were also analysed according

Abbreviations used: PEP, phosphoenolpyruvate; MWC, Monod–Wymann–Changeux; r.m.s., root mean square.

to an equation representing a Monod–Wymann–Changeux (MWC) model, at fixed NAD^+ concentration, for which the binding of PEP was only to the T-state, and the binding of glucose 6-phosphate was only to the R-state. The equation used was:

$$v = \frac{V_{max}\left(1+\frac{K_s}{s}\right)^{N-1}}{\left(1+\frac{K_s}{s}\right)^{N}+L\left[\frac{K_s}{s}\left(1+\frac{i}{K_i}\right)\right]^{N}} \quad (1)$$

where K_s is the dissociation constant for substrate s (glucose 6-phosphate), K_i is the inhibition constant for inhibitor i (PEP), L is the equilibrium constant for free T-state/free R-state, and N is the number of subunits. In this equation, it is assumed that the dissociation constants are the same for each subunit independent of occupancy of adjacent subunits, and that there is perfect co-operativity between the subunits.

Gel filtration was carried out on a Pharmacia Superdex 200 column, calibrated with lactate dehydrogenase and BSA, and with glucose-6-phosphate dehydrogenase at 1 mg/ml concentration. Samples of glucose-6-phosphate dehydrogenase at 1 μg/ml concentration in buffer B were applied, and the eluate from the column was analysed by mixing with a continuous flow of assay reagents, and detecting NADH fluorimetrically.

RESULTS

Enzyme response to glucose 6-phosphate concentration

Previous kinetic studies on *Z. mobilis* glucose-6-phosphate dehydrogenase have reported a value for the K_m for glucose 6-phosphate of 0.17 mM measured at pH 8.0, which is close to the pH at which k_{cat} is maximal [8]. Using pH 6.6 buffer B, which is closer to physiological conditions, initial results indicated that the K_m for glucose 6-phosphate was similar, about 0.15 mM. However, closer investigation of the rates at low concentrations of glucose 6-phosphate showed some non-Michaelis character, and calculation of the Hill coefficient consistently gave between 1.2 and 1.4 for several extensive sets of data in this buffer (Figure 1).

Effect of PEP on glucose-6-phosphate dehydrogenase activity

Addition of PEP resulted in marked reduction of activity, especially at low glucose 6-phosphate concentrations, with an increase in the Hill coefficient h to around 2.0 for [PEP] > 0.1 mM (Figure 2). Higher PEP concentrations suppressed activity further, but the Hill coefficient was not much increased above 2.0.

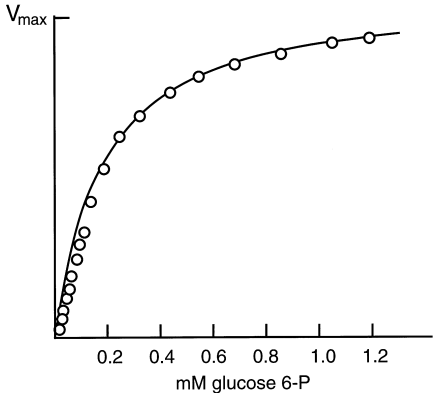


Figure 1 Reaction rate of glucose-6-phosphate dehydrogenase in buffer B, with 1 mM NAD^+

The line drawn represents perfect Michaelis–Menten behaviour. At the lower glucose 6-phosphate concentrations, the measured rates, after correction for substrate consumption, were less than theoretical. The Hill coefficient for this set of data was 1.35.

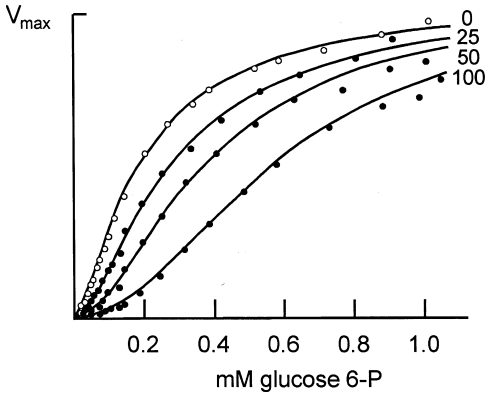


Figure 2 Response of glucose-6-phosphate dehydrogenase to glucose 6-phosphate in the presence of PEP

Buffer B was used, and the PEP concentrations were 0, 25, 50 and 100 μM. Hill coefficients h calculated for these sets of data were 1.35, 1.5, 1.7 and 2.0 respectively. Theoretical lines are plotted for $v = V_{max}\, s^h/(K^h + s^h)$, where $K = K_0(1 + i/K_i)$, $K_0 = 0.20$ mM, $K_i = 0.05$ mM (i = [PEP]).

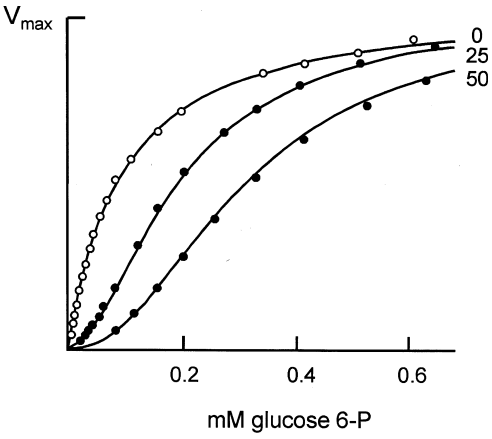


Figure 3 Data sets as for Figure 2, but using buffer A

PEP concentrations were 0, 25 and 50 μM, Hill coefficients were 1.05, 1.7 and 2.05 respectively, $K_0 = 0.08$ mM and $K_i = 0.02$ mM.

It was found that, in low-ionic-strength buffer A, the response to glucose 6-phosphate concentration was normal in the absence of PEP, that is $h = 1.0$. The K_m was 0.08 ± 0.005 mM. However, the effect of adding PEP at low I was even more marked than in buffer B, with h reaching 2.0 at 50 μM PEP (Figure 3). The best-fitting curves for this data set used values for K_0 (K_m) of 0.08 mM and for K_i of 0.02 mM, each being 2.5 times lower than the values used in Figure 2. This indicates that the salt present in buffer B is not only decreasing the strength of binding of both glucose 6-phosphate and PEP, but it is also, assuming an MWC model, shifting the equilibrium between T- and R-states more towards T-, i.e. increasing the L value.

The inhibitory effect of PEP was further demonstrated at a

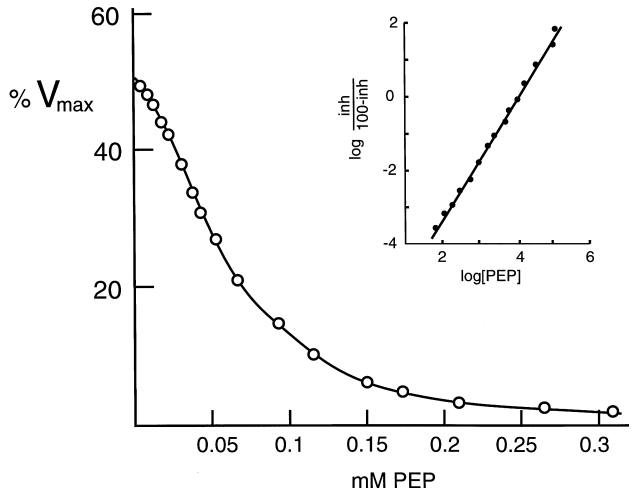


Figure 4 Inhibition of glucose-6-phosphate dehydrogenase by PEP at 0.2 mM glucose 6-phosphate

Buffer B was used with 1 mM NAD^+. Inset: Hill plot of the data as percentage inhibition (inh) with [PEP]. Slope $h = 1.6$. The x-axis is the natural logarithm of the micromolar concentration of PEP.

Table 1 Effect of ionic strength on the K_m values for NAD^+ and $NADP^+$ at pH 6.6 (buffer A) and pH 8.0 (buffer C)

V_{max} values are expressed as percentage of rate at 1 mM glucose 6-phosphate and 1 mM NAD^+ in buffer A.

	I (mol/l)	K_m (mM)	V_{max}
NAD^+			
Buffer A	0.02	0.105	100
+ NaCl	0.07	0.22	112
1.0 mM Glucose 6-phosphate	0.12	0.47	103
	0.15	0.79	101
	0.22	1.17	88
Buffer C	0.01	0.17	130
+ NaCl	0.06	0.27	126
1.0 mM Glucose 6-phosphate	0.11	0.40	128
	0.21	0.97	105
$NADP^+$			
Buffer A	0.02	0.0125	50
+ NaCl	0.12	0.023	61
1.0 mM Glucose 6-phosphate	0.22	0.047	57
Buffer C	0.01	0.022	72

fixed glucose 6-phosphate concentration, in buffer B. In Figure 4, glucose 6-phosphate concentration was 0.2 mM, NAD^+ was at 1 mM, and PEP was added at the levels indicated. 50 % inhibition occurred at 55 μM PEP, and 90 % at 150 μM. Hill analysis of the percentage inhibition against PEP concentration gave an *h* value of 1.6 (Figure 4, inset). The inhibitory effect was less at pH 8.0 (buffer C), with 150 μM PEP giving 50 % inhibition at 0.2 mM glucose 6-phosphate (results not shown).

Enzyme response to NAD^+ concentration

In most conditions the K_m for NAD^+ could be determined directly; there was no significant sigmoidicity except when PEP was strongly inhibitory, i.e. at low glucose 6-phosphate concentrations and relatively high PEP ($> 50\ \mu$M). However, the apparent K_m was increased by low concentrations of PEP, and very substantially increased by ionic strength.

Table 2 Effects of PEP on the K_m values for NAD^+

V_{max} values are expressed as percentages of rate at 1 mM glucose 6-phosphate and 1 mM NAD^+ in buffer A.

	[PEP] (μM)	K_m (mM)	V_{max}
Buffer C			
+ 0.05 M NaCl	0	0.27	132
1.2 mM Glucose 6-phosphate	100	0.39	132
Buffer A	0	0.109	105
2.0 mM Glucose 6-phosphate	25	0.117	105
	50	0.120	104
	100	0.135	104
Buffer B	0	0.47	99
0.5 mM Glucose 6-phosphate	50	1.08	93
	100	($h > 1.1$)	(90)
Buffer A	0	0.115	56
0.2 mM Glucose 6-phosphate	5	0.137	53
	10	0.185	56
	15	0.245	56
	25	($h > 1.1$)	(54)

The effect of ionic strength is illustrated in Table 1. Buffer A at pH 6.6 with 1 mM glucose 6-phosphate was used, with increasing levels of NaCl added. The K_m for NAD^+ in the absence of NaCl was 0.105 mM, and this rose to a value over 10 times higher at $I = 0.22$. (In buffer B, $I = 0.12$, the K_m was 0.47 mM.) Similar effects were found with buffer C at pH 8.0; the K_m at zero NaCl was slightly higher (0.17 mM), but did not increase quite as much with added NaCl. It should be noted that, except at the highest ionic strength, salt did not affect the value of V_{max}.

Z. mobilis glucose-6-phosphate dehydrogenase can operate with $NADP^+$ as well as with NAD^+. Although the V_{max} with $NADP^+$ is 50 % lower than with NAD^+, the K_m for $NADP^+$ is about 8-fold smaller. The increase in K_m with ionic strength is much less marked than with NAD^+ (Table 1).

The effect of PEP on NAD^+ parameters is more complex, with sigmoidicity appearing in the more potentially inhibitory conditions of low glucose 6-phosphate concentrations. Thus in buffer C at 1.2 mM glucose 6-phosphate, 100 μM PEP had no effect on V_{max}, and only increased the K_m from 0.27 to 0.39 mM. Also, in buffer A, with 2 mM glucose 6-phosphate, only a slight increase in K_m was observed with up to 100 μM PEP (Table 2). But at 0.2 mM glucose 6-phosphate, the K_m for NAD^+ rose substantially from [PEP] = 0 to 25 mM, at which point *h* became significantly greater than 1.0 (Table 2). It should be noted that in all cases the V_{max} was unaffected by PEP.

The K_m for NAD^+ also varied with glucose 6-phosphate concentration. At low *I* (buffer A), the K_m was determined for glucose 6-phosphate concentrations from 0.05 to 2 mM. Linear Eadie–Hofstee plots were obtained at all glucose 6-phosphate concentrations. The K_m value increased as glucose 6-phosphate concentration decreased (Table 3). Increasing K_m with decreasing glucose 6-phosphate concentration is characteristic of sequential reaction mechanisms; however, the data are not in concordance with a simple interpretation, as the plots (e.g. on reciprocal plotting) do not have a common intersection point. This is not surprising in view of the evidence for allosteric

Table 3 K_m and V_{max} for various NAD^+ concentrations at several fixed levels of glucose 6-phosphate in buffer A

V_{max} values are expressed as percentage of rate at 1 mM glucose 6-phosphate and 12 mM NAD^+

[Glucose 6-phosphate] (mM)	K_m (mM)	V_{max}
2	0.103	107
1	0.104	100
0.5	0.106	80
0.25	0.118	66
0.15	0.155	52
0.10	0.220	38
0.075	0.265	29
0.05	0.400	25

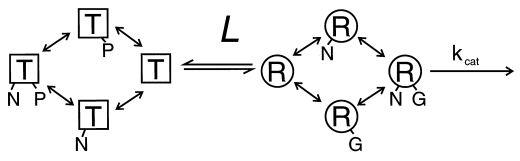
behaviour, and it suggests that, at low concentrations of both glucose 6-phosphate and NAD^+, a significant amount of free enzyme exists in the T-state even at low ionic strength.

Molecular-mass determination

The molecular mass of glucose-6-phosphate dehydrogenase was determined by gel filtration at a concentration of around 1 μg/ml, the same order of magnitude as in the kinetic experiments. This was achieved by analysing the highly dilute enzyme emerging from a gel-filtration column by mixing with assay reagents and detecting NADH formation. Three experiments were carried out using buffer B; no additions, plus 1 mM glucose 6-phosphate, and plus 0.1 mM PEP. In each case the enzyme emerged at the same point as the 1 mg/ml glucose-6-phosphate dehydrogenase control, which corresponds to a tetramer structure [8].

DISCUSSION AND PHYSIOLOGICAL INTERPRETATION

The kinetic results presented demonstrate that PEP is an allosteric inhibitor of *Z. mobilis* glucose-6-phosphate dehydrogenase, and that in its presence the response of the enzyme to glucose 6-phosphate concentration is sigmoidal. At physiological ionic strength there is evidence of some allosteric behaviour even in the absence of PEP, with a Hill coefficient of between 1.2 and 1.4 for glucose 6-phosphate. However, at low ionic strength the Hill coefficient was close to 1.0. Maximum values for h were close to 2, and on occasion slightly higher than 2. If the results are interpreted according to the MWC model for allosteric enzymes, it can be presumed that glucose 6-phosphate binds principally to the R-state, whereas for PEP the binding is to the T-state (Scheme 1). NAD^+ binds to both states, but a slight preference for the R-state could explain some sigmoidicity at high L values.



Scheme 1 Possible interpretation of glucose-6-phosphate dehydrogenase kinetic mechanism

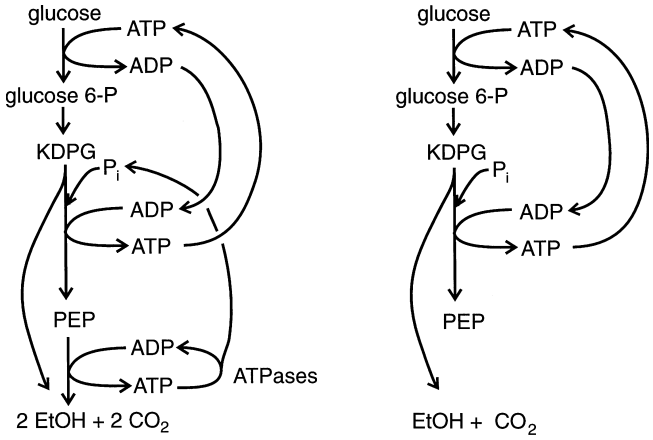
For simplicity the enzyme is shown as a single entity, but in actuality there are four subunits. G, glucose 6-phosphate; N, NAD^+; P, PEP; L, [T]/[R].

Simulating a perfect MWC model using eqn (1), the data in Figures 2 and 3 all fit best with an N value (number of subunits) of 3. However, this is inconsistent with the known structure of the enzyme. Consequently this model may be an over-simplification, with the possibility of either some mixed T/R-state conformations being present or some binding of the glucose 6-phosphate to the T-state and/or PEP to the R-state. The data actually fit the Hill expression somewhat better, despite there being no direct model to generate the Hill expression.

The fact that there was little sigmoidicity in response to NAD^+ concentration at high glucose 6-phosphate concentrations further supports the concept that glucose 6-phosphate binds to the R-state, so that at high glucose 6-phosphate concentrations, virtually all the enzyme is in the R-state. Also at low I, when it appears that little T-state exists ($h = 1.0$ for glucose 6-phosphate, Figure 3), NAD^+ showed no sigmoidicity (Table 3). However, in conditions when substantial amounts of T-state are expected to occur, sigmoidicity for NAD^+ appeared, although not as marked as with glucose 6-phosphate. The provisional interpretation of this is that NAD^+ binds preferentially, but not exclusively to the R-state.

The inhibition of glucose-6-phosphate dehydrogenase by PEP at physiological concentrations indicates that this is an important feedback inhibition on *Z. mobilis* glycolysis. The organism would naturally experience different overall demands for ATP, not necessarily coinciding with varying substrate (sugar) availability. Any organism attempts to keep its ATP levels high to maximize its ability to respond to changing conditions and to optimize the efficiency of its general metabolism. There would be times in *Z. mobilis* growth when there is plenty of substrate, but ATP demand is not maximal. Then the desirable steady state would be to maintain its ATP/ADP ratio high, but not phosphorylate the available sugar if not needed. Thus some control on glucokinase activity is needed, since glucose can permeate freely across the membrane. PEP does not have any direct effect on glucokinase, but by inhibiting glucose-6-phosphate dehydrogenase, it can slow the glucokinase activity by two means. The first is by allowing glucose 6-phosphate to accumulate and act as a product inhibitor. The second involves the reduction of P_i levels to such an extent that the glucokinase activity is depressed; we have previously shown that *Z. mobilis* glucokinase has an absolute requirement for P_i [14]. The way in which P_i is reduced is illustrated in Scheme 2. Two flux components are illustrated: one is the complete glycolysis in which glucose is converted into ethanol plus CO_2, with an exact balance of synthesis of ATP from the ADP and P_i that the cells are producing by general ATPase activity (Scheme 2, left). The second is the events that result from extra glucose being phosphorylated by ATP (Scheme 2, right). The ADP that the latter produces allows some complete glycolytic flux, but since each glucose 6-phosphate metabolized produced 2ATP molecules, there would not be enough ADP for complete conversion into ethanol plus CO_2. For every glucose phosphorylated, one ethanol plus CO_2 is produced, and the remaining half-hexose accumulates as a glycolytic intermediate, in particular PEP, which is unable to go any further because of the lack of ADP. This in turn inhibits glucose-6-phosphate dehydrogenase, so that glucose 6-phosphate becomes the chief accumulating intermediate.

The increase in glucose 6-phosphate inhibits glucokinase to a small extent (K_i = approx. 15 mM [9]). However, the second incomplete flux illustrated in Scheme 2 cannot continue indefinitely, as it involves the net consumption of P_i. Eventually the P_i level will decline to near-zero. Glucokinase has an absolute requirement for P_i; in its absence glucokinase has no activity [14]. Consequently the decline in P_i would reduce the overall



Scheme 2 Steady-state flux in *Z. mobilis*

Left, ATP breakdown by ATPases is balanced by the ATP synthesis from glucose to ethanol (EtOH) + CO_2 (complete glycolysis). Right, additional flux when ATPase activity is less than glucokinase potential. The net effect here is that PEP and other metabolites accumulate, and P_i is consumed. KDPG, 2-oxo-3-deoxy-6-phosphogluconate.

glucokinase activity to just the level required for the balancing flux in Scheme 2 (left). Thus the cell is able to adjust its glucose consumption to match its ATP demand, through the combined controls of glucokinase and glucose-6-phosphate dehydrogenase.

These results demonstrate several features relating to studies on enzyme kinetics. First, that for the understanding of the physiological behaviour of enzymes, it is important to study the kinetics in buffer conditions that are a reasonable approximation to conditions *in vivo*. In the case of glucose-6-phosphate dehydrogenase, an increase in ionic strength greatly reduces the apparent K_m values of its substrates, and the K_i of its inhibitor, PEP, but has little effect on V_{max}. On the other hand, with glucokinase, an increase in ionic strength greatly reduces the value of V_{max}, and has smaller effects on K_m values [14]. In both cases the rate of change of the parameters is rapid around physiological ionic strength. In addition, the optimum pH of an enzyme, which is usually chosen for kinetic studies, is not always the physiological pH. These points should be borne in mind when physiological interpretations are made from kinetic data.

A second feature is that accurate measurements can detect unsuspected deviations from Michaelis–Menten kinetics, which, although not necessarily affecting physiological interpretations greatly, do indicate complications in the kinetic mechanism of the enzyme. And finally, when developing a physiological role for the enzyme from kinetic studies, it is important that all relevant metabolites should be tested, before concluding that the enzyme is not subject to an allosteric inhibition or activation that might be important *in vivo*.

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